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Biochemical Characterization of Temperature-Sensitive Rabies Virus Mutants

NAIMA SAGHI AND ANNE FLAMAND*

Groupe des Laboratoires de Biologie Expérimentale, Université de Paris-Sud, 91405 Orsay Cedex, France

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Biochemical characterization of 70 temperature-sensitive (*ts*) mutants of rabies virus has been done by following the appearance of viral proteins and RNA molecules in infected cells at both permissive and nonpermissive temperature. The presence or absence of the nucleocapsid protein (N) was demonstrated by treating infected cells with anti-N fluorescent antibodies. At 33°C, all the mutants induced a fluorescence comparable to the wild type. At 39.6°C, the mutants can be classified into three groups. Three mutants induced a fluorescence comparable to the wild type (F^+ mutants); 54 mutants induced a faint fluorescence which was proportional to the multiplicity of infection and increased with time (F^{+-} mutants). No fluorescence could be detected for the 13 remaining mutants (F^- mutants). The synthesis of all viral proteins was shown to be normal for F^+ mutants, indicating that transcription and replication of the virus were normal and that the *ts* lesion was located in a protein which is not directly required for those functions. The synthesis of all viral proteins was similarly decreased for F^{+-} mutants and undetectable for the F^- mutants. This suggests that the *ts* lesion affects the transcription and/or replication of the virus. By annealing techniques it was demonstrated that the F^{+-} mutants were able to perform some amount of secondary transcription at nonpermissive temperature. No secondary transcription occurred with F^- mutants. When detectable (i.e., at higher multiplicity of infection), primary transcription of F^- mutants was normal.

Bussereau et al. (5; manuscript in preparation) have isolated 117 spontaneous or induced thermosensitive (*ts*) mutants of the CVS strain of rabies virus. Those mutants were unable to plaque at 38.6°C, although the titer at 33°C was normal. Seventy of them showed a residual growth at a nonpermissive temperature (NPT) less than 2% of wild type and were retained for further studies. These mutants failed to complement each other under conditions in which complementation could be obtained for other rhabdovirus *ts* mutants (see A. Flamand, *In* D. H. L. Bishop (ed.), *Rhabdoviruses*, in press, and reference 15 for a review). The present report describes a biochemical characterization of the rabies mutants to determine whether the mutations involve the same function.

MATERIALS AND METHODS

The Orsay stock of rabies virus used is a clone of the CVS strain of rabies, which titrates at 1.5×10^7 PFU/ml. Viral multiplication was done in BHK-21 cells. Biochemical studies of RNA and protein synthesis were performed with another hamster cell line, CER (a generous gift of T. Wiktor), in which rabies forms plaques between 30 and 38.6°C.

The following procedure was used to isolate spontaneous and chemically induced mutants. The mutagenized or control stock was titrated at 33°C, a permissive temperature (PT). Well-isolated plaques were suspended in saline medium, and this suspension was then titrated at 33 and 38.6°C, the latter being a NPT. Clones that did not yield plaques at the NPT were retained as putative *ts* mutants. Once the *ts* character was confirmed, a stock was created and stored at −70°C. The majority of mutant stocks titrated at approximately 10^7 to 2×10^7 PFU/ml. Mutants were selected at 38.6°C as NPT since wild-type plaques did not form on CER cells at higher temperatures. The wild-type viral production was normal up to 39.6°C (see Fig. 6), and this temperature was chosen as NPT for biochemical studies. Most of the mutants have been isolated after mutagenesis with 5 Fu, i.e., mutants O1 to O102, O105, and O109 to O115. Mutants O103 and O104 are spontaneous. Two mutants have been isolated after mutagenesis with nitrous acid (*ts*O106 and O107) or ethyl methane sulfonate (EMS) (*ts*O116 and O117).

Fluorescent antibody labeling of cells. Pasteur or Wistar anti-rabies nucleocapsid fluorescent antibodies were used. Lyophilized Pasteur antibodies were twice diluted in isotonic buffer before use. Wistar antibodies (a generous gift of T. Wiktor) were diluted 1/50 in isotonic buffer and stored at −70°C until used. Sterile chambers (Labtek) were seeded with BHK-21 cells and incubated overnight at 37°C, after which

they were drained and infected with 50 μl of viral suspension. The inoculum was removed after 30 min at room temperature and replaced with 0.3 ml of minimal essential medium (MEM) supplemented with 0.1% (wt/vol) bovine serum albumin (0.1% BSA-MEM). After 15, 20, or 30 h of incubation in a 5% CO_2 atmosphere at 33 or 39.6°C, the cells were washed twice with 0.2 M phosphate buffer (pH 7.2), dried with acetone, and incubated with 0.1 ml of the fluorescent antibody solution for 1 h at 37°C. The preparations were then washed several times with phosphate buffer and distilled water, and slides were microscopically observed with an UV light source.

Determination of viral protein synthesis: infection and labeling of cells. Growth medium was removed from confluent CER monolayers (3×10^6 cells) in 60-mm-diameter culture dishes. Cells were then infected with 0.2 ml of either viral suspension (multiplicity of infection [MOI] between 1.2 and 2.3 PFU/cell, see legends to the figures) or sham infected with 0.2 ml of MEM. Adsorption was for 30 min at room temperature, and cells were then incubated for 15, 20, or 24 h at 33 or 39.6°C in 3 ml of 0.1% BSA-MEM in 5% CO_2. Cells were then treated with hypertonic amino acid-free medium (Earle salt solution supplemented with Earle vitamins, 10% fetal calf serum, and an excess of NaCl (600 mosM), as described by Madore and England (14) for 30 min.

Cells were labeled for 120 min in 1 ml of hypertonic amino acid-free medium containing 25 μCi of [^{35}S]-methionine at a final specific radioactivity of 50 to 100 Ci/mol.

