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# Virion Transcriptase Activity Differences in Host Range Mutants of Vesicular Stomatitis Virus

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Three types of conditional lethal mutant were isolated from wild-type vesicular stomatitis virus, New Jersey serotype, after mutagenization by 5-fluorouracil: (i) conventional temperature-sensitive (*ts*) mutants, which form plaques at 31 C but not at 39 C; (ii) conventional host range mutants (*hr* CE), which grow in BHK but not in secondary chicken embryo cells; and (iii) temperature-dependent host range mutants (*td* CE), which form plaques both at 31 and 39 C on BHK cells but only at 31 C on chicken embryo cells. To determine whether the mutation in *hr* CE and *td* CE mutants affected the virion-associated RNA transcriptase, this enzyme was assayed in vitro at 31 and 39 C, and the results were compared with those obtained for the wild-type virus. The RNA transcriptase activity of *hr* CE mutants did not appear to be affected by the mutation. The *td* CE mutants fall into two classes: those that synthesised RNA at 39 C similar to the wild-type virus and those that did not. One mutant of the latter category, *td* CE 3, had heat-sensitive transcriptase regardless of whether it was grown in BHK or chicken embryo cells. A revertant to the wild-type phenotype isolated from this mutant had regained the ability to synthesize RNA at 39 C. These results strongly suggest that a polypeptide that is either the transcriptase itself or part of the transcriptase complex was made temperature sensitive by the mutation in the second class of *td* CE mutants. The inhibition of the transcriptase activity of the mutant *td* CE 3 was fully reversible by lowering the temperature of incubation from 39 to 31 C, and both inhibition and reactivation appeared to be instantaneous.

Vesicular stomatitis virus (VSV), the prototype of the rhabdovirus group, is an enveloped RNA virus which contains five major structural proteins (12). A glycoprotein (G) and a matrix protein (M) make up the outer coat of the virion, and the L, N, and NS proteins are components of the ribonucleoprotein core. The virion contains RNA transcriptase activity presumed to be responsible for the synthesis of mRNA in infected cells (1). The virion can be degraded to a transcribing ribonucleoprotein complex (TNP) which is still infectious but contains only the L, N, and NS polypeptides (11). It is now considered that polypeptide L is either the transcriptase or at least an essential component of the transcriptase complex (2).

Temperature-sensitive (*ts*) mutants of VSV Indiana fall into six complementation groups (5, 8), and one of these groups is now considered to represent the cistron coding for the transcriptase L protein (3, 6). Comparison of representative mutants from five groups showed certain mutants in group I to be irreversibly inactivated by incubation at 39 C. The thermolability of these mutants was considered to be a direct consequence of heat inactivation of the transcriptase (10).

In this paper we examine the effect of mutation on the virion-associated RNA transcriptase of conventional host range and temperature-dependent host range mutants of VSV New Jersey.

The development of the mutant *td* CE 3 is considered to be restricted in chicken embryo (CE) cells at 39 C, because mRNA synthesis is inhibited; in addition, the heat sensitivity of the RNA transcriptase of *td* CE 3 in BHK cells at 39 C is believed to be compensated by a cell factor present in BHK cells. However, the presence of such a factor in cell extracts so far could not be detected.

## MATERIALS AND METHODS

**Cell culture. (i) CE cells.** Primary CE cells were prepared weekly from leucosis-free eggs supplied by the Poultry Research Centre, Edinburgh, from a flock

of Thornber 909 Brown Leghorn fowl. The cells were used for virus assay and propagation as secondary monolayers. Infectivity assays were carried out under agar overlay, and assay plates were incubated for 48 h at 39 C or 72 h at 31 C.

**(ii) BHK-21 clone 13 cells.** Cells were routinely supplied by the Institute's cytology unit. Infectivity assay plates were incubated for 48 h at both 31 and 39 C.

**(iii) Mixed-cell indicator plates.** Mixed-cell plates were used only in the mutant screening program. Plastic petri dishes (60 mm; Nunclon) were seeded with $2 \times 10^6$ secondary CE cells and $2 \times 10^6$ BHK-21 cells. They were confluent and ready for use after 24 h at 37 C.

**Isolation of td CE and hr CE mutants.** The isolation of *td* CE and *hr* CE mutants from a cloned, wild-type strain of VSV New Jersey will be described separately. The origin of the mutants described here is as follows.

Mutant *td* CE 1 was isolated from wild-type virus grown in BHK cells in the presence of 50 μg of 5-fluorouracil (Koch-Light Ltd.) per ml. Mutant *td* CE 2 was isolated from the same mutagenized stock plated on mixed indicator plates of BHK and CE cells. Mutant *td* CE 3 was isolated from the wild-type stock mutagenized with 100 μg of 5-fluorouracil per ml and plated on CE cells. Mutant *td* CE 4 was obtained from the wild-type mutagenized with 50 μg of 5-fluorouracil per ml and plated on BHK cells, as were mutants *hr* CE 1 and *hr* CE 2. Mutant *hr* CE 3 was obtained from the wild-type mutagenized with 200 μg of 5-fluorouracil per ml and plated on BHK cells.

Mutant *ts* 103/92 was a normal temperature-sensitive mutant isolated during the same screening program from virus mutagenized with 50 μg of 5-fluorouracil per ml and plated on mixed indicator BHK/CE monolayers.

The revertant clone *td* CE/R1 was a spontaneous mutant isolated from *td* CE 3 plated on CE cells at the permissive temperature. One hundred and twenty clones of *td* CE 3 were isolated and screened. One of these clones produced a few plaques on monolayers of CE cells incubated at 39 C. The revertant clone *td* CE/R1 was established from one of these plaques.

