# Maintenance of the u Mutant of Sindbis Virus by 40° Passage (33583)

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Plaque mutants of Sindbis virus have been reported (1–3), of which the unusually large-plaque (u) mutant isolated in our laboratory was unique in that it was more sensitive to interferon than the parent type virus (3). Serial passage of this mutant by the ordinary method resulted in its replacement by the parent type virus, probably because the latter appearing as a back mutant excluded the u mutant owing to the higher resistance of the parent virus to interferon. Hence, maintenance of the u mutant required cloning from a typical plaque at every passage (3). It was later observed that, even with this method of passage, some changes of properties occurred after several subculturings: the size of plaques formed at 40° became smaller than before, although plaques formed at 37° remained as large as 8–10 mm, and virus titer determined at 40° was about 1 log lower than that determined at 37°.

The present work was begun with dual purposes of (i) finding a simpler method of maintenance of the u mutant to obviate the cumbersome cloning passage, and (ii) finding a way to regain its earlier characteristics of plaque morphology, i.e., formation of larger plaques at 40° as well as at 37° with an equal plating efficiency. Both the purposes were fulfilled by adopting 40° passage, as described herein. The virus so maintained was then examined for interferon sensitivity, baby mouse pathogenicity, and resistance to agar inhibitor.

*Materials and Methods. Virus.* Passage history of the parent Sindbis virus, and isolation of different clones therefrom were detailed earlier (3). The u mutant had been maintained by the cloning passage described below up to the ninth passage before starting the present experiments. The lp (large-plaque) clone representing the parent type was recloned once; it formed plaques 2–5 mm in diameter at both 37 and 40°.

*Plaque titration.* Preparation of chick embryo cell monolayers, virus diluent, and method of inoculation were detailed previously (3); 60-mm dishes seeded with $1 \times 10^7$ cells in 5 ml of growth medium were used after 1 day's incubation at 37°. After virus inoculation, the dishes were incubated at 37° for 1 hr for adsorption and overlaid with 4 ml/dish of Earle's medium containing 1% Noble agar (Difco), 2% calf serum and 0.0025 *M* Tris buffer of pH 7.2. After 3 days' incubation, at either 37 or 40°, a second overlaying was done with 2 ml of 1% agar containing 1:10,000 neutral red. Virus titers were expressed by pfu (plaque-forming units).

*Standard passage.* Culture bottles, $4 \times 7 \times 2.5$ cm, were seeded with $5 \times 10^6$ chick embryo cells in 5 ml of growth medium, and were used next day. An inoculum from the previous passage fluid containing about 1000 pfu was used as seed. Maintenance medium was Earle's medium containing 2% calf serum. Incubation temperatures are specified for each experiment in the text. When cytopathic effect was evident the bottles were stored in a deep freezer.

*Cloning passage.* This had been used only for the u mutant. Plaques were formed at 37° with a limiting dilution of the previous passage fluid, and a typical u plaque was rinsed with diluent and used as seed for the next passage. The subsequent steps were the same as in the standard passage.

*Interferon.* Interferon was prepared in the manner described previously (3). Fluids harvested from chick embryo cell cultures infected with the parent Sindbis virus were dialyzed against KCl–HCl buffer at pH 2.5, and then against pH 7.2 buffered saline, and sterilized by filtration. Storage was at —20°. Assay for interferon sensitivity of virus was done as follows. Monolayer dishes were given 2 ml/dish of maintenance medium (Earle's

TABLE I. Effect of Different Temperatures at Which the u Mutant Was Passaged.

| | Titer of progeny virus in the third passage fluid (pfu/ml) | |
|---|---|---|
| Temp (°) | lp type ($\times 10^8$) | u type |
| 35 | 3.4 | $3.6 \times 10^7$ |
| 37 | 3.6 | $2.0 \times 10^7$ |
| 40 | 0 | $3.2 \times 10^8$ |

solution with 2% calf serum and 0.0025 *M* Tris buffer at pH 7.2) containing the above interferon preparation at 1:8, and incubated at 37°. Control dishes were given interferon-free medium. After 24 hr, the fluids were aspirated and virus titration was performed using the interferon-treated and control dishes in the manner of the usual plaque assay.

