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# Temperature-Sensitive Mutants of Vesicular Stomatitis Virus: In Vitro Studies of Virion-Associated Polymerase

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The temperature dependence of the virion-associated polymerase activity of six temperature-sensitive (ts) mutants of vesicular stomatitis virus (tsW10, 11, 14, 16B, 28, and 29) has been examined in vitro and compared to the heat-resistant parent (HR). The polymerase of five of the mutants (tsW10, 11, 14, 16B, and 28) appears to be significantly more ts than that of HR. Because certain pairs of these five mutants can complement each other's in vitro polymerase activity, it appears that in vitro some components involved in the polymerase of one virion can be utilized by another virion. Examination of 19 revertants of tsW11 and tsW16B which had regained their ability to replicate at 38 C showed that their in vitro polymerase activity had also become less ts. Furthermore, it was found that the pairs of mutants which showed in vitro complementation of polymerase activity at 38 C were those which had shown complementation in yielding infectious progeny in mixedly infected cells. These two observations suggest that the ts behavior of the in vitro polymerase activity of the five mutants is related to their inability to replicate at the nonpermissive temperature.

Temperature-sensitive (ts) mutants of vesicular stomatitis virus (VSV) have been isolated in this laboratory to investigate the replication of VSV in mouse L cells (3). One step in such a study is to ascertain for each mutant which, if any, viral activities show altered temperature dependence. One activity demonstrated in VSV virions is an RNA-dependent RNA polymerase (1) which appears to catalyze the synthesis of messenger RNA rather than virion RNA (1, 4). We are investigating polymerase activity in our mutants both in infected cells and in vitro and have demonstrated that one of our mutants, tsW11, has a defective virion-associated polymerase (2). This paper describes the in vitro behavior of five additional mutants, and further results for tsW11, as well as the ability of certain pairs of mutants to enhance each other's polymerase activity in vitro.

## MATERIALS AND METHODS

**Viruses and cells.** The isolation of ts mutants from a heat-resistant (HR) parent strain of VSV (Indiana Serotype), together with the preparation and assay of stocks in L cells (L-60 line), has been previously described (3). The permissive and nonpermissive temperatures were 30 and 38 C, respectively.

For the experiments described below, virus stocks were grown on confluent monolayers of L cells which had been infected with an input multiplicity of 10 plaque-forming units (PFU) per cell. Virus stocks used for this infection had been derived from isolated plaques and were examined on the electron microscope to ensure that the content of T particles was less than 5%. The monolayers were incubated for 24 hr at 30 C, and the lysate was clarified by centrifuging at 13,000 × *g* for 10 min. The supernatant fluid was centrifuged at 33,000 × *g* for 60 min to give a virus pellet which was resuspended in 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, *p*H 7.9, and dispersed by sonic treatment. The virus concentration of the resulting suspension was about 60 times that of the lysate and had $10^{10}$ to $10^{11}$ PFU/ml.

Some virus preparations were further purified by three additional centrifugation procedures. First the virus, suspended in Tris-hydrochloride, was layered onto a 9 to 29% (solute weight percentage of solution weight) continuous gradient of potassium tartrate in 3 mM mercaptoethanol and centrifuged at 23,000 rev/min in an SW27 rotor for 60 min. The visible band was collected and diluted to 40 ml in 0.1 M Tris-hydrochloride, *p*H 7.9. This virus suspension was then pelleted by centrifuging at 33,000 × *g* for 60 min, resuspended in 0.55 ml of Tris-hydrochloride, and dispersed by sonic treatment. The virus suspension was then layered onto a continuous 5-ml gradient of 16.5 to 62.5% sucrose in 3 mM

mercaptoethanol and centrifuged to equilibrium for 24 hr at 50,000 rev/min in an SW65 rotor. The virus band was collected with a syringe and assayed for PFU and pclymerase activity.