**Growth and purification of virus.** Mutants and wild type were propagated in monolayer cultures of secondary CE fibroblasts grown in 2-liter "burrler" bottles. Virus was inoculated at low multiplicity (~0.01 PFU/cell) and allowed to adsorb for 30 min at 31 C. The infected monolayer was then incubated in 50 ml of Eagle medium (Glasgow modification) containing 2% calf serum until the cytopathic effect was well advanced (about 48 h after inoculation). The medium was decanted and clarified by centrifugation at 2,000 rpm for 5 min in an MSE Mistral centrifuge at +4 C. The clarified supernatant was centrifuged at 18,000 rpm for 1.5 to 2 h in the rotor (8 by 50 ml) of an MSE high speed 18 centrifuge. The tubes were carefully drained, and the virus-containing pellet was soaked in 0.25 ml of 20 mM Tris-hydrochloride (pH 8.0) buffer containing 1 mM EDTA (Analar) for 16 to 24 h at +4 C. The pellets were suspended with a narrow-bore pipette, and 0.5 ml was layered onto a 5-ml preformed gradient of 15 to 40% sucrose in Tris-EDTA buffer. The gradients were centrifuged at 30,000 rpm in a Beckman SW50.1 rotor for 50 min at +4 C. The visible virus zone in the gradient was removed by side-puncture of the tube. This purified virus was concentrated by sedimentation in a Beckman SW50.1 rotor at 40,000 rpm for 30 min. The tubes were drained, and the virus pellet was suspended immediately in 0.1 ml of Tris buffer.

Purified virus from 4 to 10 burrler bottles was used immediately for enzyme assay or stored at −70 C.

**"TC preparation."** The TC preparation of wild-type virus and mutants was prepared as described by Szilágyi and Pringle (10). Suspension of purified virus containing approximately 0.5 mg of protein was diluted with 20 mM Tris-hydrochloride buffer (pH 8.0) to 1.8 ml, and then 0.1 ml of 70 mM dithiothreitol and 0.1 ml of 0.8% Triton-N101 were added. After standing for 10 min at 0 C, the disrupted virus particles were centrifuged for 1 h at 50,000 rpm at 2 C, with a Beckman SW50.1 rotor, through a 5 to 25% sucrose gradient made up in 20 mM Tris-hydrochloride buffer (pH 8.0) against an 80% sucrose cushion. The resulting virus material (TC preparation) was withdrawn by side puncture (approximately 0.5 ml), and aliquots of this were used for the assay of the RNA transcriptase.

**"TNP preparation."** The TNP preparation of wild-type virus and mutants was prepared by the method of Szilágyi and Uryvayev (11) with minor modifications. Purified virus was diluted with 20 mM Tris-hydrochloride buffer (pH 8.0) to obtain a suspension of exactly 1 mg of protein per ml. To 10 ml of this suspension were added: 0.75 ml of glycerol, 0.5 ml of 0.2 M Tris-hydrochloride buffer (pH 8.0), 0.75 ml of 0.1 M EDTA, 0.75 ml of 70 mM dithiothreitol, 0.75 ml of 2% Triton-N101, and finally 1.5 ml of 5 M CsCl in small aliquots during rapid mixing when a marked drop in the turbidity of the preparation was observed. After standing for 20 min at 0 C, it was diluted with 7.5 ml of 20 mM Tris-hydrochloride buffer (pH 8.0) that contained 3.5 mM dithiothreitol. Equal volumes of this suspension were placed on two 30 to 50% glycerol gradients (made up in 20 mM Tris-hydrochloride buffer [pH 8.0], 1 mM EDTA, 3.5 mM dithiothreitol, and 0.1 M NaCl) and centrifuged for 4 h at 50,000 rpm at 2 C with a Ti 35 rotor in an MSE superspeed 65 centrifuge. After centrifugation, the liquid layers were discarded, the surface of the centrifuge tubes and the pellets were rinsed with 20 mM Tris-hydrochloride buffer (pH 8.0), and finally each pellet was gently suspended in 2.5 ml of 20 mM Tris-hydrochloride buffer (pH 8.0). After these suspensions were combined (5 ml), 2.5 ml of glycerol was added and this TNP preparation was stored at −70 C.

**Assay of RNA transcriptase.** The assay conditions for RNA transcriptase were the same as those described for VSV (1, 10, 11) with the following modifications. The 0.2-ml reaction mixtures consisted of (in order of addition) TC or TNP preparations, 20 mM Tris buffer (pH 8.0) to make the final volume up to 0.2 ml, 0.01 ml of 70 mM dithiothreitol, and finally 0.05 ml of "reagent mixture" which contained 400 mM Tris-hydrochloride buffer (pH 8.0), 400 mM

NaCl, 3.2 μg of actinomycin D, 2.56 mM ATP, 2.56 mM CTP, 2.56 mM GTP, 256 μM cold UTP, and 12 μCi of [5,6-$^3$H]uridine 5'-triphosphate (Radiochemical Centre, Amersham; specific activity 49 Ci/mmol; in 50% ethanol which was evaporated before use). After mixing, a 0.02-ml zero-time sample was taken, the suspension was brought up to incubation temperature (31 or 39 C) for 1 min (unless stated otherwise), and the reaction was started by the addition of 0.005 ml of 220 mM $MgCl_2$ during rapid mixing. If the reaction mixture was 0.4 ml, the amount of every ingredient was doubled and the reaction was started with 0.005 ml of 440 mM $MgCl_2$. During the incubation, 0.02-ml samples were taken and placed on Whatman no. 1 filter paper disks.The disks were dried and washed seven times with 5% trichloroacetic acid containing 0.04 M sodium pyrophosphate, three times in ethanol, and twice in diethyl ether. After drying, toluene-based scintillant was added and radioactivity was counted in an Intertechnique SL 30 liquid scintillation spectrometer. The washed, zero-time samples (0.02 ml) gave approximately 35 counts/min, and the unwashed samples (0.02 ml) gave approximately 150,000 counts/min.