Preparation of cell extracts. Labeled cells were washed twice with ice-cold TD buffer (0.15 M NaCl–0.01 M Tris-hydrochloride, pH 7.4) and were scraped from the tissue culture dish with 2 ml of the same ice-cold buffer. A 10-ml amount of ethanol was added to the suspension, which was stored overnight at −20°C. A 15-min centrifugation at 9,000 rpm in a Sorvall swinging-bucket rotor was then performed. The pellets were dried and then dissolved in 150 μl of protein dissociation buffer (62.5 mM Tris-hydrochloride [pH 6.8], 2% sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue). Samples were kept at room temperature for 1 h, boiled for 1 min, and stored at −70°C until used. The protein concentration was determined as described by Bramhall et al. (4). Appropriate amounts of buffer were added so that all samples were at the same protein concentration.

Polyacrylamide gel electrophoresis. Samples containing 20 μl of cell lysate were electrophoresed in 10% discontinuous slabs containing sodium dodecyl sulfate (13). After electrophoresis for 4 h at 80 V, the gels were fixed in methanol–acetic acid–water (3:1:6, vol/vol/vol), dehydrated in dimethylsulfoxide, infiltrated with 20% 2,5-diphenyloxazole in dimethylsulfoxide, dried, and finally exposed to RP Royal "X-Omat" film at −70°C, as described by Bonner and Laskey (3).

Preparation of unlabeled RNA from infected cells. Cells were pretreated 30 min before infection with 3 ml of 0.1% BSA-MEM containing 100 μg of cycloheximide per ml or mock treated with 0.1% BSA-MEM. They were then infected with 0.2 ml of the wild type or mutant viral suspension as described above and incubated in 0.1% BSA-MEM in the presence or absence of cycloheximide (100 μg/ml). Efficiency of cycloheximide treatment was controlled before use: at a dose of 100 μg/ml, a 96% inhibition of cellular protein synthesis was found. Reversibility of the action of the drug was controlled as well to see whether a 24-h treatment with 100 μg of cycloheximide per ml was not toxic for the host cell. It was found that 30 min after removal of the drug, protein synthesis in 24-h-treated cells increased to 66% of synthesis in the control non-treated cells.

After varying periods of time at 33 or 39.6°C, cells were drained, washed in Eagle medium, and solubilized with 5 ml of 0.01 M Tris-hydrochloride (pH 7.4), 0.4 M NaCl, 1% sodium dodecyl sulfate, 0.1 ml of diethylpyrocarbonate (as RNase inhibitor), and 5 ml of phenol-cresol (500 g of redistilled phenol, 70 ml of redistilled *m*-cresol, 0.5 g of 8-hydroxyquinoline saturated with 200 ml of 0.01 M Tris-hydrochloride [pH 7.4], and 0.15 M NaCl) was then added to the suspension. The mixture was then sonicated to fragment DNA, reduce the viscosity of the solution, and aid recovery of RNA from the phenol-water interface. An MSE ultrasonic disintegrator was used at full power for 40 s. After centrifugation, the phenol phase and the interface were reextracted with 1 ml of buffer, and the combined aqueous phases were reextracted with 5 ml of phenol-cresol before a precipitation with 2 volumes of ethanol in a siliconized Corex centrifuge tube (Corning Glass, Corning, N.Y.). After an overnight storage at −20°C, nucleic acids were recovered by centrifuging for 30 min in a Sorvall HB4 swinging-bucket rotor at 9,000 rpm. The nucleic acid pellet was dissolved in 1 ml of 0.01 M Tris-hydrochloride (pH 7.4)–0.4 M NaCl and was reprecipitated with 3 ml of cold ethanol to remove residual phenol and sodium dodecyl sulfate. After 3 h at −20°C, nucleic acids were recovered by centrifugation as above, drained, dried, dissolved in 0.2 ml of 0.01 M Tris-hydrochloride (pH 7.4)–0.4 M NaCl, and finally frozen at −20°C until used.

Preparation of labeled viral RNA: preparation of labeled virus. Three bottles of confluent BHK-21 cells (3×10^7 cells) were infected with the Orsay stock of rabies virus at an MOI of 0.1 PFU/ml. After adsorption at room temperature for 30 min, infected cells were incubated in 0.1% BSA-MEM (80 ml/bottle) for 24 h at 37°C in 5% CO_2. The medium was then replaced with an equal volume of 0.1% BSA-MEM containing 4 mCi of [^{3}H]uridine (Commissariat à l'Energie Atomique, Saclay, France), and incubation was continued for an additional 48 h. The supernatant was removed and clarified by centrifugation at 1,500 rpm for 15 min. Virus particles were concentrated by precipitation with polyethylene glycol (70 g of polyethylene glycol-6000 per liter + 23 g of NaCl per liter). The suspension was kept at 4°C for 3 h and was centrifuged in a Sorvall HB4 rotor for 30 min at 5,000 rpm. The pellets were then dried and dissolved in 2 ml of isotonic buffer (0.01 M Tris-hydrochloride [pH 7.4]–0.15 M NaCl). The viral suspension was then loaded onto a 10 to 60% sucrose velocity gradient and centrifuged in a SW 25.1

rotor at 23,000 rpm for 30 min. The material in the gradient above the viral band was carefully removed, and the viral material was then removed with a pipette. No bands corresponding to defective particles could be observed in the gradients.

Extraction of labeled RNA. A 4-ml amount of the phenol-cresol mixture was added to the viral suspension, and RNA was extracted as above except that the sonication step was omitted. The final RNA preparation was dissolved in 0.5 ml of 0.01 M Tris-hydrochloride (pH 7.4)–0.4 M NaCl, and 50-μl portions were stored at −20°C until used. This preparation contained 48 μg of RNA per ml with a specific radioactivity of 1.5×10^8 cpm/mg of RNA.