**In vitro RNA polymerase assay.** RNA synthesis was detected by the conversion of $^3$H-guanosine triphosphate (GTP) (Schwarz BioResearch, Inc.), 1 $\mu$Ci per 0.3 ml of reaction mixture, into an acid-insoluble product by using an assay which is essentially that of Baltimore et al. (1). The samples were filtered through Whatman glass filters and washed with 30 ml of cold 5% trichloroacetic acid. The filters were placed in glass scintillation vials, dried, and 0.5 ml of a 3:1 mixture of NCS tissue solubilizer (Amersham/Searle) and toluene was added to each vial. After 30 min at room temperature, 7 ml of a toluene mixture containing 1% glacial acetic acid and 4.2% Liquifluor (New England Nuclear Corp.) were added. The vials were then counted in a liquid scintillation counter to give a statistical accuracy better than $\pm$5%. The acid-insoluble $^3$H activity in an unincubated sample was found to be approximately the same as that of a sample with no virus incubated for 20 min. This nonspecific background (50 $\pm$ 25 counts/min) was subtracted to give the values for net incorporation.

## RESULTS

**Temperature dependence of virion-associated polymerase.** The virion-associated polymerase activity of the parental type VSV (HR) and several ts mutants (tsW10, 11, 14, 16B, 28, and 29) was tested in vitro at 30 and 38 C. The results of two such experiments are shown in Fig. 1 and 2, in which the relative incorporation of $^3$H-GTP into an acid-insoluble product is plotted against time.

In Fig. 1, it can be seen that mutants tsW16B and 28 show relatively less incorporation at 38 C than does HR or mutant tsW29. For the experiment whose results are plotted in Fig. 2, another preparation of tsW16B shows a similar temperature dependence to that in Fig. 1, whereas the temperature dependence of tsW10 is intermediate between that of tsW16B and HR.

To compare the temperature dependence of the virion-associated polymerase activity from experiment to experiment, a temperature-dependence index (TDI) was used where: TDI = (net incorporation in 20 min of incubation at 38 C)/(net incorporation in 20 min of incubation at 30C) $\times$ 100%. Average values of TDI, with 95% confidence limits where sufficient data exist, are shown in Table 1 for HR and the six ts mutants tested. The polymerase activities of mutants tsW10, 16B, 11, and 28 are significantly more ts than that of HR. The low plaquing efficiency of tsW29 at 38 relative to 30 C ($5 \times 10^{-4}$) indicates that its relatively high TDI is a characteristic of the mutant and not due to "leakiness" or the presence of ts+ revertants.

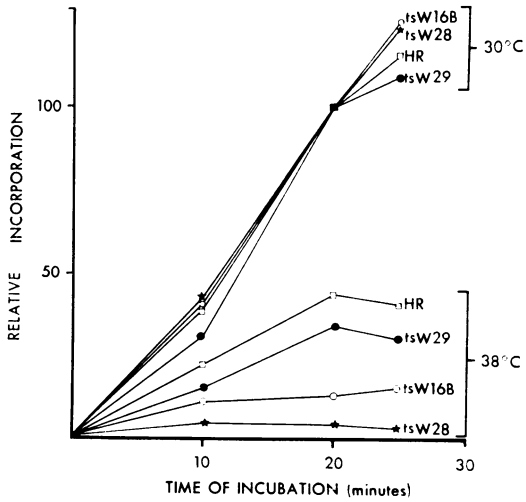


FIG. 1. *RNA polymerase activity of HR, tsW16B, tsW28, and tsW29. The acid-insoluble $^3$H activity was corrected for nonspecific background by subtracting the average value for a nonincubated sample (~50 counts/min). The values of the 30 C incorporation in 20 min and for the number of infectious virus in the reaction mixture were as follows: HR, 770 counts/min, $10^9$ PFU; tsW16B, 1,660 counts/min, 3 $\times$ $10^9$ PFU; tsW28, 780 counts/min, 7 $\times$ $10^9$ PFU; tsW29, 780 counts/min, 3 $\times$ $10^9$ PFU.*