**Preparation of cell extracts from BHK and CE cells.** Approximately 8 × $10^7$ BHK-21 C13 and secondary CE cells were suspended in 4 ml of 20 mM Tris-hydrochloride buffer (pH 8.0) and homogenized, with a Teflon homogenizer, by hand (20 strokes). The cell debris was removed by centrifugation at 2,500 rpm for 10 min, and the supernatant was used as the cell extract.

**Determination of protein.** Protein was determined by the method of Lowry et al. (4), with bovine serum albumin as the standard.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.** Sodium dodecyl sulfate-discontinuous gradient 5 to 15% polyacrylamide slab gels were prepared by the method of Marsden and Subak-Sharpe (manuscript in preparation) with the modifications of McCrae and Szilágyi (manuscript in preparation). Samples containing 6 to 12 μg of protein were prepared by boiling in a 2% sodium dodecyl sulfate–5% 2-mercaptoethanol solution for 2 min. The conditions of electrophoresis were: 30 mA at 4 C until the dye front reached the bottom of the gel. Gels were stained for 2 h in 0.025% Coomassie brilliant blue R-250 in water-methanol-acetic acid (91:91:18.2, vol/vol/vol) and then destained in a solution containing 10% acetic acid and 2.5% ethanol.

## RESULTS

**Conventional and temperature-dependent host range mutants.** Three types of conditional lethal mutant have been isolated from wild-type stocks of VSV Cocal. VSV Indiana, and VSV New Jersey after mutagenization by 5-fluorouracil. The origin and properties of these mutants will be described in detail separately; this paper is concerned only with mutants of VSV New Jersey (Table 1).

Mutants designated *ts* are conventional temperature-sensitive mutants, which are unable to form plaques at 39 C on either monolayers of BHK cells or secondary CE cells. These mutants are analogous to those isolated previously (7) and have not been characterized further.

Mutants designated *td* CE are temperature-dependent host range mutants, i.e., they form plaques on BHK cells at 31 and 39 C, and on secondary CE monolayers only at 31 C. Such mutants occurred comparatively frequently, although this phenotype has not been described previously. Attempts to place these mutants in complementation groups have not been successful so far, and their relationship to the six established groups of VSV New Jersey *ts* mu-

TABLE 1. *Mutants of VSV New Jersey*

| Mutant | PFU on BHK cells | | | PFU on CE cells | | |
|---|---|---|---|---|---|---|
| | 31 C | 39 C | 39 C/31 C | 31 C | 39 C | 39 C/31 C |
| *ts*$^+$ [a] | $6.9 \times 10^7$ | $8.7 \times 10^7$ | 1.26 | $4.1 \times 10^7$ | $9.8 \times 10^6$ | 0.24 |
| *ts* (103/92) | $3.2 \times 10^7$ | $<10^3$ | 0.0003 | $1.1 \times 10^7$ | $<10^3$ | <0.001 |
| *td* CE 1 | $2.0 \times 10^7$ | $10^7$ | 0.50 | $9.0 \times 10^6$ | $<10^2$ | <0.0001 |
| *td* CE 2 | $1.5 \times 10^8$ | $2.0 \times 10^7$ | 0.13 | $2.0 \times 10^7$ | $<10^1$ | <0.00005 |
| *td* CE 3 | $7.2 \times 10^7$ | $4.0 \times 10^7$ | 0.56 | $9.0 \times 10^6$ | $<10^2$ | <0.0001 |
| *td* CE 4 | $2.3 \times 10^8$ | $10^7$ | 0.04 | $9.0 \times 10^6$ | $<10^1$ | <0.00001 |
| *hr* CE 1 | $1.9 \times 10^8$ | $2.0 \times 10^8$ | 1.05 | $<10^3$ | $<10^1$ | |
| *hr* CE 2 | $2.7 \times 10^8$ | $3.0 \times 10^8$ | 1.11 | $<10^3$ | $<10^1$ | |
| *hr* CE 3 | $6.0 \times 10^7$ | $10^6$ | 0.17 | $<10^3$ | $<10^1$ | |
| *td* CE/R1 | $5.0 \times 10^7$ | $4.4 \times 10^7$ | 0.88 | $1.5 \times 10^8$ | $3.0 \times 10^7$ [b] | 0.20 |

[a] Mean of three determinations.
[b] Minute plaques.

tants is unknown. However, the mutants are not identical, since various differences can be discerned among individual mutants. Two pairs of mutants have been used in this study, representing distinguishable classes on the basis of temperature shift experiments. Mutants *td* CE 1 and *td* CE 2 represent one class; *td* CE 3 and *td* CE 4 represent the other.

Mutants designated *hr* CE are conventional host range mutants, which are able to form plaques in BHK cells at both 31 and 39 C, but not in CE cells at either temperature. Mutants of this type occurred infrequently, and only three (*hr* CE 1, *hr* CE 2, *hr* CE 3) have been isolated. They were not complemented in CE cells by either *ts* or *td* CE mutants.