Annealing of RNA and RNase digestion. Unlabeled RNA from infected cells was annealed with labeled viral RNA as previously described (7). Briefly, 20, 5, or 1 μl of unlabeled RNA extracted from infected cells was mixed with 730, 1,460, or 3,650 cpm of labeled viral RNA. The volume of the reaction was adjusted to 25 μl with 0.01 M Tris-hydrochloride (pH 7.4)–0.4 M NaCl. The mixture was incubated in sealed tubes for 36 h at 60°C. Each sample was then transferred to 0.6 ml of 0.01 M Tris-hydrochloride (pH 7.4)–0.4 M NaCl, and a 0.3-ml portion was precipitated with trichloroacetic acid before or after digestion with pancreatic RNase A at 20 μg/ml at 37°C for 30 min. Controls consisted of labeled viral RNA annealed with extracts from uninfected cells under the same conditions. Each determination was made in duplicate, and a mean value was calculated from the results. Viral RNA demonstrated a 12% resistance to RNase before annealing, and this figure was not significantly increased after incubation (17.5%). A relatively high RNase resistance of RNA from purified rabies virions has been observed on several occasions (2; Flamand, unpublished data). Postannealing RNase resistance was considered to be significant when it was more than 20% (and less than 80%) of the total radioactivity. When RNase resistance after hybridization was included between these two values, the total radioactivity hybridized by unlabeled RNA extracted from one petri dish (3×10^6 cells) was calculated and corrected for the background RNase resistance of viral RNA (17.5%).

RESULTS

Initially, it was verified whether the 70 *ts* mutants could induce the synthesis of rabies nucleocapsid protein in infected cells at both PT and NPT. The presence or absence of N protein was demonstrated by treating infected cells with anti-N protein fluorescent antibodies. Subsequently, mutant-induced protein and RNA syntheses were examined with electrophoresis and RNA-RNA hybridization.

Fluorescence induction in infected cells. When cells infected with wild-type rabies were treated with anti-rabies nucleocapsid fluorescent antibodies, fluorescence began to appear after 11 h at 37 or 39.6°C. Fluorescence became visible after 15 h at 33°C. This early fluorescence was a function of the MOI. It was decided to classify the mutants on the basis of fluorescence induction in infected cells after 15, 20, or 30 h at PT and NPT at an MOI lower than 1 PFU/cell. Fluorescence intensity was estimated on BHK-21 monolayers in which less than 70% of the cells had been infected. In this case, most cells were infected with only 1 PFU.

At 33°C, all the mutants induced a fluorescence which was comparable to that induced by the wild type (Fig. 1). At a temperature of 39.6°C, three *ts* mutants, *ts*O22, *ts*O34 and *ts*O94, exhibited a fluorescence similar to that of the wild type and were classified as fluorescent-positive mutants (F^+). The remainder of the 70 mutants tested exhibited either a weak or undetectable fluorescence after 15 or 20 h of incubation at NPT and were termed F^{+-} or F^-, respectively. When incubation was for longer periods, i.e., 30 h, or when cells were infected with a greater MOI, most of these mutants (F^{+-}) exhibited fluorescence. Thirteen F^- mutants exhibited a barely detectable fluorescence after 30 h of incubation or at an MOI of 10: *ts*O6, *ts*O12, *ts*O16, *ts*O18, *ts*O31, *ts*O42, *ts*O48, *ts*O55, *ts*O83, *ts*O90, *ts*O96, *ts*O101, and *ts*O104. When detectable, the fluorescence of NPT was not due to leakiness of the mutants since residual maturation at 39.6°C was less than 2%.

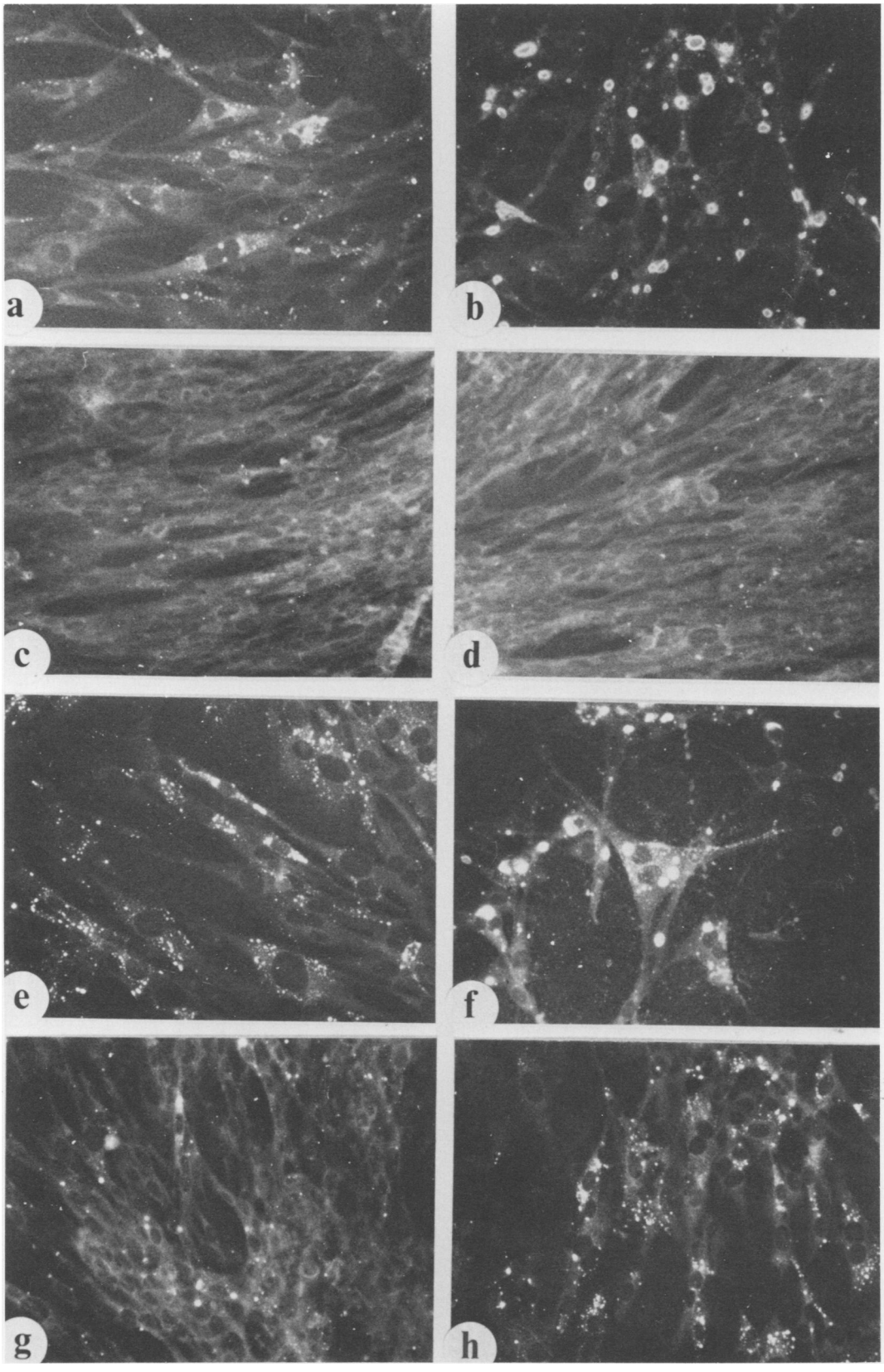
Conclusions based on the results of fluorescence induction lack a certain precision, since they depend on incubation time, MOI, and other factors, such as the quality of the UV light source and the microscope and the concentration of antibody preparations. Thus, with a more powerful light source or with more concentrated preparations of fluorescent antibodies, it was possible to detect some fluorescence, whereas the test was negative in other conditions.