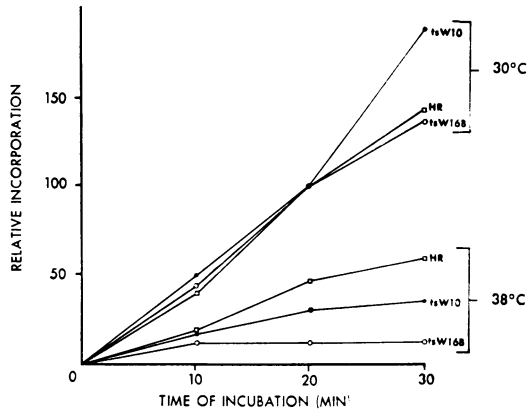


FIG. 2. *RNA polymerase activity of HR, tsW16B (different preparations from those in Fig. 1), and of tsW10. Values of 30 C incorporation and infectious virus were: HR, 6,200 counts/min, $10^{10}$ PFU; tsW10, 1,900 counts/min, 2 $\times$ $10^9$ PFU; tsW16B, 1,900 counts/min, $10^9$ PFU.*

The values of TDI appearing in Table 1 were obtained by using virus preparations which had been purified by differential centrifugation only. To determine the effect of further purification on the TDI, stocks of HR and tsW16B were further purified by rate zonal centrifugation

followed by isopycnic centrifugation (see Materials and Methods). A comparison of the incorporation of preparations purified by the different procedures is shown in Table 2 and indicates that the additional purification has little effect on the temperature dependence.

In addition to comparisons of the temperature dependence, the polymerase activity at 30 C for each mutant has been compared with that for HR. Table 3 shows average values for the incorporation in 20 min by using 50-$\mu$liter samples of undiluted virus stocks having titers of approximately $10^{11}$ PFU/ml. It will be noted that those mutants whose polymerase was most ts (tsW11, 14, and 28) showed considerably reduced incorporation even at 30 C. This low incorporation, which is comparable to that of the nonspecific background, tends to increase the uncertainty in the TDI, as seen for tsW11 in Table 1.

**Temperature shift from 30 to 38 C.** Experiments were performed in which the reaction mixture, containing one of the ts mutants or HR, was shifted to 38 C after an initial 10-min incubation period at 30 C. The results from one such experiment, shown in Fig. 3, demonstrate that the polymerase activity of tsW16B or of tsW28 ceases shortly after the temperature is raised to 38 C, while the polymerase activity of HR continues. Additional experiments have confirmed this behavior for these two mutants and have indicated that, for tsW10 and tsW11, like tsW16B and tsW28, the polymerase activity at 38 C is not increased by a preincubation period at 30 C. It would appear, therefore, that the temperature sensitivity of the polymerase activity of tsW10, 11, 16B, and 28 is not due to a defect in a function which must precede polymerase activity, since with such a defect one would expect the incorporation to be enhanced by prior incubation at 30 C.

TABLE 1. *Temperature dependence of in vitro polymerase activity*

| Mutant | Plaque ratio[a] | TDI[b] | No. of experiments |
|---|---|---|---|
| HR | 1 | 60 ± 6% | 40 |
| tsW29 | $5 \times 10^{-4}$ | 52 ± 21% | 6 |
| tsW10 | $1 \times 10^{-2}$ | 34 ± 3% | 8 |
| tsW16B | $1 \times 10^{-3}$ | 13 ± 2% | 36 |
| tsW11 | $1 \times 10^{-3}$ | 8 ± 11% | 16 |
| tsW28 | $1 \times 10^{-3}$ | 5 ± 2% | 8 |
| tsW14 | $1 \times 10^{-3}$ | —[c] | 1 |

[a] Ratio of number of plaques formed in 24 hr at 38 C to that formed in 48 hr at 30 C.

[b] TDI = (incorporation in 20 min at 38 C)/(incorporation in 20 min at 30 C) × 100%. Intervals give 95% confidence limits.

[c] Insufficient data to determine (see text).

TABLE 2. *Effect of purification on polymerase activity*

| Virus | Method of purification[a] | Net counts/min (30 C) | Net counts/min (38 C) | TDI (%) |
|---|---|---|---|---|
| HR | A | 2,490 | 1,970 | 79 |
| | A + B + C | 1,160 | 720 | 62 |
| 16WB | A | 2,830 | 340 | 12 |
| | A + B + C | 790 | 80 | 10 |

[a] A, Differential centrifugation; B, rate-zonal centrifugation; C, isopycnic centrifugation.