Mutant *td* CE/R1 is a revertant clone isolated from a stock of mutant *td* CE 3. The temperature-sensitive phenotype has been lost, but *td* CE/R1 is distinguishable from wild-type virus by the morphology of the plaques at 39 C.

The mutant screening procedure would have detected clones with the reciprocal phenotypes, i.e., mutants unable to replicate in BHK cells (*hr* BHK) and mutants conditionally temperature sensitive in BHK cells (*td* BHK). No mutants of these types have been isolated, and BHK cells appear to be generally permissive.

The *hr* CE mutants may be similar to the single *hr* mutant described by Simpson and Obijeski (9), but the *td* CE mutants are different from their *hr/ts* mutants because they were temperature sensitive in the permissive cells (CE or BHK) but did not grow at either temperature in the restricted cells (HeLa or HEp-2). The ability of the *hr* CE and *td* CE mutants to form plaques in other types of cells is being studied.

The *hr* CE and *td* CE mutants were specifically neutralized by VSV New Jersey antiserum.

**In vitro RNA synthesis by hr CE and td CE mutants.** The enzyme activity was assayed at 31 and 39 C by using "TC preparations" of the *hr* CE and *td* CE mutants to determine whether virion-associated RNA transcriptase activity is affected by the mutation. The results were compared with those obtained for the wild-type virus (Fig. 1).

Wild-type VSV New Jersey synthesized RNA linearly at 31 C for about 60 min, and RNA synthesis continued during the entire 3 h of the assay. The initial rate of RNA synthesis at 39 C was approximately half of the initial rate at 31 C, and the duration of the RNA synthesis was shorter, reaching a plateau after 60 to 90 min. The amount of RNA synthesized at 39 C during the 3 h of incubation was approximately 20 to 25% of that synthesized at 31 C.

Mutant *hr* CE 1 synthesized RNA both at 31

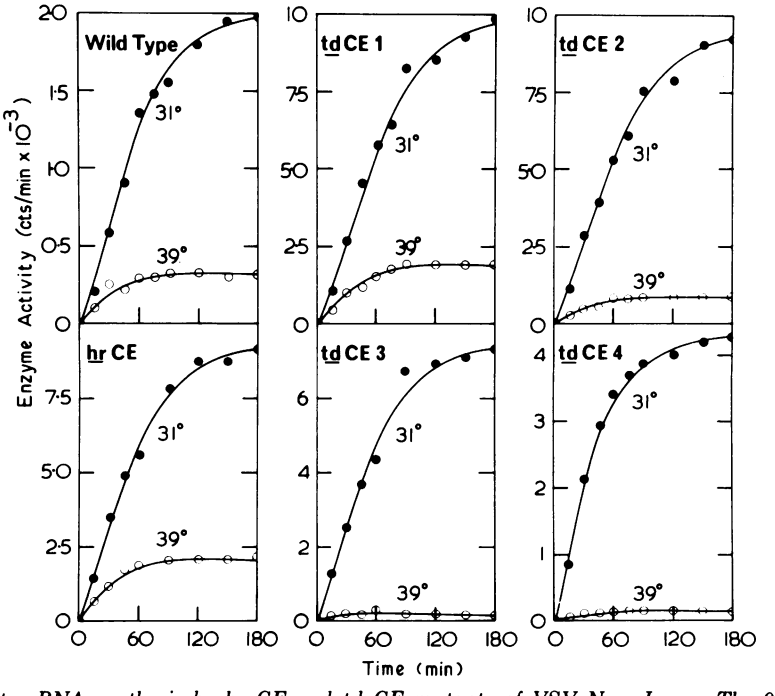


FIG. 1. *In vitro RNA synthesis by hr CE and td CE mutants of VSV New Jersey. The 0.2-ml reaction mixtures contained 0.025 ml of TC preparations.*

and 39 C, similar to the wild-type virus, indicating that the RNA transcriptase of this mutant did not become heat sensitive by the mutation.

Of the four temperature-dependent host range mutants examined, two (*td* CE 1 and *td* CE 2) synthesized RNA at both temperatures. Although the other two mutants (*td* CE 3 and *td* CE 4) synthesized RNA normally at 31 C, only slight RNA synthesis was observed at 39 C. The total RNA synthesized by mutants *td* CE 3 and *td* CE 4 amounted to not more than 2 to 3% of that synthesized at 31 C during the 3 h of incubation. Thus, the mutation appears to affect the in vitro RNA transcriptase activity of some, but not all, of the *td* CE mutants.

The polypeptides of *td* CE mutants, as well as those of wild-type VSV New Jersey, were analyzed by electrophoresis with a discontinuous sodium dodecyl sulfate-5 to 15% polyacrylamide gradient gel. The wild-type virus contained the well-established polypeptides L, G, N, NS, and M (12), and no difference in the electrophoretic mobility of the polypeptides of the wild-type virus and the *td* CE mutants was observed.

**In vitro RNA synthesis by the mutant td CE 3 grown in BHK and CE cells.** Mutant *td* CE 3 and the wild-type virus were grown in both BHK and CE cells to establish whether RNA transcriptase activity was affected by the cells in which the virus had been propagated (Fig. 2).