Viral protein synthesis in cells infected

FIG. 1. *Fluorescence of cells infected by wild-type and ts mutants of rabies virus. After 20 or 30 h of incubation at 33 or 39.6°C, infected cells were treated with fluorescent antibodies directed against the rabies nucleocapsid protein as explained in the text. This figure shows successively: fluorescence induced by tsO55 (F^- mutant) after 20 (a) and 30 h (b) of incubation at 33°C (MOI = 0.7); fluorescence induced by tsO55 (F^- mutant) after 20 (c) and 30 h (d) of incubation at 39.6°C (MOI = 0.7); 3°C fluorescence induced by tsO22 (F^+ mutant) after 20 (e) and 30 h (f) of incubation at 39.6°C (MOI = 0.7); fluorescence induced by tsO23 (F^{+-} mutant) after 20 (g) and 30 h (h) of incubation at 39.6°C (MOI = 0.7). Fluorescence induced by the wild type, F^{+-}, and F^+ mutants at 33°C or the wild type at 39.6°C resembles that of F^- mutants at 33°C and is not shown in this figure.*

20 h

30 h



with F^+, F^{+-}, and F^- mutants. Slab gel electrophoresis was used to study viral protein synthesis in CER cells infected with the three mutant classes and with the wild type at an MOI of 1 to 2 PFU/cell after 15, 20, and 24 h of incubation at PT and NPT. Cellular protein synthesis was selectively depressed by high-salt treatment (14). Under these conditions, proteins N, M_1, and M_2 are clearly visible, whereas proteins L and G are less easily detectable. Autoradiographs of these gels appear in Fig. 2 through 5. The residual growth of mutants was measured in the same experiment as a control, and it was found that this parameter was less than 0.1% of

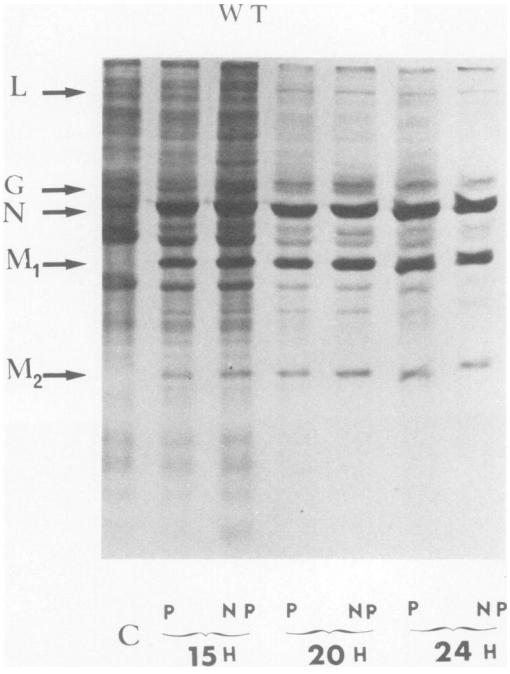


FIG. 2. *Intracellular protein synthesis after infection with wild-type rabies virus. Autoradiographs of 10% slab gel electrophoresis. Cells were infected with the wild type at a MOI of 1.2 PFU/cell. After 15, 20, or 24 h of incubation at 33 (PT) or 39.6°C (NPT), infected cells were treated with hypertonic amino acid-free medium as explained in the text. This treatment selectively depresses cellular protein synthesis and allows the detection of viral proteins L, G, N, M_1, and M_2. Labeling was for 2 h with 25 μCi of [^{35}S]methionine. A 20-μl amount of cell lysate (from 3×10^5 cells) was loaded onto each well and electrophoresed for 4 h at 80 mA. Positions of the five viral proteins determined by comigration of proteins from purified labeled virus mixed with unlabeled cellular extracts are indicated by arrows. C, Residual intracellular protein synthesis in uninfected cells (hypertonic treatment and labeling as for infected cells).*

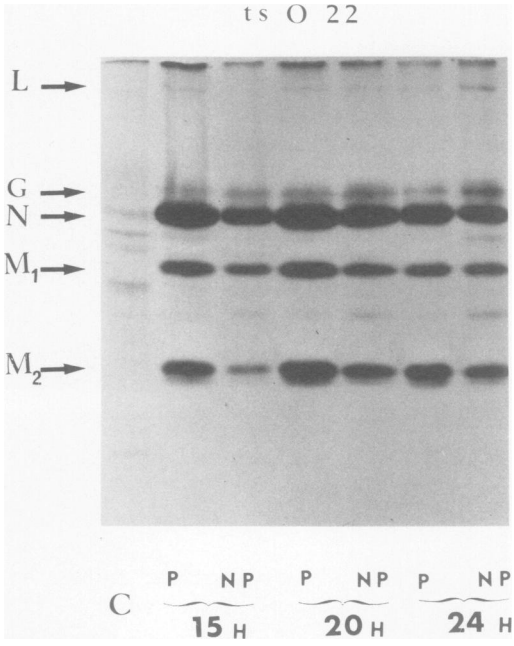


FIG. 3. *Intracellular protein synthesis after infection with an F^+ ts mutant of rabies virus. Autoradiograph of 10% slab gel electrophoresis. Labeling and electrophoresis as explained in the text and the legend to Fig. 2. The MOI was equal to 2.1 PFU/cell.*

that of the wild type at NPT. Mutant multiplication at PT was normal (cf. Fig. 6 for a typical result).

