Study of the Transcription and the Replication of Vesicular Stomatitis Virus by Using Temperature-Sensitive Mutants

CHRISTIANE PRINTZ-ANÉ, ARLETTE COMBARD, AND CLAIRE MARTINET
Institut de Microbiologie, Université de Paris-Sud, Centre d’Orsay, 91405-Orsay, France

Received for publication 6 September 1972

The viral ribonucleic acids (RNA species) synthesized in HeLa cells infected with temperature-sensitive (ts) mutants and with the wild type of vesicular stomatitis virus at permissive (30 C) and nonpermissive (39.2 C) temperatures were compared by sucrose gradient centrifugation. Two ts mutants (ts 5 and ts 100) representing two separate complementation groups (respectively, groups I and IV), each concerned with viral RNA synthesis, were chosen. Mutant ts 5 failed to synthesize any RNA at 39.2 C. Under the same conditions, mutant ts 100 showed a low, but easily detectable, synthesis of RNA without characteristic peaks. The in vitro transcriptase activity was tested with mutants ts 5 and ts 100 at 39.2 C: normal activity, compared with wild-type virus, was detected with purified ts 100, but no activity was detected with purified ts 5. From all our data we conclude that mutant ts 5 is defective in transcription. The defect could be in the structural transcriptase enzyme or at the level of template for transcription. Results with mutant ts 100 were not so clear-cut. However, we suggest that this mutant is concerned with some aspect of transcription in vivo. In addition, our results lead us to postulate some linkage between transcription and replication.

Conditional lethal mutants of vesicular stomatitis (VS) virus should provide probes for studying the intracellular events in virus development. The isolation and genetic characterization of temperature-sensitive (ts) mutants, Indiana serotype, have been previously described (7, 8, 11, 23). Whereas these mutants multiply quite well at the permissive temperature of 30 C, their growth is markedly reduced at the restrictive temperature of 39.2 C, which completely supports growth of the parental wild type. Among the five complementation groups described by Flamand (8), two of them appear unable to induce actinomycin-resistant ribonucleic acid (RNA) synthesis at 39.2 C and therefore are called RNA− ts mutants (16).

These two groups of ts mutants, which may be defective in different aspects of RNA synthesis, should provide a good system to investigate the complex events occurring during transcription and replication of VS virus RNA species, as described by several authors (13, 19–21, 26, 28, 29, 33). Genetic data alone are not sufficient to characterize the altered function of each ts mutant; therefore, we have undertaken a biochemical analysis of the RNA species synthesized by each mutant under various conditions.

MATERIALS AND METHODS

Cells and media. HeLa cells (S3) obtained from the Institut Pasteur were used for all the experiments and were grown in monolayer culture in Eagle medium provided by Eurobio (Laboratoires Eurobio, Paris), supplemented with 10% calf serum, 250 units of penicillin per ml, and 0.01 g of streptomycin per ml. Monolayer cultures were obtained by seeding approximately 3 × 10^6 cells in 75-cm² Falcon plastic flasks and incubating them at 37 C for 24 hr prior to use. At this time, the number of cells has roughly doubled, giving a confluent monolayer.

Viruses. The VS viruses used in these experiments were of the Indiana serotype. The origin and genetic characteristics of the ts mutants have previously been described (7, 8). The two RNA− mutants studied here were ts 5 (Pringle's group I) and ts 100 (group IV) (9). The permissive and nonpermissive temperatures were 30 C (±0.1) and 39.2 C (±0.1), respectively.

Chicken embryo cells (CEC) were used to prepare virus stocks. They were infected with 0.1 plaque-forming unit (PFU) per cell. After 45 min of adsorption at 30 C, Porterfield medium (8) was added and cells were incubated at 30 C. VS virus was harvested after 35 hr of infection and titrated by the plaque assay method with the appropriate medium on CEC monolayers. Before using mutant stocks, we made
sure their reversion rate was lower than 0.01% and their leakiness less than 0.02%.

In addition, our mutant stocks were cloned and never gave rise to T particles even after three passages at high multiplicity.