*DEAE-dextran and protamine sulfate.* A 1% solution was made with each drug and pH 7.2 buffered saline; and were sterilized by autoclaving. They were incorporated into the agar overlay medium to the desired final concentrations.

*Baby mouse titration.* The DD/n strain of Swiss mice aged 1–3 days were inoculated with virus intracerebrally in 0.03-ml amounts. Two to three litter mates were used per dilution. The animals were observed for 2 weeks, and $LD_{50}$ was calculated by the formula of Reed and Muench (4).

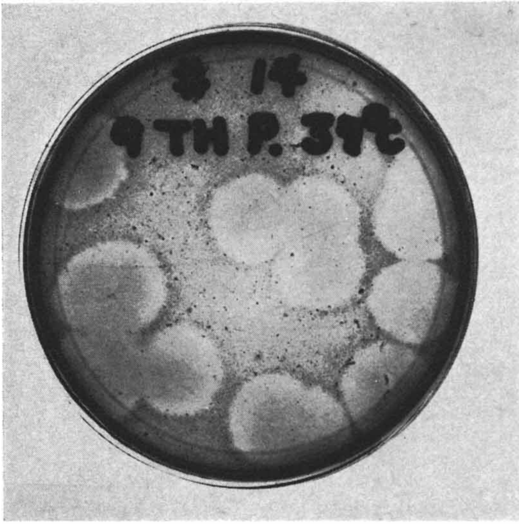
*Results. Passage of the u mutant at different temperatures.* The ninth subculture of the u mutant maintained by cloning passage was used as the seed to start three parallel serial passages in the manner of standard passage. In one series the infected cultures were incubated at 35° at each passage, in another at 37° and in the other at 40°. After three passages, progenies were titrated for pfu, plaque dishes being incubated at 37°. Plaques smaller than 5 mm and those measuring more or less than 8 mm were called lp (parent type) and u type plaques, respectively, and counted separately. As indicated in Table I, the lp type virus predominated in the two lower temperature series, while the progeny of the 40° passage contained u type virus exclusively. This result was not ascribable to a higher stability of the u mutant at 40°, because an *in vitro* degradation test showed about equal rates of degradation at 40°: —1.48 and —1.55 $\log_{10}$ /day with the u and lp viruses, respectively.

The reason for the replacement of the u mutant by the parent lp type virus during standard passage at 37° was considered earlier to be due to appearance of a back mutant capable of excluding the u mutant (3). If so, the above phenomenon would mean either that such a back mutation seldom occurs at 40° or that such a back mutant if present cannot exclude the u mutant virus at this temperature for some reason. The following experiment denied the second explanation. A large quantity of the u mutant virus was mixed with a small amount of lp virus to start serial standard passage employing the incubation temperature of 40°. A control series was set without addition of lp virus in the starting seed. Progenies of the third passage, recorded in Table II, clearly demonstrated that, once introduced into the u mutant culture, the lp virus could replace it even at 40°.

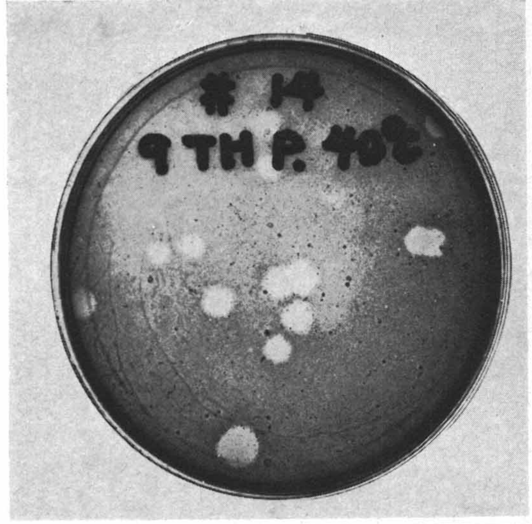
*Changes in plaque characteristics of the u mutant occurring during 40° passage.* After being transferred from the previous cloning passage to serial 40° passage, the u mutant showed a gradual adaptation to the growth at