Protein synthesis induced by *ts*O22 and *ts*O94 (F^+) under these conditions was compared to each other at PT and NPT and was comparable to that of the wild type. The results concerning *ts*O22 and the wild type are shown in Fig. 2 and 3. For F^+ mutants protein synthesis seems to be normal at NPT.

At NPT, protein synthesis was detectable for two F^{+-} mutants, *ts*O23 (Fig. 4) and *ts*O21 (data not shown), although it was depressed in comparison to that at 33°C or to that of the wild type. These results are consistent with the hypothesis that the syntheses of all viral proteins are similarly depressed.

Although protein synthesis in cells infected with five F^- mutants, *ts*O55, *ts*O31, *ts*O16, *ts*O101 and *ts*O104, was normal at PT, it was undetectable at NPT. Results for *ts*O55 are shown in Fig. 5.

Since the synthesis of all viral proteins was similarly affected in the F^{+-} and F^- mutants, this suggests that the replication and/or transcription of the virus is affected at NPT.

RNA synthesis induced by F^{+-} and F^-

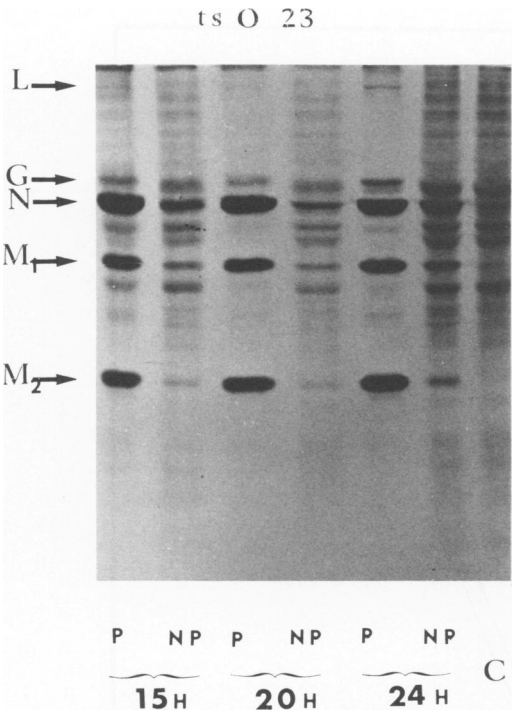


FIG. 4. *Intracellular protein synthesis after infection with an F^{+-} ts mutant of rabies virus. Labeling and electrophoresis as explained in the text and the legend to Fig. 2. The MOI was equal to 1.7 PFU/cell.*

mutants. To determine at which step of the viral cycle, i.e., at the level of primary transcription or secondary transcription, F^{+-} and F^{-} mutants were blocked, viral RNA synthesis was studied at PT and NPT in the presence or absence of cycloheximide. It is well documented that the primary transcription of vesicular stomatitis virus (VSV) (the transcription of the infecting genome by the structural enzyme), the first step of the viral cycle, occurs normally in the presence of cycloheximide. Replication and secondary transcription, however, are blocked by the drug when added early as a result of the inhibition of protein synthesis. It has been shown that rabies, as other rhabdoviruses, contains a particle-associated transcriptase (10, 12) and that the primary transcription of this virus takes place in infected cells in the presence of cycloheximide (1).

The levels of RNA synthesis are low, especially in the presence of cycloheximide. Thus, the more sensitive technique of hybridization was used to demonstrate it (7, 8). Unlabeled RNA from infected CER cells incubated at PT and NPT for increasing periods (0 to 18 h) was extracted and hybridized with labeled RNA of known specific activity extracted from purified rabies virions (see Materials and Methods and the legend to Fig. 7 for details). This technique enabled us to determine the level of synthesis of molecules which were complementary to the viral genome, i.e., primarily viral messengers, which represent the majority of RNA molecules synthesized during the cycle of viral infection (6).

These biochemical studies involved two F^{+-} mutants (*ts*O23 and *ts*O2), three F^{-} mutants (*ts*O55, *ts*O42, and *ts*O31) and the wild type. Results obtained with wild-type virus are shown in Fig. 7. In the presence of 0.1 mg of cycloheximide per ml, primary transcription was undetectable at an MOI of approximately 1.2 PFU/cell. At an MOI of 10, it was clearly detectable and proceeded for at least 18 h.

In the absence of the drug considerable amplification of RNA synthesis occurred. For instance, RNA extracted from 3×10^6 cycloheximide-treated cells after 18 h of infection could hybridize 4.3×10^3 cpm, and RNA extracted from the same number of nontreated cells could hybridize 7×10^4 cpm, although the starting MOI was 10 times lower (Fig. 7).