TABLE 3. *Incorporation of GTP at 30 C*

| Virus | No. of experiments | Acid-insoluble $^3$H activity (counts/min)[a] |
|---|---|---|
| HR | 20 | 4,300 ± 1,300 |
| tsW29 | 5 | 1,800 ± 600 |
| tsW10 | 9 | 4,800 ± 1,200 |
| tsW16B | 9 | 1,700 ± 450 |
| tsW11 | 12 | 70 ± 40 |
| tsW28 | 5 | 730 ± 120 |

[a] Acid-insoluble activity after 20 min of incubation at 30 C. Reaction mixtures contained $5 \times 10^9$ PFU of virus and 1 $\mu$Ci of $^3$H-GTP (9 Ci/mmole). Intervals indicate standard deviations.

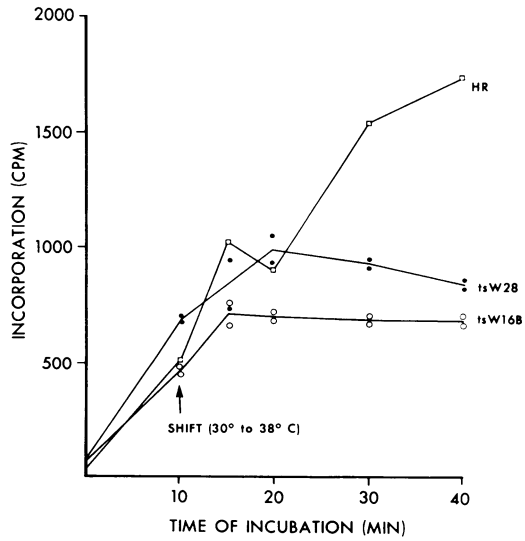


FIG. 3. *Effect of temperature shift-up on polymerase activity of HR, tsW16B, and tsW28. Reaction mixtures were incubated for 10 min at 30 C and then shifted to 38 C. Number of infectious virus in each reaction mixture was as follows: HR, $7 \times 10^8$ PFU; tsW16B, $5 \times 10^8$ PFU; tsW28, $5 \times 10^9$ PFU.*

**Enhanced in vitro incorporation in mixtures of ts mutants.** Since some of our mutants can complement each other at the nonpermissive temperature in mixed infections to yield infectious progeny, pairs of mutants were combined in vitro to determine whether the polymerase activity would be enhanced to an extent greater than was expected for the sum of the polymerase activities of each of the two mutants separately.

The results of one such experiment are illustrated in Fig. 4 and show that the polymerase activity of tsW11 and tsW16B when incubated together at 38 C is considerably enhanced over the polymerase activity of either mutant alone.

The results of similar experiments, using these and other pairs of mutants, are shown in Table 4. The results given in the body of the table are for single experiments. The figure in the right-hand column shows the number of experiments performed for each pair, all of which gave consistent results. At 38 C, tsW16B, when mixed with tsW11, tsW14, or tsW28, shows enhanced polymerase activity, whereas mixtures of any two of tsW11, 14, and 28 do not. This enhanced polymerase activity of certain pairs of mutants may be described as an in vitro complementation. Mutant tsW10 also appears to complement the polymerase activities of tsW11 and tsW28, whereas no evidence of such in vitro complementation can be found in mixtures of tsW10 and tsW16B. For those pairs of mutants which show complementation at 38 C, some enhancement of incorporation is also seen at 30 C. This suggests, as do the results shown in Table 3, that the polymerase activity of some of these mutants is defective even at 30 C. Mixtures consisting of tsW11 × tsW14 or tsW11 × tsW28 also show indication of complementation at 30 C, although none at 38 C. Increased incorporation by virus mixtures does not appear to be due to the increase in the virus concentration since an increase from 50 to 100 μliters per mixture for any one virus gave somewhat less than a twofold increase in incorporation. The increased incorporation at 30 C may represent the mutual alleviation of

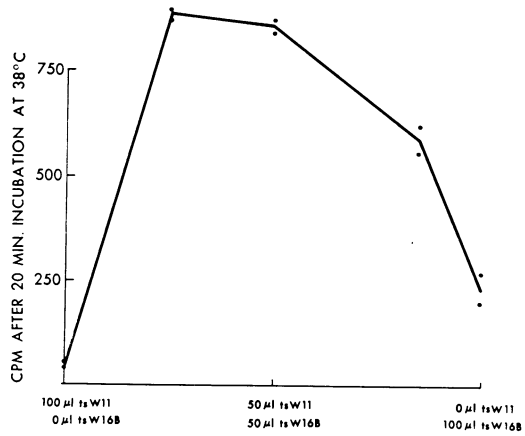


FIG. 4. *RNA polymerase activity in mixtures of tsW11 and tsW16B. The tsW11 stock had 5 × $10^{10}$ PFU/ml, and the tsW16B stock had $10^{10}$ PFU/ml. The total volume of virus stock in each mixture was 100 μliters.*