TABLE II. Effect of Addition of lp Virus into the u Mutant Seed with Which to Start Serial 40° Passage.

| | Virus contained in the starting seed (pfu/bottle) | | Titer of progeny virus in the third passage fluid (pfu/ml; $\times 10^8$) | |
|---|---|---|---|---|
| Passage series | lp ($\times 10^4$) | u ($\times 10^8$) | lp type | u type |
| 1 | 1.3 | 2.1 | 8.0 | 0 |
| 2 | 0 | 2.1 | 0 | 3.8 |



# 14
9TH P. 37°C

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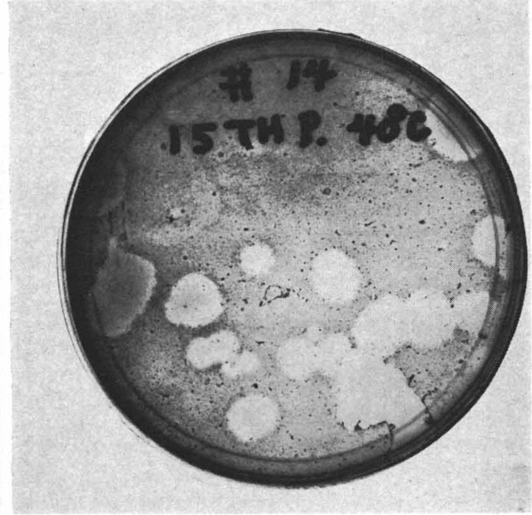
15TH P. 37°C

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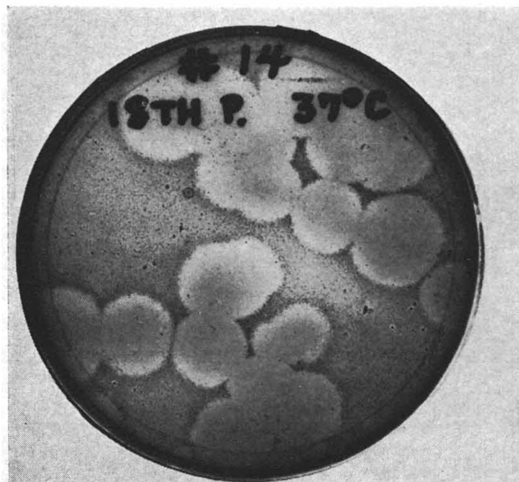

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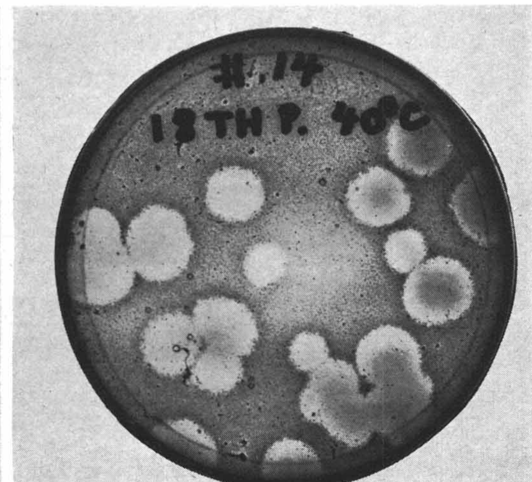

# 14
18TH P. 40°C

FIG. 1. Plaques formed by the u mutant virus at 37 and 40° before and after 40° passage: 9th P. = ninth cloning passage receiving no 40° passage; 15th P. = sixth 40° passage starting from the above virus; 18th P. = ninth 40° passage.