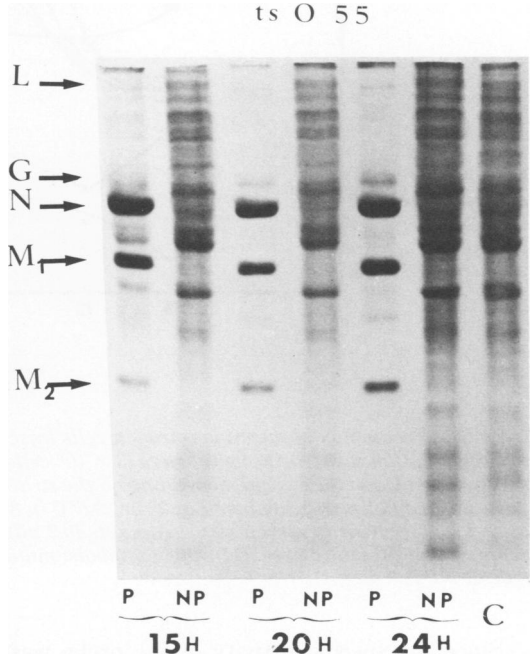


FIG. 5. *Intracellular protein synthesis after infection with an F^{-} ts mutant of rabies virus. Labeling and electrophoresis as explained in the text and the legend to Fig. 2. The MOI was equal to 2.3 PFU/cell.*

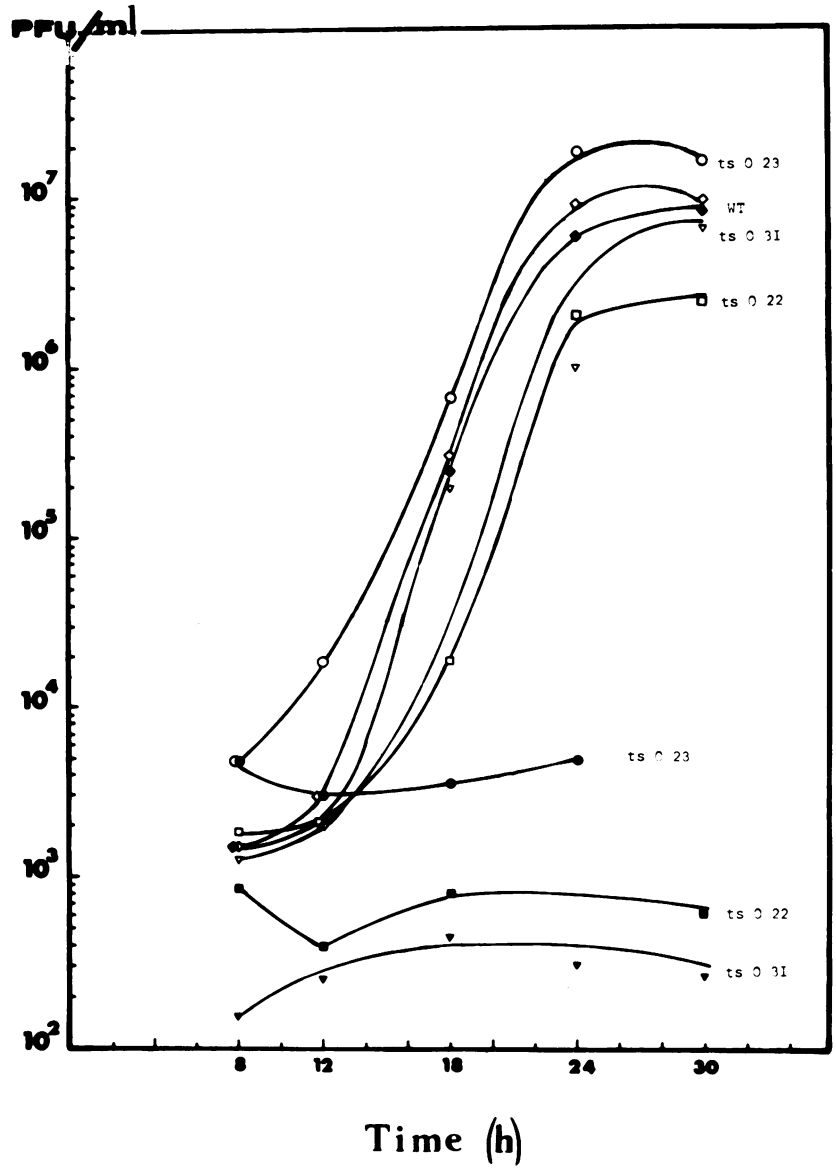


FIG. 6. *Production of infectious virus in cells infected by the wild type and ts mutants of rabies virus at 33 and 39.6°C. Confluent CER monolayers (3 × 10^6 cells) were infected with 0.2 ml of the viral suspension (MOI was between 1 and 5 PFU/ml, depending on the strain). Adsorption was for 30 min at room temperature; cells were washed twice and incubated at 33 or 39.6°C in 3 ml of 0.1% BSA-MEM in 5% CO_2. Medium was changed after 1 h to remove desorbed virus. Aliquots (0.2 ml) were taken at varying periods of time and titrated as explained in Flamand et al. (11). Open symbols: mutants or wild type at PT; closed symbols: mutants or wild type at NPT.*

Since the specific activity of the probe was known (1.5 × 10^8 cpm/mg of RNA or 1 cpm = 8.6 × 10^5 molecules), the quantity of + strands present in the cytoplasm was calculated from the number of counts per minute hybridized. Results were expressed in "genome equivalent" mass copies per infected cell (Table 1). In the presence of cycloheximide, synthesis was linear for at least 5 h, giving an average of 120 genome equivalents per infected cell per h. Since the

