A Stable Syncytial Mutant of Herpes Simplex Type 2 Virus

(Accepted 4 February 1974)

SUMMARY

A stable mutant of type 2 herpes simplex virus has been obtained with plaques which consist entirely of flat syncytial cells. The \( \text{syn} \) plaques could easily be differentiated from wild-type \( \text{syn}^+ \) plaques and they retained their \( \text{syn} \) morphology on prolonged incubation and when assayed in different cell lines. Serologically, \( \text{syn} \) virus reacted as type 2 herpes simplex and showed a rate of reversion from \( \text{syn} \) to \( \text{syn}^+ \) of \( 3.6 \times 10^{-4} \). The \( \text{syn} \) marker should be a valuable third marker for the preparation of a preliminary linkage map of the type 2 virus genome.

Temperature-sensitive (ts) mutants are proving of value for the genetic analysis of animal viruses. A number of these mutants have been isolated from both type 1 and type 2 herpes simplex virus (Subak-Sharpe, 1969; Schaffer et al. 1970; Timbury, 1971; Zygraich & Huygelen, 1973; Esparza et al. 1974), but only in the case of type 1 virus has it been possible to order the markers on the basis of recombination frequencies from 3-factor crosses (Brown, Ritchie & Subak-Sharpe, 1973). Type 1 virus can be obtained in either non-syncytial (\( \text{syn}^+ \)) or syncytial (\( \text{syn} \)) form and Brown et al. (1973) used this \( \text{syn}/\text{syn}^+ \) plaque morphology marker as a non-selective third marker in addition to the temperature-sensitive defects of the mutants in the crosses. Plaques of HSG 52, the wild-type strain of type 2 virus from which ts mutants have been isolated, show the mixed type of c.p.e. (\( \text{syn}^+ \)) characteristic of type 2 virus. Elongated syncytial cells are present in the edge of the plaque and there are numerous rounded cells seen both at the periphery and in the centre of the plaque. Unlike type 1 virus, however, HSG 52 does not spontaneously produce stable \( \text{syn} \) plaques with measurable frequency. However, the importance of 3-factor crosses for the preparation of a reliable linkage map and the ease with which the \( \text{syn}/\text{syn}^+ \) plaque morphology marker can be scored makes the isolation of a \( \text{syn} \) plaque virus from the type 2 wild-type strain of considerable potential value for genetic studies with type 2 virus.

This communication describes the isolation of a mutant from HSG 52 which forms large flat syncytial (\( \text{syn} \)) plaques that can easily be differentiated from the wild-type \( \text{syn}^+ \) plaques. All experiments were done using BHK 21 cells and Eagle's medium with 10% (v/v) of tryptose phosphate broth and 10% (v/v) calf serum; plaque assay was as previously described (Timbury, 1971). Fig. 1 gives a summary of the history of the isolation of the \( \text{syn} \) virus. This began with the plating out under fluid overlay, and after dilution \( 10^{-4}, 10^{-5} \), and \( 10^{-6} \), of HSG 52 (titre \( 3.9 \times 10^8 \) p.f.u./ml) to yield approx. 17000 plaques on the titration plates. No plaques composed of flat syncytia were seen but three plaques, in which the syncytia which are normally seen at the edge of the plaque were more prominent than usual, were picked and subcultured. After four further passages at which 5, 10, 4 and 7 plaques, respectively, were picked and replated, two of the plaques obtained at the fourth passage were grown up into stocks of virus. Neither stock, however, was suitable for use as a third marker because, although the plaques were considerably more syncytial than wild-type virus and showed an absence of rounded cells, they consisted of small retracted syncytia that could at first glance be mistaken for clumps of rounded cells. Both stocks were then replated and a
Type 2 $syn^+$
(HSG 52)
Plated 31 °C