40°. This relation can be seen in Table III. before the 40° passage, its pfu titer determined at 40° was about 1 log lower than that determined at 37°, and the plaque size at 40° was about 4 mm on the average. After the serial 40° passage, two changes were appreciated. First, the plating efficiencies at 37 and 40° became almost equal, and secondly the size of plaques formed at 40° increased up to the level of 7 mm. The change of the plaque size is illustrated in Fig. 1.

TABLE III. Titration at 37 and 40° of the u Mutant Virus at Different Levels of 40° Passage.

| | Titration at 37° | | Titration at 40° | |
|---|---|---|---|---|
| No. of 40° passage | Fluid virus titer (pfu/ml) | Plaque size (mm) | Fluid virus titer (pfu/ml) | Plaque size (mm) |
| 0[a] | $8.0 \times 10^8$ | 10.8[b] | $5.6 \times 10^7$ | 3.8 |
| 3 | $3.2 \times 10^6$ | 8.0 | $2.2 \times 10^6$ | 4.0 |
| 6 | $6.0 \times 10^6$ | 10.7 | $6.0 \times 10^6$ | 6.6 |
| 9 | $4.6 \times 10^7$ | 9.2 | $4.4 \times 10^7$ | 7.0 |

[a] Before the serial 40° passage, the virus had received 9 successive cloning passages at 37°.

[b] Average of 10 plaques randomly selected from 3 parallel dishes.

*Sensitivity to interferon.* One characteristic of the u mutant exhibited earlier was a higher sensitivity to interferon as compared with the parent lp type virus. Whether or not this property had changed after the 40° passage was examined. The u and lp viruses were titrated using interferon-pretreated and control monolayer dishes. As shown in Table IV, the sensitivity to interferon of the u mutant, as expressed by reduction in pfu, was higher than that of the lp virus. However, the difference between the interferon sensitivities of the two viruses was not so marked as seen before the 40° passage (3).

TABLE IV. Titration of the u Mutant and the Parent lp Virus in Interferon-Treated and Control Untreated Monolayers.

| Virus | Titer in interferon-treated dishes (a) (pfu/ml) | Titer in control dishes (b) (pfu/ml) | Ratio of (a)/(b) |
|---|---|---|---|
| u | $2.1 \times 10^6$ | $2.0 \times 10^7$ | 0.1 |
| lp | $1.6 \times 10^8$ | $1.1 \times 10^9$ | 0.15 |

*Baby mouse pathogenicity.* The above result implied that, although the 40° passage made the u mutant regain its earlier plaque morphology, the progeny tended to lose the high interferon sensitivity. In parallel with this tendency, the pathogenicity for baby mice of this virus also showed some change. Table V lists the baby mouse pathogenicities of the parent lp and the u mutant before and after the 40° passage. In the case of the lp virus, almost one pfu corresponded to one LD, and with the u mutant the pfu:LD ratio was much greater before but showed a diminishing trend after the 40° passage.

*Effects of DEAE-dextran and protamine sulfate upon the plaque size.* The u mutant and the lp virus were titrated in triplicate using (i) ordinary overlay, (ii) overlay containing 0.01% DEAE-dextran, and (iii) overlay containing 0.05% protamine sulfate. The result is presented in Table VI, which indicates that the plaque-enhancing effects of those polyions were more marked upon the lp virus, proving that the u mutant was more resistant to agar inhibitor (5).

*Discussion.* The present results indicate first of all that the simple 40° passage could maintain the u mutant without recourse to the cumbersome cloning passage. This success seems to be due to absence of back mutation toward the parent type at 40°. In connection with this, the virus maintained by 40° passage showed also a low frequency of appearance of small-plaque virus. This is in contrast to the earlier u mutant which, like the giant-plaque mutants of Hannoun *et al.* (1) and of Nagata *et al.* (2), was always accompanied by a few percent of small-plaque virus (3).