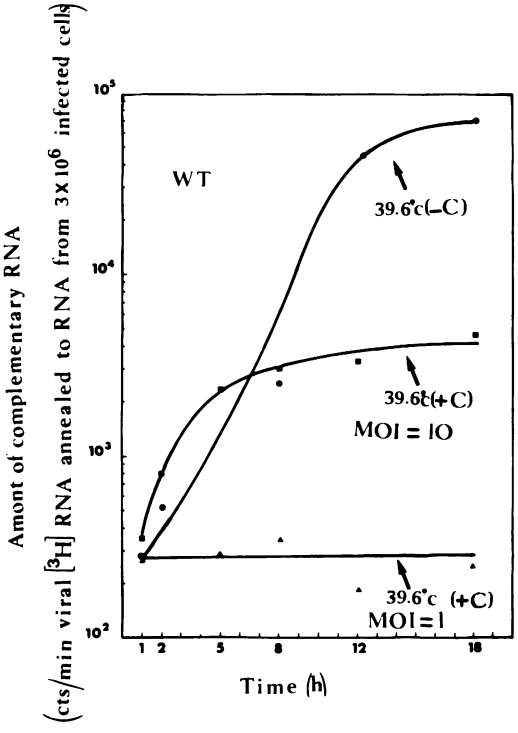


FIG. 7. *Production of viral complementary RNA in cells infected by wild-type rabies virus at 39.6°C in the presence or absence of cycloheximide. Total unlabeled RNAs were extracted from rabies-infected CER cells at various periods of time after infection. The MOI was either equal to 1.2 or to 10 PFU per cell. In this latter case the virus has been concentrated by centrifugation (30,000 rpm for 90 min in a Beckman 42 rotor). The pellet was dissolved in 0.1% BSA-MEM and titrated as usual. Since pelleting could cause aggregation of the virus, the evaluation of the MOI based on the titer in PFUs was a minimal figure. The production of rabies complementary RNA was measured in the presence and absence of cycloheximide by annealing the unlabeled RNAs extracted from infected cells to ^{3}H-labeled RNA extracted from purified rabies virions, as explained in the text. Specific activity of the RNA was 1.5 × 10^8 cpm/mg of RNA (1 cpm = 8.6 × 10^5 molecules). Symbols: ●, 39.6°C in the absence of cycloheximide at an MOI of 1.2 PFU per cell; ▲, 39.6°C in the presence of cycloheximide at an MOI of 1.2 PFU per cell; ■, 39.6°C in the presence of cycloheximide at an MOI of 10 PFU per cell.*

MOI was equal to 10 PFU/cell, the rate of synthesis was equal to 12 genome equivalents per cell per infectious particle per h. The rate is therefore in the order of one genome equivalent per 5 min.

In the absence of cycloheximide, an average of 9,860 and 20,880 genome equivalents were present in the cytoplasm 12 and 18 h after infection, respectively.

Clearly, this numeration gives no indication about the relative amount of mRNA species. It is probable that some are more abundant than the others, since the five viral cistrons may not be transcribed with the same efficiency, depending on their position on the viral genome (9, 14).

The results obtained with the two F^{+-} mutants (Fig. 8A and B) were comparable. RNA synthesis at NPT is lower than that at 33°C or than that of wild type. Nevertheless, RNA synthesis is greater in the absence of cycloheximide than in its presence, indicating that at least some secondary transcription occurred at NPT. The amount of mRNA present in the cytoplasm represents around 1/10 of what is normally found with the wild type at NPT.

The results concerning the three F^- mutants are shown in Fig. 8C and D and 9. RNA synthesis induced by mutants *ts*O55 and *ts*O31 at NPT is considerably lower than that of wild type or of the mutants at PT. In addition, it is lower than that induced by F^{+-} mutants at NPT. When it is detectable, RNA synthesis is not higher in the absence than in the presence of cycloheximide. At higher MOIs, where primary transcription at NPT is clearly detectable, RNA synthesis in the presence or absence of cycloheximide is comparable, confirming that only primary transcription occurred at this temperature (Fig. 9). It is also comparable to the wild type at similar MOI (Fig. 7; wild type + cycloheximide).

DISCUSSION

Numerous reports confirm that the molecular biology of rabies virus is similar to that of other rhabdoviruses (6, 9–12). Although modality of the transcription resembles that of VSV, the rate is different. It takes 5 min with rabies and only 90 s with VSV (8) to obtain a set of messengers equivalent in length to the genome. The number of messengers regularly increases during

TABLE 1. *Number of + strands in rabies-infected cells, in the presence and absence of cycloheximide*[a]

Time after infection (h)	+ Cycloheximide[b]	− Cycloheximide[c]
1	101	78
2	232	145
5	638	
8	870	638
12	957	9,860
18	1,334	20,880

[a] Expressed in genome equivalents per infected cell.
[b] MOI = 10.
[c] MOI = 1.2.