**Chemicals.** \(^{2}H\)-uridine (20 Ci/m mole) was purchased from CEA, Saclay, France, and \(^{3}H\)-guanosine triphosphate (GTP) (13.3 Ci/m mole) from Radiochemical Centre, Amersham, England. Actinomycin D was a generous gift from Merck Sharp and Dohme, through the French branch. Sodium dodecyl sulfate (SDS) was obtained from Serva, Heidelberg, Germany; Triton N101 was from Sigma Chemical Co., St. Louis, Mo.

**Infection of HeLa cells and \(^{3}H\)-labeling of virus-specific RNA.** HeLa cell monolayers were infected with VS virus at an input multiplicity varying from 4 to 5 PFU/cell and incubated for 45 min at room temperature to allow adsorption. Actinomycin D (10 μg/ml) was then added and the cells were brought to appropriate temperature. \(^{3}H\)-uridine (20 μCi/ml) was added at the indicated time. Incorporation of \(^{3}H\)-uridine was stopped by chilling the cells on ice and washing them twice with cold saline medium.

**Preparation of RNA.** Cells were harvested by scraping, suspended in saline medium at 4°C, and centrifuged at 600 × g for 5 min. Two different methods were used to extract RNA. (i) RNA was solubilized from cytoplasmic extracts (22). For this purpose, the cell pellet was resuspended in hypotonic reticulocyte standard buffer (10^{-2} M NaCl, 10^{-2} M tris(hydroxymethyl)aminomethane [Tris]-hydrochloride, pH 7.4, 1.5 mM MgCl\(_2\)) at 4°C for 15 min. Swollen cells were disrupted by six strokes of a Dounce homogenizer and immediately centrifuged at 800 × g for 5 min. The nuclei pellet was discarded and SDS was added to the supernatant fluid to a final concentration of 1% to solubilize RNA. If not used immediately, cytoplasmic extracts were stored at −20°C for a few days. (ii) In some experiments, RNA was extracted from whole cells by a modification of Scherrer's method (27) with SDS (0.5%)-phenol in acetate buffer at 40°C. The extracted RNA was solubilized by dialysis overnight at 17°C against gradient buffer.

**Sucrose gradient analysis of RNA.** RNA extracts were layered onto either 5 to 20% or 15 to 30% sucrose gradients in TNE buffer (0.01 M Tris-hydrochloride, pH 7.4, 0.1 M NaCl, 0.001 M ethylenediaminetetraacetic acid) containing 0.5% SDS. Centrifugation was performed at 17°C in a Spinco rotor SW65 at 40,000 or 50,000 rev/min for 2 hr, or in a SW27.1 rotor at 21,000 rev/min for 15 hr. Gradients were collected as 0.1- or 0.3-ml fractions, depending on the length of the centrifugation tube, by an Isco density gradient fractionator; absorbance at 254 nm was monitored with an Isco ultraviolet analyzer. Fractions were precipitated with an equal volume of cold 10% trichloroacetic acid, filtered on 0.45-μm nitrocellulose filters (Millipore Corp.) and washed twice with cold 5% trichloroacetic acid. Radioactivity was measured in 10 ml of scintillation fluid of Bray (5) in an Intertechnique SL40 spectrometer. Each radioactive sample was counted several times, and the activity reported was obtained by averaging the results.

**RESULTS**

To detect alterations of normal RNA synthesis relative to each mutant, actinomycin D-treated cells were infected either with wild-type virus, ts 5, or ts 100 and incubated at 30°C or 39.2°C. As differences between RNA− mutants could not be demonstrated by total uridine incorporation (17, 24), viral RNA species were extracted at various times after infection and analyzed by sucrose gradient centrifugation. HeLa cells were chosen because they possess low ribonuclease activity and support identical uridine incorporation both at 30°C and 39.2°C.