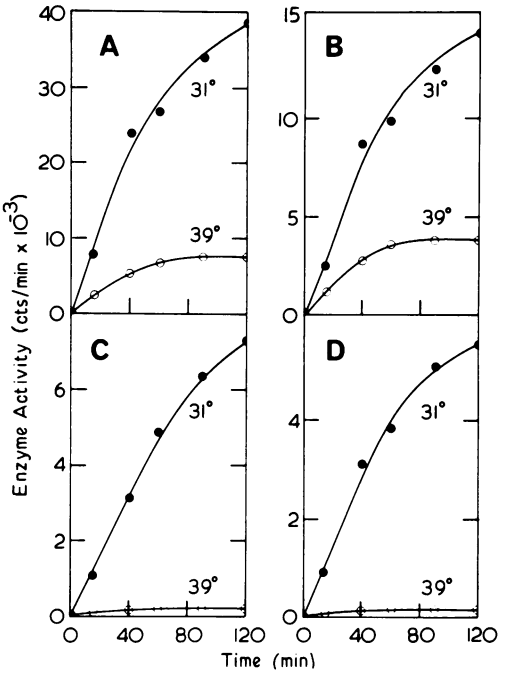


FIG. 2. *In vitro RNA synthesis by wild-type VSV New Jersey and the mutant td CE 3 grown in BHK and CE cells. The 0.2-ml reaction mixtures contained 0.025 ml of TC preparations. A, Wild-type VSV grown in BHK cells; B, wild-type VSV grown in CE cells; C, td CE 3 grown in BHK cells; D, td CE 3 grown in CE cells.*

Irrespective of whether the wild-type virus was grown in BHK or CE cells, the initial rate of in vitro RNA synthesis at 39 C was always about half that at 31 C. At 39 C synthesis reached a plateau by about 60 to 80 min, and the RNA synthesized by 120 min was only 20 to 27% of the 31 C value (Fig. 2A and B). The rate of RNA synthesis with mutant *td* CE 3 was also independent of the cell of origin, but total RNA synthesis at 39 C in both cases amounted to only 2 to 3% of that at 31 C (Fig. 2C and D). Thus, there is no evidence that the type of cell in which the *td* CE 3 had been propagated itself influences the in vitro RNA synthesis.

**Effect of reversion of temperature-sensitive phenotype on in vitro RNA transcriptase activity.** The revertant clone *td* CE/R1 was isolated from a stock of mutant *td* CE 3 as described above. This clone formed plaques on CE monolayers at 39 C as efficiently as the wild-type virus (Table 1), but they were much reduced in size compared to wild-type or *td* CE plaques. The in vitro RNA transcriptase activities of wild-type VSV, mutant *td* CE 3, and revertant *td* CE/R1 were compared in a single experiment (Fig. 3).

Wild-type virus and mutant *td* CE 3 synthesized RNA in vitro at 39 C with relative efficiencies of 20% and 2 to 3%, respectively, compared with the 31 C result, just as was found previously (Fig. 3A and B); revertant *td* CE/R1 at 39 C produced about 30% of the RNA synthesized at 31 C (Fig. 3C).

Thus, in *td* CE/R1 reversion of the *td* CE 3 phenotype is accompanied by restoration of wild-type enzyme activity. This implies that the original *td* CE 3 mutation involves the virion transcriptase.

**In vitro RNA synthesis by TNP preparation of mutant td CE 3.** A ribonucleoprotein complex (TNP) was prepared from purified virus particles for further investigation of in vitro RNA synthesis by the mutant *td* CE 3. TNP preparation was preferred to TC preparation since it retained only three of the virus polypeptides (L, N, and NS); the two polypeptides of the envelope (G and M) had been removed (Fig. 4). Most RNA transcriptase activity of the virion was also still in the TNP. In the case of wild-type virus and of mutant *td* CE 3, in vitro RNA synthesis by TNP and by TC preparations was similar.

In both cases, RNA synthesis by TNP at 31 C

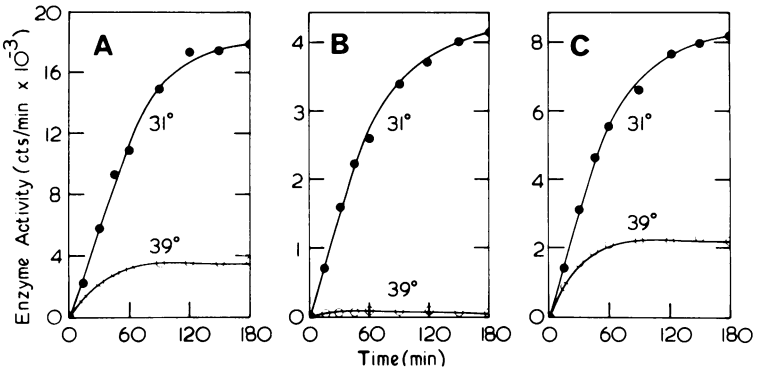


FIG. 3. *In vitro RNA synthesis by the revertant clone td CE/R1. The 0.2-ml reaction mixtures contained 0.025 ml of TC preparations. A, RNA synthesis by wild-type VSV New Jersey; B, RNA synthesis by temperature-dependent host range mutant td CE 3; C, RNA synthesis by revertant clone td CE/R1 isolated from td CE 3.*

was linear for about 60 to 80 min, whereas at 39 C the wild-type virus reached a plateau value after about 40 to 60 min (Fig. 5A and C). Once more, very little RNA was synthesized by the mutant at this temperature, representing not more than 2 to 3% of the amount of RNA it synthesized during 140 min at 31 C.