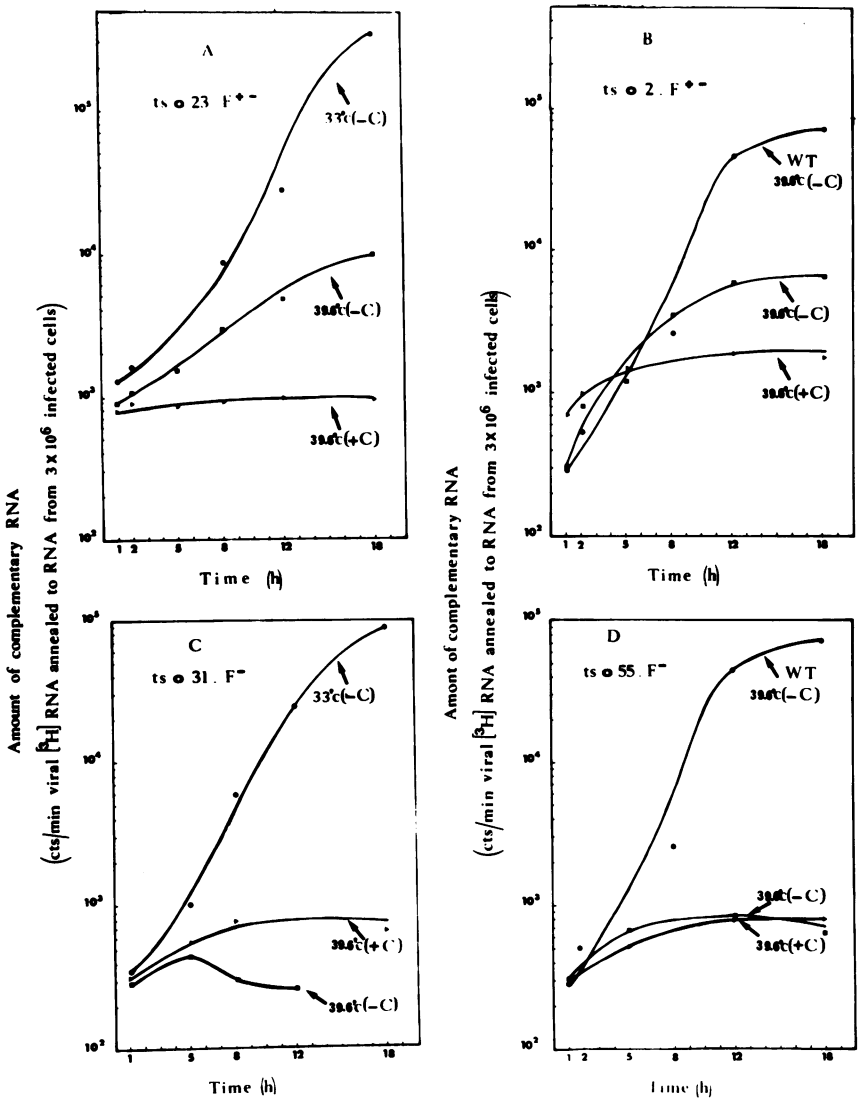


FIG. 8. *Production of viral complementary RNA in cells infected by F^{+-} or F^{-} mutants at 33 and 39.6°C in the presence and absence of cycloheximide. Total unlabeled RNAs were extracted from rabies-infected cells at various periods of time after infection and annealed to ^{3}H-labeled RNA extracted from purified rabies virions, as explained in the text and in the legend to Fig. 7. MOIs were respectively equal to 1.7 (tsO23), 4.2 (tsO2), 1.3 (tsO31) and 2.3 PFU per cell (tsO55). Symbols: ●, mutants at 33°C (left panels), wild type at 39.6°C (right panels) without cycloheximide; ■, mutants at 39.6°C without cycloheximide; ▲, mutants at 39.6°C with cycloheximide (100 μg/ml).*

more than 18 h in the case of rabies and during 6 h for VSV. At maximum production there are 2×10^4 genome equivalents of messengers per rabies-infected cell and 2×10^5 per VSV-infected cell (8). This indicates that transcription (and probably replication) is slower and less efficient for rabies than for VSV.

On the basis of fluorescence induction in infected cells at NPT, the *ts* mutants of rabies virus studied were classified into three groups, F^{+}, F^{+-}, and F^{-}. Approximately 80% of the *ts* mutants were F^{+-}, although the borderline between the most affected F^{+-} and F^{-} was not precise since the classification depended on the levels of viral proteins experimentally detected. Our biochemical studies showed that synthesis

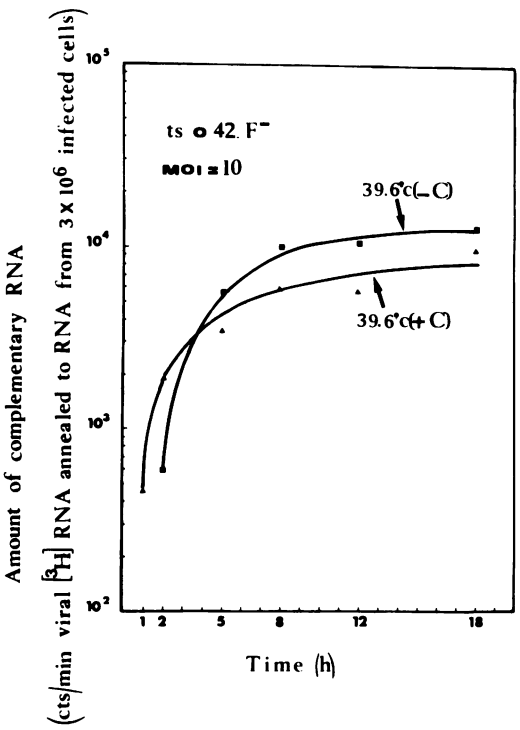


FIG. 9. *Production of viral complementary RNA in cells infected by an F⁻ mutant at 39.6°C at an MOI of 10 PFU per cell, in the presence and absence of cycloheximide. Total unlabeled RNAs were extracted from rabies-infected cells at various periods after infection and annealed to ^{3}H-labeled RNA extracted from purified rabies virions, as explained in the text. Since the virus was concentrated by centrifugation, the evaluation of the MOI based on the titer in PFU is a minimal figure as explained in Fig. 7. Treatment by cycloheximide (100 μg/ml) was as explained in the legend to Fig. 7.*

of the five viral proteins was either normal for F^+ mutants, similarly inhibited for F^{+-} mutants, or undetectable for F^- mutants. A unique mutational event could therefore depress the synthesis of all viral proteins, indicating that this event was at the level of viral transcription and/or replication.

Our hybridization experiments indeed demonstrated that F^{+-} were capable of performing at least some secondary transcription (and therefore some replication) at NPT. F^- mutants, however, could perform primary transcription but not replication and/or secondary transcription at NPT.

Since F^+ mutant protein synthesis is normal at NPT, the *ts* lesion must be located in a protein which is not directly required for transcription and replication, i.e., probably in proteins M_1, M_2, or G. In the absence of complementation, it is not possible to determine whether F^+ mutants are affected in the same function. The characterization of the mutated protein is currently under study.

It has been well documented that 87% of VSV *ts* mutants belong to complementation group I and are mutated in the transcriptase (for reviews see 15; Flamand, in press; and C. R. Pringle and J. Szilagyi, *In* D. H. L. Bishop, *Rhabdoviruses*, in press). In most cases VSV mutants of group I are able to perform primary transcription, but replication and secondary transcription are completely inhibited at NPT. In this respect they resemble F^- mutants of rabies virus.

In the case of rabies, the majority of *ts* mutants are of the F^{+-} phenotype. Secondary transcription of F^{+-} mutants is depressed at NPT compared with the wild type, although some amplification of RNA synthesis could be clearly detected. The transcribing structure of the rhabdovirus is the nucleocapsid. Rabies nucleocapsid is composed of two viral proteins: L, which is likely to be the transcriptase, and N (16). The hypothesis that F^{+-} and F^- mutants could be affected in the N or L protein is under investigation.

We have shown that all our *ts* mutants of rabies virus do not have the same phenotype. Presumably, they are not mutated in the same function. Why it was impossible to demonstrate complementation in tests involving F^+, F^{+-}, and F^- mutants is still an intriguing question.

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