The earlier u mutant was characterized by

TABLE V. Baby Mouse Pathogenicities of the Parent lp Virus and the u Mutant.

| Virus | Plaque titer (a) (pfu/ml) | Mouse infectivity (b) ($LD_{50}$/ml) | Ratio of (a)/(b) |
|---|---|---|---|
| lp | $1.2 \times 10^8$ | $9.2 \times 10^7$ | 1.3 |
| u before 40° pass.[a] | $1.0 \times 10^8$ | $2.1 \times 10^6$ | 47.6 |
| u at tenth 40° pass. | $4.0 \times 10^7$ | $4.1 \times 10^6$ | 9.8 |
| u at eleventh 40° pass. | $5.0 \times 10^6$ | $5.0 \times 10^5$ | 10.0 |

[a] At the seventh cloning passage.

two distinct properties: it formed very large plaques not only at 37° but also at 40°, and was more sensitive to interferon than the parent type virus. The latter characteristic may have been carried over from the small-plaque clone from which it was derived. Namely, it may be that the u mutant is an agar inhibitor-resistant daughter variant of the interferon-sensitive small-plaque virus. However, the ability to form large plaques may not be ascribable solely to the increased resistance to agar inhibitor, because earlier growth curve experiments (3) demonstrated a quicker rise and a higher peak of the u mutant in agar-free circumstances than the parent type virus. The question of why the more interferon-sensitive virus grew better than the parent type virus has not yet been solved.

The above-stated characteristics of the u mutant were both labile. The cloning passage adopted earlier could maintain the plaque morphology at 37° but not that at 40°. The reason for this remains unknown. The 40° passage, on the other hand, rendered it possible to regain the ability to form very large plaques at 40°, but, at the same time, tended to deprive the progeny of the high sensitivity to interferon. It may be conceivable that the two markers are stable at different temperatures. More detailed studies on the effects of different passage methods upon individual markers are under way.

An observation of importance was that the u mutant showed an attenuated pathogenicity for baby mice despite the unusually large-plaque size. There have been many cases in which the large-plaque virus was more pathogenic than the small-plaque virus (6–21), but the present case is just contrary to those examples. In some of those cited cases (14, 17, 18, 20, 21) the resistance to agar inhibitor also paralleled the animal pathogenicity. In the present case, the animal pathogenicity revealed rather a close parallelism with the resistance to interferon. This fact suggests that the relation between these two markers is more direct than the relation between the animal pathogenicity and the resistance to agar inhibitor.

*Summary.* The unusually large-plaque (u) mutant previously isolated from a small-plaque clone of Sindbis virus was maintained first by cloning passage, because the ordinary passage resulted in replacement of the u mutant by the more interferon-resistant parent type virus. After 9 cloning passages, it was found that the virus did not form such large plaques at 40° as it did before, and the pfu titer determined at 40° was lower than

TABLE VI. Effects of Incorporation of DEAE-Dextran and Protamine Sulfate into Agar Overlay upon the Plaque Size and Titer of the u and lp Viruses.

| Virus | Plaque size (mm) | | | Titer (pfu/ml) | | |
|---|---|---|---|---|---|---|
| | Control | DEAE-dex. | Prot. sulf. | Control | DEAE-dex. | Prot. sulf. |
| u | 8.7[a] | 10.6 | 13.3 | $3.0 \times 10^7$ | $3.2 \times 10^7$ | $3.6 \times 10^7$ |
| lp | 3.6 | 11.4 | 12.1 | $2.2 \times 10^8$ | $5.0 \times 10^8$ | $4.0 \times 10^8$ |

[a] Average of 10 plaques randomly selected from 3 parallel dishes.

that determined at 37°. However, serial passage of this virus in a standard passage manner adopting incubation at 40° allowed it to regain the original plaque morphology, i.e., formation of very large plaques at 40° as well as at 37° with about equal plating efficiencies. It seems likely that back mutation toward the parent type seldom occurs at 40°. The virus so maintained was found to be slightly more sensitive to interferon, less pathogenic for intracerebrally inoculated baby mice, and more resistant to agar inhibitor than the parent type virus.

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