To observe the effect of preincubation on the transcriptase activity at 39 C, we incubated reaction mixtures containing all the ingredients except $MgCl_2$ at 39 C for various lengths of time before RNA synthesis was started by the addition of $MgCl_2$ (Fig. 5B and D). Increasing the time of preincubation at 39 C resulted in gradual loss of total transcriptase activity at rates similar for the wild-type virus and for the mutant *td* CE 3, although the total RNA synthesized by the wild-type virus was approximately 10 times that synthesized by mutant *td* CE 3. The time of RNA synthesis until the plateau values were reached remained constant at about 60 min irrespective of the preincubation period.

These results show: first, that even prolonged preincubation at 39 C does not inhibit completely the in vitro RNA synthesis by mutant *td* CE 3; second, that at 39 C its transcriptase activity was instantaneously inhibited; and third, that the remaining activity had a normal survival time.

**Effect of temperature shift from 39 to 31 C on the transcriptase activity of mutant td CE 3.** The experiment was designed to determine whether the temperature sensitivity of the *td* CE 3 polymerase was due to inhibition of enzyme function or simply a consequence of irreversible thermal inactivation of the transcriptase as had been observed with some of the group I mutants of VSV Indiana (10). Two series of reaction mixtures were used in the experiment (Fig. 6).

In the first series, the reaction mixtures containing all of the ingredients except $MgCl_2$ were preincubated at 39 C for 40, 70, and 130 min and then transferred to 31 C, and RNA synthesis was initiated by the addition of $MgCl_2$ at the time of the transfer (Fig. 6, broken lines). Comparison with the results of a reaction mixture that was not preincubated (Fig. 6, line and dot) showed that approximately 40% of the transcriptase activity was lost during the first 40 min, 65% during 70 min, and 90% during 130 min of preincubation. Since similar losses were observed with the wild-type virus (not shown), these losses are not peculiar to *td* CE 3.

The second series of reaction mixtures was preincubated at 39 C for 40 min to reduce residual RNA synthesis drastically. $MgCl_2$ was added after preincubation, and two reaction tubes were transferred to 31 C after 30 and 90 min, while a third was incubated at 39 C throughout the experiment (Fig. 6, solid lines). After the temperature of incubation was shifted from 39 to 31 C, RNA synthesis immediately resumed and the amount of RNA synthesized was comparable to the values obtained with the first series.

Thus, the inhibition of the RNA transcriptase activity of mutant *td* CE 3 at 39 C was fully and immediately reversible when the losses of enzyme activity associated with incubation at 39 C were taken into account, showing that inactivation was due to the inhibition of enzyme function and not due to irreversible thermal inactivation.

**Effect of BHK and CE cell extract on the in vitro RNA synthesis by mutant td CE 3.** To assess the role of host factors in the expression

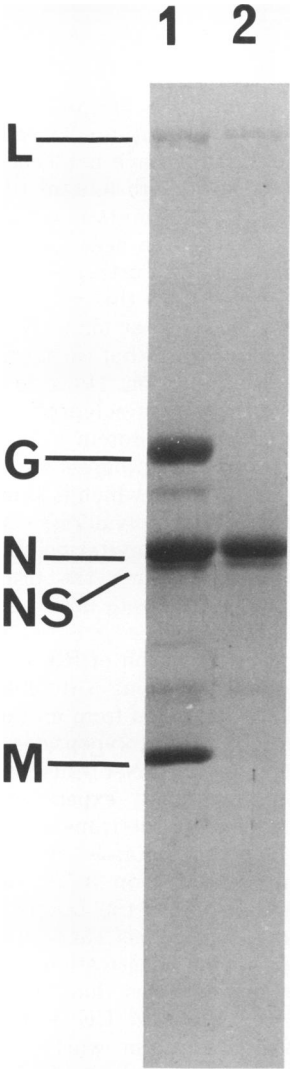


FIG. 4. *SDS-polyacrylamide gel electrophoresis of the polypeptides of the mutant td CE 3 and of its TNP. 1, Virion of td CE 3; 2, TNP of td CE 3. The polypeptides L, G, N, NS, and M are indicated.*

of the *td* CE phenotype, we tested the effects of cell extracts of BHK and CE cells on the in vitro RNA synthesis.

The in vitro RNA synthesis at 31 C by this mutant was increased by about 50% upon addition of BHK extract, and in other experiments the increase was 100 to 200% (Fig. 7). At 39 C also there was observed a very small increase (Fig. 7A and B); however, this increased total was considerably less than the control level of RNA synthesis obtained with the wild-type virus at 39 C.

There was a moderate, but repeatable, increase in the in vitro RNA synthesis at 31 C by the mutant when CE cell extract was added (Fig. 7C), although this increase was always less than that obtained with the BHK cell extract. However, 39 C RNA synthesis was not detectably increased by the CE cell extract (Fig. 7C and D).

Therefore, although BHK and, to a lesser extent, CE extracts enhance in vitro RNA synthesis at 31 C, they have very little effect at 39 C.

## DISCUSSION

The RNA transcriptase activity of the *hr* CE and *td* CE mutants has been compared in vitro with that of the wild-type virus to determine whether mutation affected this enzyme.

Since RNA synthesis by *hr* CE 1 at both temperatures was like that of the wild-type virus, it is unlikely that the host range mutation affects the transcriptase of *hr* CE mutants.