TABLE 4. *In vitro polymerase activities of mixtures of mutants*

| Mutant (tsW___) | | Incorporation at 30 C[a] (counts/min) | | | Incorporation at 38 C[a] (counts/min) | | | No. of experiments[b] |
|---|---|---|---|---|---|---|---|---|
| A | B | A[c] | B[c] | A + B[d] | A[c] | B[c] | A + B[d] | |
| 10 | 16B | 4,320 | 2,000 | 4,820 | 1,230 | 230 | 1,270 | 4 (all −) |
| 10 | 11 | 4,320 | 70 | 4,680 | 1,230 | 20 | 1,710 | 3 (all +) |
| 10 | 28 | 4,320 | 460 | 4,900 | 1,230 | 50 | 2,070 | 2 (both +) |
| 16B | 11 | 2,000 | 70 | 3,140 | 230 | 20 | 1,200 | 8 (all +) |
| 16B* | 11 | 1,310 | 120 | 2,030 | 230 | 40 | 930 | |
| 16B* | 14* | 1,310 | 120 | 2,380 | 230 | 90 | 1,150 | 1 (+) |
| 16B | 28 | 2,000 | 460 | 3,560 | 230 | 50 | 1,300 | 4 (all +) |
| 16B* | 28* | 1,310 | 600 | 2,380 | 230 | 40 | 1,160 | |
| 11* | 14* | 120 | 120 | 260 | 40 | 90 | 20 | 1 (−) |
| 11 | 28 | 70 | 460 | 490 | 20 | 50 | 40 | 5 (all −) |
| 11* | 28* | 120 | 600 | 1,050 | 40 | 40 | 40 | |

[a] Acid-insoluble $^3$H-GTP after 20 min of incubation.

[b] Number of experiments in which the given pair of mutants was tested. A plus sign indicates an observed in vitro complementation whereas a minus sign indicates no evidence of complementation.

[c] Reaction mixtures (300 μliters) contained 50 μliters of a suspension of either A or B. The results shown are from two experiments. The suspensions tested in one experiment are marked with an asterisk whereas those used in the other experiments are unmarked.

[d] Reaction mixtures (300 μliters) contained 50 μliters of A and 50 μliters of B.

defects which may or may not be related to the ts defect in viral replication.

**Temperature dependence of virion-associated polymerase of ts+ revertants.** Wild-type appearing plaques were picked from 38 C assay plates of tsW11 and tsW16B and were used to grow stocks of virus. These stocks were assayed at 30 and at 38 C and, if found to be ts+, were tested for in vitro polymerase activity. The results for one revertant of tsW11 and five revertants of tsW16B are shown in Table 5 together with data for the tsW11, tsW16B, and for the heat-resistant parental virus, HR. The results of two such experiments are shown in Table 5. In one, revertant 11R1 was compared with tsW11. In the second revertants 16R5, 16R6, 16R7, 16R11, and 16R14 are compared with tsW16B. Data for wild-type virus, HR, are shown for comparison. It will be noted that each of these revertants appears less ts than the mutant from which it was derived. An additional 13 revertants of tsW16B were also tested for polymerase activity, and, although the results are less well-established than those shown in Table 5, the polymerase activities of all 13 appeared less ts than that of tsW16B. Thus, the revertant studies suggest that the ts nature of the in vitro polymerase activity of tsW11 and ts16B is responsible for the inability of these viruses to replicate at 38 C.

## DISCUSSION

Of the six ts mutants of VSV whose virion-associated polymerase activity has been examined, all but one, tsW29, appear to have a temperature dependence significantly different from that of the HR parent virus. Unlike the other mutants, tsW29 has been found to be RNA+ in infected cells, that is, it stimulates the incorporation of labeled uridine in actinomycin D-treated cells, even at 38 C, the nonpermissive temperature, and at low multiplicites (P. K. Y. Wong, A. F. Holloway, D. V. Cormack, Virology, *in press*).