**RNA species synthesized by ts RNA− mutants.**

At 30°C, RNA profiles observed during each infection period with ts 5 and ts 100 were similar to those obtained with the wild type (Fig. 1). Five major RNA species (38S, 30 to 25S, 23S, 19S, and 13 to 15S) were found. 38S RNA was always present but was sometimes difficult to detect in 5 to 20% sucrose gradients. A well-defined peak was observed in 15 to 30% sucrose gradients. At 39.2°C for both mutants, the synthesis of specific viral RNA species was negligible (Fig. 1). This reduced incorporation of \(^{3}H\)-uridine was always lower for the ts mutants at 39.2°C than at 30°C even at an early period of labeling. In this respect, the mutants differed from the wild type.

Figure 2 illustrates, on an expanded scale, the viral RNA profile of HeLa cells infected at 39.2°C with ts 5 (Fig. 2a) compared with ts 100 (Fig. 2b). In the case of ts 5 infection at 39.2°C, all RNA species were depressed. Regardless of the infection period considered, the RNA pattern was quite similar to residual RNA synthesis found in uninfected actinomycin-treated cells. On the other hand, ts 100 RNA synthesis at 39.2°C appeared to be quite heterogeneous and showed gradually increasing labeling of RNA species which are found between fractions 30 and 50. The same ts 100 RNA profile was observed qualitatively and quantitatively at each 1-hr pulse from the first to the fifth hour after infection; in Fig. 2b is shown the profile obtained after a pulse label from 2 to 3 hr postinfection (pi). These data reveal that the same RNA species were synthesized at a constant rate throughout...
the cycle of infection with ts 100 at restrictive temperature.

Restriction effect of shift-up from 30 C to 39.2 C on ts RNA− mutants. The low yield of RNA detected at nonpermissive temperature for both mutants might result from a restricted number of templates due to the relatively low input multiplicity. To avoid changes in profiles expected when the multiplicity is increased (29), infection was begun at permissive temperature to allow the cycle of replication to start. Shift-up experiments from 30 to 39.2 C were performed either at 2 or 3 hr pi, at which time all viral RNA species have been fully synthesized. The 3H-uridine was added at the time of the temperature shift to 39.2 C to study viral RNA synthesis only at restrictive temperature. Figure 3 shows that cells infected with ts 5 did not exhibit significant RNA synthesis after being shifted to 39.2 C. The time in the infectious cycle of shift-up did not influence the results. The small amount of radioactive incorporation detected, when compared to the cellular back-
ground, might be due to slight contamination with leaky virus. This incorporated radioactivity was confined to the upper part of the gradient (less than 20S) even if the labeling period at 39.2 C was extended to 7 hr pi (not shown).

Also shown in Fig. 3 is the radioactive profile of cells infected with ts 100 after shift-up to 39.2 C at 2 hr pi. The results were qualitatively different from the profile obtained when infected cells were maintained at 39.2 C and labeled during the 3 to 5 hr pi period. Instead of the heterogeneous and diffuse RNA profile obtained in the latter case, after shift-up at 2 hr, all specific viral RNA species became recognizable (Fig. 3). The same qualitative changes were observed when the profile of RNA synthesized at 39.2 C from 3 to 6 hr (Fig. 4) was compared to the profile obtained after a shift-up at 3 hr (Fig. 4). However, far more 3H-uridine was incorporated into RNA when infected cells at 30 C were shifted at 3 hr to 39.2 C. Nevertheless, the amount of RNA synthesized after shift-up was always below the corresponding labeling found when cells were maintained throughout infection at 30 C (Fig. 4). When ts 100-infected cells were pulse-labeled from 3 to 6 hr pi at 30 C, it must be pointed out that the major peaks were not 13 to 15S or 28S RNA messengers but progeny 38S and 23S RNA. We also found in the 23S region a completely double-stranded RNA, not precipitated in 2 M LiCl (2) and resistant to ribonuclease, representing around 50% of the total radioactivity in the peak (unpublished data).

Therefore, after shift-up of ts 100-infected cells at 3 hr pi, all RNA species present at 30 C were observed, but in lesser amounts, except the 28S broad region which was completely missing. The less affected peaks were 19S and 23S. The RNA species present in the 23S band were all precipitable in 2 M LiCl.