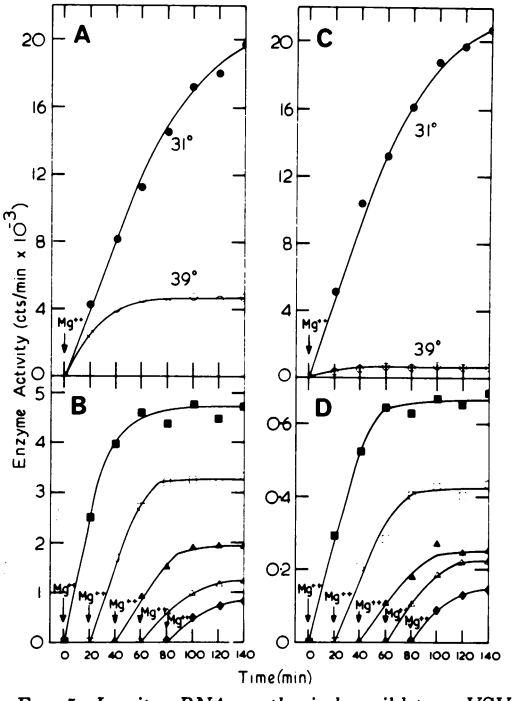


FIG. 5. *In vitro RNA synthesis by wild-type VSV New Jersey and the mutant td CE 3, and the effect of preincubation at 39 C on the transcriptase activity at 39 C. The 0.2-ml reaction mixtures contained either 0.008 ml of TNP of wild-type VSV or 0.02 ml of TNP of td CE 3. A, RNA synthesis by wild-type VSV; C, RNA synthesis by td CE 3, both after 1 min of warming up to incubation temperature (31 or 39 C) before the addition of $MgCl_2$. B, RNA synthesis by wild-type VSV; D, RNA synthesis by td CE 3, both at 39 C after a preincubation at 39 C for 1 min (■), 20 min (□), 40 min (▲), 60 min (△), or 80 min (◆) before the addition of $MgCl_2$.*

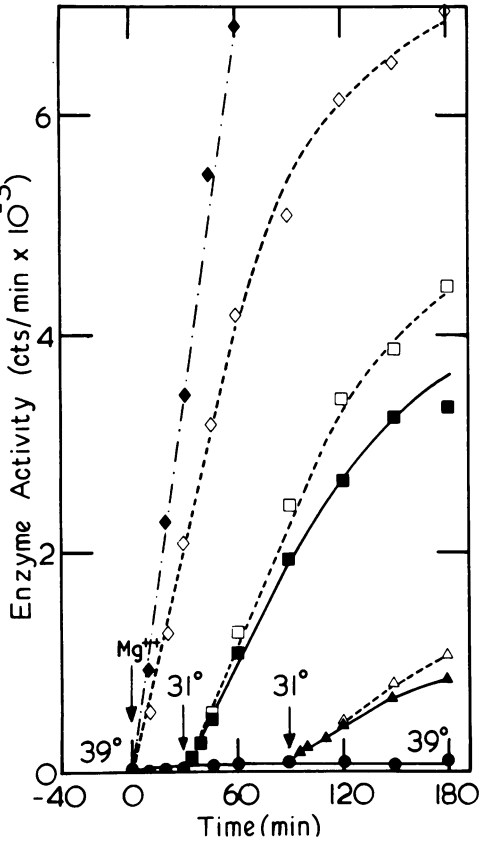


FIG. 6. *Effect of temperature shift from 39 to 31 C on the transcriptase activity of the mutant td CE 3. The 0.4-ml reaction mixtures contained 0.02 ml of TNP of td CE 3. Three reaction mixtures were preincubated at 39 C for 40 min, and then $MgCl_2$ was added. The first one was incubated at 39 C for a further 30 min (■); the second was incubated for a further 90 min (▲) and then down-shifted to 31 C. The third reaction mixture was incubated at 39 C for the entire 180 min of the experiment (●). As controls, identical reaction mixtures were preincubated for 1 min (◆), 40 min (◇), 70 min (□), or 130 min (△) at 39 C, $MgCl_2$ was added, and residual transcriptase activity was assayed at 31 C.*

On the basis of the in vitro RNA synthesis, the temperature-dependent host range mutants appeared to fall into at least two classes. Synthesis at 39 C was similar to that of the wild-type virus in the case of mutants *td* CE 1 and *td* CE 2 but was very strongly inhibited in the case of *td* CE 3 and *td* CE 4. Separation of the four mutants into two classes on the basis of RNA synthesis at 39 C agrees with results obtained from temperature shift experiments (Pringle and Szilágyi, manuscript in preparation). The results strongly suggest that in the *td* CE 3 class mutants the mutation affected one of the polypeptides involved in transcription, whereas some other polypeptide may be affected in the mutants of the *td* CE 1 class. Attempts to complement the *td* CE mutants inter se or to place these *td* CE mutants in existing complementation groups have not been successful so far. It was shown with mutant *td* CE 3 that the cell in which the mutant was propagated has no influence on the heat sensitivity of the transcriptase (Fig. 2). Furthermore, in a revertant clone derived from this mutant, the RNA transcriptase activity at 39 C was restored. These findings confirm that the transcriptase is affected by the mutation. However, we do not know which of the three polypeptides (L, N, or NS) of the ribonucleoprotein is involved, and experiments are in progress to determine whether polypeptide L, which is known to be at least part of the RNA polymerase (2), is the one that is affected by the mutation. Likewise, we are investigating whether the primary transcription at 39 C in CE cells infected with *td* CE 3 is inhibited or not.