Five serial passages of single plaques at 31 °C

Type 2 with small distorted $syn$
plaques: replated 31 °C

Four serial passages of single plaques at 31 °C

Type 2 $syn$: found to be $ts$:
replated at 36 °C

Three serial passages of single plaques at 38 °C

Type 2 $syn$ $ts^+$

Fig. 1. Diagram of the isolation of $syn$ type 2 herpes simplex virus.

total of 22 plaques picked and replated. Three further passages were then carried out at
which 5, 7 and 15 plaques, respectively, were picked and subcultured. All the 15 plaques
at the last passage were completely composed of flat syncytial cells and had derived from one
of the original 22 plaques. A stock of virus was prepared from one of the 15 $syn$ plaques but was
found on titration to be temperature-sensitive with a relative efficiency of plating at 38 °C
compared to 31 °C of $2.0 \times 10^{-4}$. After replating at 36 °C followed by three serial passages
of single plaques at 38 °C, a stock of virus (titre, $2 \times 10^8$ p.f.u./ml) was obtained which had
wild-type temperature resistance (the efficiency of plating at 38 °C/31 °C was $4.5 \times 10^{-4}$) and
which formed large flat $syn$ plaques which could easily be distinguished from the $syn^+$
plaques of the parental virus. The history of isolation of $syn$ virus, however, makes it pos-
sible that more than one mutational event was involved in the change from $ts^+syn^+$ to
$ts^+syn$. Fig. 2(a) and (b) shows plaques of $syn$ and $syn^+$ type 2 virus. It can be seen that there
are no rounded cells in the $syn$ plaque, which is entirely composed of flat syncytial cells. In
contrast, the $syn^+$ plaque consists largely of rounded cells. Fig. 3(a) and (b) show the edges of
$syn$ and $syn^+$ plaques at higher magnification. The aggregation of nuclei in the syncytia into
club-shaped projections which can be seen in Fig. 3(a) is a characteristic feature of $syn$
plaques. The $syn$ phenotype was not a transient phenomenon and $syn$ plaques retained their
characteristic morphology on continued incubation, for example, for 5 days in fluid medium.
Fig. 2. (a) Syn plaque with large flat syncytia and total absence of rounded cells. Magnification $\times 35$. (b) Syn$^+$ plaque with numerous darkly stained rounded cells; a small secondary syn$^+$ plaque is also present. Magnification $\times 35$. 
Fig. 3. (a) Aggregations of nuclei at the edge of a syn plaque: no rounded cells are present. Magnification $\times 120$. (b) Mixture of rounded cells and elongated small syncytial cells at the edge of a syn$^+$ plaque. Magnification $\times 120$. 
Table 1. Mean $k$ values* of herpes simplex virus strains in neutralization tests with type 1 and type 2 antisera

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Type 1 $syn^+$</th>
<th>Type 2 $syn^+$</th>
<th>Type 2 $syn$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1</td>
<td>39.2†</td>
<td>3.3</td>
<td>2.5</td>
</tr>
<tr>
<td>Type 2</td>
<td>2.1†</td>
<td>5.3</td>
<td>3.4</td>
</tr>
</tbody>
</table>

* Based on the results of four experiments.
† This value is significantly different ($P < 0.001$) from the $k$ values of type 1 antiserum with both type 2 $syn^+$ and type 2 $syn$ values but the $k$ values of type 1 antiserum with type 2 $syn^+$ and type 2 $syn$ viruses are not significantly different: none of the $k$ values with the type 2 antiserum are significantly different from each other.

Table 2. Rate of reversion from $syn$ to $syn^+$ plaque morphology

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Total no. of plaques</th>
<th>Mean no. of p.f.u. in progeny per plaque</th>
<th>No. of plaques with $syn^+$ plaques in progeny</th>
<th>Rate of reversion from $syn$ to $syn^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>86</td>
<td>20.6</td>
<td>1</td>
<td>$3.9 \times 10^{-4}$</td>
</tr>
<tr>
<td>2</td>
<td>99</td>
<td>213.9</td>
<td>8</td>
<td>$2.7 \times 10^{-4}$</td>
</tr>
<tr>
<td>3</td>
<td>99</td>
<td>122.4</td>
<td>7</td>
<td>$4.2 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

When the $syn$ virus was plated on to Vero monkey kidney cells and human embryo lung cells the plaques produced were clearly $syn$ and easily distinguishable from the wild-type $syn^+$ plaques which had been plated on parallel cultures for comparison.