The virion-associated polymerase of each of the other five mutants, tsW10, 11, 14, 16B, and 28, appears to be more ts than that of the HR parent. Temperature shift-up experiments (Fig. 3) indicate that this increased temperature sensitivity is in the polymerase itself rather than in some preceding function.

Furthermore, the in vitro complementation of the structural RNA polymerase activity in mixtures of certain pairs of mutants (Table 4) suggests that the virion-associated polymerase system of VSV consists of at least two components and that there can be an exchange of components among detergent-treated virions. According to this model, when two mutants which have defects in different components of the polymerase system are mixed together, complementation occurs. However, when two mutants have a defect in the same component, no complementation of polymerase activity is detected. Hence, if this model is correct, tsW11, tsW14, and tsW28 have a defect in a different component of the transcriptase system than do tsW10 and tsW16B.

These five mutants also have been tested for their ability to complement each other in vivo at the nonpermissive temperature in L cells to produce infectious progeny (reference 7; P. K. Y. Wong, A. F. Holloway, and D. V. Cormack, Virology, *in press*). It was found that those pairs of mutants which complement each other in the in vitro polymerase test also complement each

TABLE 5. *Polymerase activity of mutants tsW11 and tsW16B and of their revertants 11R1, 16R5, 16R6 16R7, 16R11, and 16R12*

| Virus | Plaque[a] ratio (38 C/30 C) | PFU in reaction mixture | $^3$H-GTP incorporation[b] | | |
|---|---|---|---|---|---|
| | | | 30 C | 38 C | TDI[c] (%) |
| HR | 1 | $1 \times 10^9$ | 3,270 | 2,320 | 70 |
| tsW11 | $10^{-3}$ | $1 \times 10^9$ | 125 | 13 | 10 |
| 11R1 | 1 | $2 \times 10^9$ | 1,420 | 1,020 | 72 |
| tsW16B | $5 \times 10^{-4}$ | $4 \times 10^9$ | 620 | 70 | 11 |
| 16R5 | 0.8 | $1 \times 10^{10}$ | 1,360 | 430 | 30 |
| 16R6 | 0.6 | $1 \times 10^{10}$ | 1,590 | 480 | 30 |
| 16R7 | 1 | $1.2 \times 10^{10}$ | 2,240 | 620 | 28 |
| 16R11 | 0.9 | $1.5 \times 10^{10}$ | 2,760 | 820 | 30 |
| 16R14 | 1 | $2 \times 10^{10}$ | 3,200 | 2,750 | 87 |

[a] Ratio of number of plaques formed in 24 hr at 38 C to that formed in 48 hr at 30 C.
[b] Net acid-insoluble $^3$H activity in counts per minute after 20 min of incubation.
[c] Temperature dependence index: ratio of 38 C incorporation to 30 C incorporation.

other in vivo to produce virus at the nonpermissive temperature, whereas those mutants which fail to complement each other's polymerase in vitro at 38 C also fail to complement each other in vivo. This correspondence of the in vitro complementation of structural polymerase activities and the in vivo complementation of infectivity suggests that the ts polymerase activity of these mutants observed in vitro reflects the reason for the inability of these mutants to replicate in L cells at the nonpermissive temperature. The observation that the polymerase activity of all the revertants of tsW16B and tsW11 have a decreased temperature sensitivity adds further support to this idea. Hence, the indications are that, in tsW11, tsW14, tsW28, tsW10, and tsW16B but not in tsW29, there is a defect in the structural polymerase system which prevents the replication of these mutants in L cells at the nonpermissive temperature. To confirm these indications, studies are in progress of the RNA polymerase products made by the mutants in infected L cells at the permissive and nonpermissive temperature.

A collaborative study of the in vivo complementation between our ts mutants and those of Pringle (5) indicates that mutants tsW11, 14, and 28 belong to complementation group I; tsW10 and 16B to group IV; and tsW29 to group III. These homologies and the RNA synthesis by the Glasgow mutants (6) are consistent with the above interpretation of the results of the in vitro experiments.

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