The mode of inhibition of RNA synthesis at 39 C was studied by using a ribonucleoprotein complex (TNP) prepared from mutant *td* CE 3, which contained three polypeptides (L, N, and NS) and retained the RNA transcriptase activity. Temperatures shift experiments showed that the inhibition of the transcriptase activity was fully and immediately reversible. This differs from the inhibition at 39 C of the RNA transcriptase of some of the group I mutants of VSV Indiana, which was the consequence of irreversible thermal inactivation (10). The inhibition presumably was due to a configurational change of the *td* CE 3 mutant polypeptide. Experiments in which the reaction mixture was preincubated at 39 C, as well as temperature shift experiments, indicated that both inhibition and reversion of activity were instantaneous.

Simpson and Obijeski (9) have reported variation in the transcriptase activity of their *hr/ts* mutants at the permissive temperature in vitro. How this relates to our findings is unclear at present.

The replication of the mutant *td* CE 3 is restricted in CE cells at 39 C presumably because of the heat sensitivity of the transcriptase, which cannot synthesise mRNA at this temperature. Since the heat sensitivity of the transcriptase of *td* CE 3 appeared to be unaffected by the cell in which the mutant was grown, we postulate that virus development in BHK cells at 39 C is due to the presence of a host factor which, in some way, helps the RNA transcriptase to overcome its heat sensitivity.

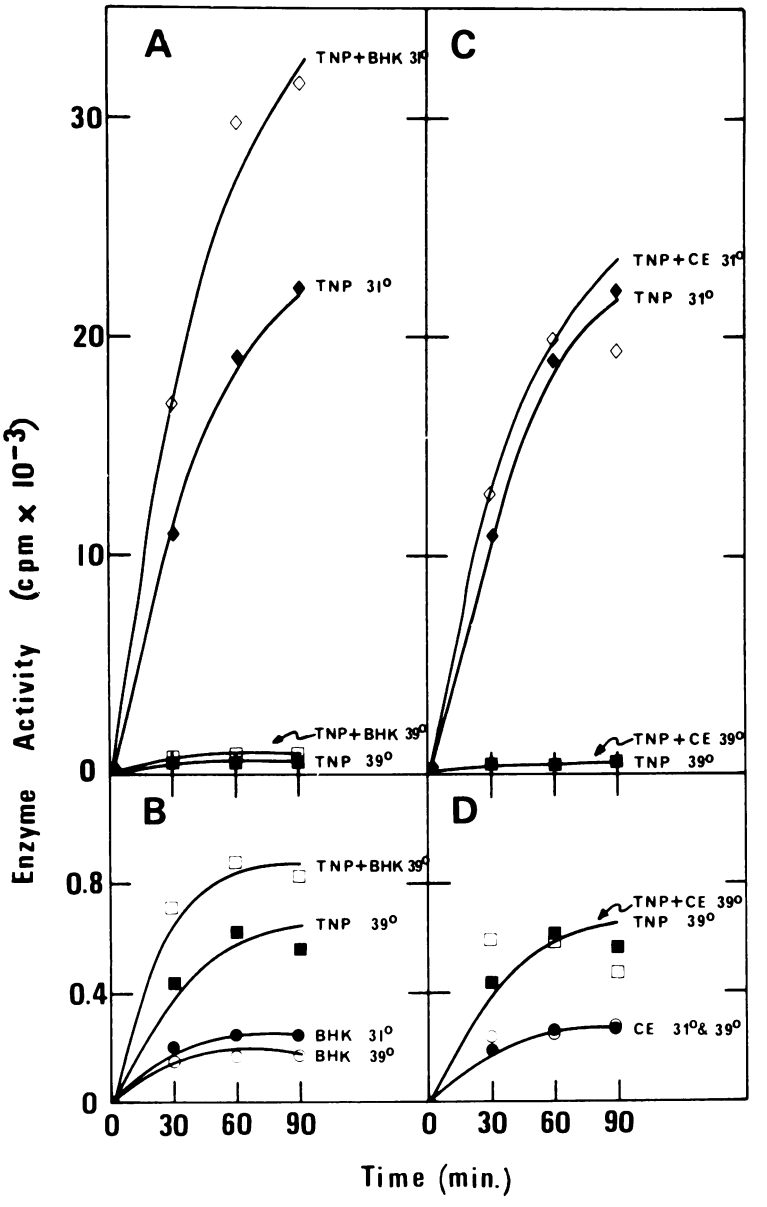


FIG. 7. *Effect of BHK and CE cell extracts on the in vitro RNA synthesis by the mutant td CE 3. The 0.2-ml reaction mixtures contained 0.04 ml of TNP of the mutant td CE 3 and 0.04 ml of cell extracts. A, RNA synthesis at 31 C by TNP alone (◆) and in combination with BHK cell extract (◇), and RNA synthesis at 39 C by TNP alone (■) and in combination with BHK cell extract (□). (B) The same results at 39 C on a different scale and also the background RNA synthesis by BHK cells at 31 C (●) and 39 C (○). (C and D) Results with the CE cell extract instead of the BHK cell extract.*

This host factor is presumably present in BHK cells, but missing from CE cells (or present in lower concentration). If such a factor does exist in BHK cells, it is presumably a cytoplasmic component since the virus can grow in enucleated BS-C-1 cells, which are permissive for both the *td* CE and *hr* CE mutants. We examined the effect of cell extracts of BHK and CE cells on the transcription of mutant *td* CE 3, but the very slight increase in RNA synthesis at 39 C in the presence of BHK cell extract was nowhere near the level of RNA synthesized by the wild-type virus at this temperature. Experiments to try to isolate and identify such a factor are continuing.

If the need for a host factor in the transcrip-

tion by *td* CE 3 proves to be correct, it may be an indication of the involvement of a host factor in the transcription of the wild-type virus.

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