The $syn$ virus was also tested by neutralization with both type 1 and type 2 antisera to confirm that it was serologically type 2 herpes simplex. The antisera were each prepared by inoculating a rabbit with one injection of the appropriate virus emulsified with Freund's complete adjuvant followed by a single intravenous injection of virus 1 month later. The results of these tests are shown in Table 1. The type 1 antiserum used was a good serum for discriminating between the two types of virus but the type 2 antiserum, which neutralizes even the homologous type 2 virus poorly, was not. The results of $k$ value estimations with type 1 antiserum show that $syn$ virus is serologically type 2 and confirms its origin from HSG 52.

Experiments of the fluctuation test type were also done to calculate the rate of reversion of the virus from $syn$ to $syn^+$. $Syn$ virus was diluted and plated on to monolayers of BHK 21 cells under an overlay of Eagle's medium containing 5% (v/v) calf serum and 0.6% (w/v) agarose. After 5 to 6 days incubation at 31 °C, neutral red was added and individual plaques were picked through the agarose into 1 to 2 ml of medium, frozen and thawed once and replated at 31 °C. The progeny plaques derived from each plaque were counted and the number of $syn^+$ plaques was recorded. On the basis of these results, the rate of reversion from $syn$ to $syn^+$ was calculated using the formula originally derived for bacteria by Luria & Delbruck (1943) and used for the estimation of the mutation rate from small-to-large plaque forming virus with EMV virus by Breeze & Subak-Sharpe (1967). This formula uses the proportion ($p$) of picked plaques not resulting in revertant progeny and the mean plaque count ($N$) in the replated virus. The reversion rate per duplication is then estimated by

$$-(\log_2 p) \times (\log N).$$

The formula, originally applied to bacterial mutations, estimates the number of rounds of genome duplication from the ratio of final to original titre. In a virus such as herpes, in
which not all daughter DNA molecules are finally encapsidated in infectious virus particles, the formula can be shown to overestimate the true reversion rate per DNA replication. A ‘duplication’ in this case is more realistically interpreted as that series of molecular events that results in the doubling of the infectious titre. The results of three separate experiments are shown in Table 2. In the first experiment counts of plaques in the progeny of the plaques picked were low so that in the second and third experiments plaques were picked into 1 ml instead of 2 ml medium and the amount plated was doubled from 0.4 ml to 0.8 ml. The plaques from which the second experiment were picked, however, were larger than usual and the counts much higher than expected. The results of the three experiments were nevertheless in close agreement and the mean rate of reversion to syn+ was \(3.6 \times 10^{-4}\). Despite the very low rate of mutation from syn+ to syn, syn virus therefore shows a relatively high rate of reversion to syn+. Revertant syn+ plaques were picked and were found to breed true as syn+ on subculture. However, the syn plaque marker has proved to be sufficiently stable on passage for the preparation without difficulty of high titre stocks of virus with a low incidence of syn+ plaques. It is of value to have some reversion so that mutants prepared from syn virus can also be obtained in syn+ form thus allowing 3-factor crosses to be carried out reciprocally. Work is already in hand to back cross the syn plaque marker into the temperature-sensitive mutants and also to isolate mutants directly from the syn virus. In this way it is hoped to prepare a preliminary linkage map for type 2 herpes simplex virus based on 3-factor crosses.

The help of Mrs Ann Weir with the photography is gratefully acknowledged.

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REFERENCES


(Received 26 November 1973)

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