Similar results, both at nonpermissive temperature and after shift-up, have been obtained with other members within each of the two complementation groups (I and IV).

In vitro polymerase assay. We have compared the in vitro RNA polymerase activity of VS wild type, ts 5, and ts 100 viruses at optimal temperature (28 C) by the method of Aaslestad et al. (1) and at 39.2 C (Table 1). At 28 C, linear kinetics of incorporation of nucleoside triphosphates were observed during 1 hr for each of the three viruses. The initial rates of 3H-GTP incorporation, as measured by the slopes of the curves, were different, depending on the virus, but this seemed to be related to the starting titer of the virus.
Table 1. In vitro polymerase activity of wild-type VS virus, ts 5, and ts 100 mutants

<table>
<thead>
<tr>
<th>Virus</th>
<th>30 min Net 4H incorporation (counts/min)</th>
<th>60 min Net 4H incorporation (counts/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>28 C</td>
<td>39.2 C</td>
</tr>
<tr>
<td>Wild type</td>
<td>3,000</td>
<td>800</td>
</tr>
<tr>
<td>ts 5</td>
<td>4,000</td>
<td>50</td>
</tr>
<tr>
<td>ts 100</td>
<td>5,000</td>
<td>700</td>
</tr>
</tbody>
</table>

a Each assay (200 μl) contained 12.8 μmoles of Tris-hydrochloride (pH 8.2), 21.3 μmoles of NaCl, 1.6 μmoles of MgCl2, 0.8 μmol of mercaptoethanol, 0.16 μmol of unlabeled adenosine triphosphate, cytidine triphosphate, and uridine triphosphate, 0.016 μmol of 3H-GTP, 36 μg of Triton N-101, and ~10^6 PFU either of wild-type VS virus, ts 5, or ts 100 mutant. Each assay was done in duplicate. An unincubated control reaction mixture, containing either wild type or one ts mutant, was treated in parallel, and its radioactivity was subtracted from the experimental data to give the values reported.

At 39.2°C, the wild type and ts 100 showed significant preservation of activity (25 and 15% of their maximal activity at 28°C, respectively). On the other hand, with ts 5, no RNA polymerase activity was detected.

DISCUSSION

RNA synthesis by cells infected with temperature-sensitive mutants and wild-type virus was compared at both permissive and restrictive temperatures. The VS virus ts mutants were spontaneous mutants, selected from individual wild-type clones to be sure that they were independent (7, 8). The genetic instability of VS virus prevents use of the same wild-type strain for experiments performed over a long period of time. The control wild-type strain described here was probably not identical to all the original clones used for isolation of mutants. It was, however, very near to the original stock and appeared as a satisfactory control since its RNA profile at 30°C is identical to that of the different mutants at 30°C. The RNA species synthesized at 39.2°C by the wild type were similar to those made at 30°C and to those described by other authors (13, 19–21, 26, 28, 33). Furthermore, complementation at 39.2°C between mutants of all the different complementation groups is relatively efficient, a finding that suggests that our mutants arose by single point mutation and that no viral polypeptide other than the polypeptide corresponding to the specific cistron is likely to be altered at the restrictive temperature.

If we analyze the RNA profiles of the ts 5 (group I) mutant, we can conclude that this mutant does not synthesize any detectable RNA at 39.2°C in vivo. It has been demonstrated (3) that the structural polymerase of VS virions acts as a transcriptase in vitro (4). The messenger RNA species obtained under these conditions and those present in polysome fractions of infected cells (13, 28) show the same complementary to virion RNA. This transcriptase was not active in vitro at 39.2°C for mutants of complementation group I. Therefore, mutants in complementation group I represent either a mutation in the transcriptase gene, or a defect of template which appears at nonpermissive temperature; one region of the ts 5 template may not be "accessible" to the polymerase at 39.2°C. This phenomenon could be related to the one observed by S. V. Emerson and R. R. Wagner (in press) with T particles. In good agreement with our data, Printz and Wagner (25) and, recently, Wunner and Pringle (34) did not detect any viral protein synthesis at the restrictive temperature with ts mutants of group I. Transcriptase-defective VS virions have also been identified by several authors (6, 30) although it has not yet been proved that these mutants belong to the same complementation group.

The complete lack of RNA synthesis at 39.2°C by ts 5 has additional implications. Since no synthesis of 38S viral RNA or any other anti-complementary RNA has been observed in an in vitro polymerase reaction (4), we can postulate that another protein is necessary for replication, a "replicase." The ts 5 mutant did not allow 38S virion RNA to be made in cells at 39.2°C, although its single defect was the transcriptase activity. Thus, the hypothetical replicase should not be present in the virion, at least as a functional component. This protein could be either coded directly by some part of the viral genome, as it has been suggested for early proteins of Sendai virus (18), or a protein translated from messenger RNA transcribed by the transcriptase, as has been suggested by Huang and Manders (14). Whatever the conditions in which this replicase is translated, we should expect 38S RNA synthesis after a shift-up to 39.2°C from ts 5-infected cells, since after 3 hr at 30°C a sufficient amount of templates and of all proteins (15, 32) is present. That was never observed even if the labeling period at 39.2°C was extended to 7 hr pi. Therefore, neither of these two hypotheses seems to be satisfactory. A possible explanation of this transcriptase-dependent replication could be the existence of a common subunit in both enzymes,
a subunit which is altered in group I mutants and which carries the in vitro transcriptase activity.

Mutant ts 100 (group IV) differs from ts 5 in several respects. First, transcriptase activity of ts 100 is detected in vitro at 39.2°C. Second, low, but discernible, viral RNA synthesis was observed in vivo at 39.2°C at a constant rate as measured by 1-hr pulses at all periods in the cycle of infection. Thus, no amplification of RNA synthesis can be observed. Qualitatively, the ts 100 mutant produced very little RNA of the characteristic sizes of the usual viral RNA species. This profile of ts 100 RNA species synthesized at 39.2°C could be compared with the slowly sedimenting and heterogeneous profile found by Bishop et al. (4), by Huang et al. (12) and by us for 90-min in vitro transcription products (unpublished data). Third, although normal synthesis of ts 100 viral RNA species and proteins has taken place for the first 3 hr at 30°C, an abnormal RNA pattern appears after a temperature shift-up to 39.2°C. This profile could be compared with that obtained in cells infected with B and T particles (14); however, we must point out that we never detected T particles in our preparations, even after a shift-up experiment. However, in both cases, the 23S peak is predominant. Its exact nature is not known. We have only observed that the double-stranded 23S RNA present at 30°C accumulates late in the cycle and is detected after a long labeling period (3 hr), perhaps as a by-product of the transcription-replication process. This double-stranded RNA was not synthesized by ts 100 at 39.2°C even in a temperature shift-up experiment.

In cells infected by ts 100, in vivo transcription seems to be affected since the 13 to 15S RNA peak was lower and both 28S messenger RNA and transcriptive intermediates sedimenting in this region were almost completely absent. This might suggest that a transcription cofactor could be present. Our data on ts 100 are consistent with this hypothesis. The structural transcriptase of ts 100 is active in vitro as is the wild-type strain at 39.2°C. This in vitro enzyme is able to transcribe up to 94% of the viral genome (4) but not necessarily to give the true messengers found in vivo. This is supported by the analogy between the in vitro transcriptase products pattern and the ts 100 RNA profile at 39.2°C, which are both different from the in vivo messenger RNA profile.

The absence of normal transcription, especially of 28S RNA, and the absence of RNA synthesis amplification at 39.2°C during infection with mutant ts 100 might be related in two alternative ways. First, one of the messenger RNA species not synthesized by ts 100 could code for replicase. Second, as reported by Wagner et al. (31), progeny virion-like RNA could appear in the region of 28S and 23S messengers. Therefore, the absence of 28S messenger RNA in cells infected with ts 100 at 39.2°C could prevent formation of new 28S virion RNA even in the presence of a functional replicase. On the other hand, after a temperature shift-up, labeling in the 38S peak could be the consequence of previous 28S messenger RNA synthesis at 30°C.