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Transcription and Replication of Vesicular Stomatitis Virus: Effects of Temperature-Sensitive Mutations in Complementation Group IV

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Temperature-sensitive (ts) mutants of vesicular stomatitis virus belonging to the RNA^- complementation group IV were investigated under various conditions to study both their RNA and protein syntheses. In infected cells maintained at 39.2 C, viral RNA species were recovered only in the 13 to 15*S* region of the gradient in an amount depending on the ts mutant used. In the presence of cycloheximide at 39.2 C, the primary transcription was deficient, especially for 28*S* mRNA production. When mutant-infected cells were shifted to nonpermissive temperature, a shutoff of 28*S* mRNA synthesis occurred as a general feature. On the contrary under this condition, the two mutants chosen, ts IV100 and ts IV111, behaved very differently in their 13 to 15*S* and 38*S* RNA production. However, treatment with cycloheximide at the time of the transfer to 39.2 C resulted in a similar recovery of 13 to 15*S* RNA in both mutants, whereas the 28*S* remained very depressed. The viral proteins synthesized by cells infected with the same two mutants also showed a distinct pattern, especially regarding the N protein; a correlation between 38*S* RNA and protein N syntheses was tentatively drawn. The whole set of data suggested that the lesion in group IV mutants concerned a viral structural protein required for the process of in vivo transcription and which probably intervened in the replication mechanism.

Progress has recently been made in the study of processes involved in the production of vesicular stomatitis virus (VSV), making use of mutants isolated in various laboratories (9, 11, 30). Temperature-sensitive (ts) mutants, unable to synthesize sizable amounts of viral RNA at high temperature (RNA^-), have been actively investigated as being the most useful tools to delineate the mechanisms of both viral transcription and replication. New facts emerged from our previous studies (16, 20) of two mutants, ts I and ts IV100, representative of two RNA^- complementation groups, I and IV (F. Lafay, thesis, University of Paris, France, 1972) respectively. Firstly, transcription and replication appear to be tightly linked, and we suggest that a common subunit intervenes in vivo in transcription and replication. In addition, results obtained with ts IV100 prompted a new hypothesis, in which the enzyme which functions in vitro as a transcriptase (2) would require another virus-coded polypeptide to produce faithful transcription in vivo. In this paper, evidence obtained by studying various group IV mutants and which further supports the hypothesis is presented.

MATERIALS AND METHODS

Cells. In all RNA analyses, HeLa confluent monolayers were used under conditions already described (20). For some protein studies, chicken embryo fibroblasts (CEF) were cultured as reported (9).

Virus. Temperature-sensitive (ts) mutants of VSV (Indiana strain), herein investigated, belong exclusively to complementation group IV. Virus stocks containing only standard B particles were obtained and were checked for low percentage of revertants, no residual growth at 39.2 C, and by complementation test as described (20). The wild-type virus grew at 39.2 C, as well as at 30 C.

Chemicals. Actinomycin D, a generous gift from Merck Sharp and Dohme, French branch, was used at a final concentration of 10 μg/ml. Cycloheximide, diluted in medium to 100 μg/ml, and sodium dodecyl sulfate (SDS) were purchased from Serva, Heidelberg, Germany. RNase T_1 was obtained from Sankyo (Tokyo, Japan) and pancreatic RNase A was obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio). All labeled compounds were from C.E.A. (Saclay, France): [^{3}H]uridine (20 Ci/mmol), [^{3}H]leu-

cine (33 Ci/mmol), [^{3}H]tyrosine (49 Ci/mmol), and a mixture of [^{14}C]amino acids (100 mCi/mmol).

Analysis of viral RNA. HeLa cells were infected (~10 PFU/cell, unless otherwise specified) and treated with actinomycin D as previously described (20). When cycloheximide was used, addition of radioactive nucleoside occurred 15 min later. After labeling with [^{3}H]uridine (20 μCi/ml unless otherwise specified in figure legends), cells were harvested at the end of the indicated incubation periods. From cytoplasmic extracts in reticulocyte standard buffer, made 1% with respect to SDS, solubilized RNAs were analyzed by centrifugation in the Spinco SW 27.1 rotor in 15 to 30% sucrose-SDS gradients. All methods and buffer compositions were previously described (20). After centrifugation (21,000 rpm at 17 C for 15 h), the gradients were fractionated and monitored as previously described (20).

Annealing conditions. Annealing of RNA species was achieved as follows: portions (1,500 ^{3}H-counts/min) were mixed with virion RNA (4 to 10 μg/ml) extracted according to the method of Montagnier (17). Duplicate samples were made to a final volume of 400 μliters and a final concentration of 2 × standard saline citrate (0.15 M NaCl, 0.015 M sodium citrate). They were boiled for 3 min, hybridized 1 h at 70 C, and then divided into two equal portions. One of them was digested for 30 min at 37 C by pancreatic RNase A (5 μg/ml) and T_1 (30 units/ml). All samples were then acid-precipitated in the presence of 0.08 M sodium pyrophosphate and 100 μg of yeast carrier RNA per ml. Materials trapped on membrane filters, type HA, were assayed for radioactivity as usual. Controls simultaneously conducted lacked added virion RNA.

Labeling and analysis of viral proteins. CEF or HeLa cells were first exposed for 90 min at 37 C to actinomycin D (1 μg/ml) and, after viral adsorption (40 min at room temperature), were covered with diluted 1:10 (vol/vol) Eagle minimal essential medium (Eurobio) containing 1% calf serum. A mixture of [^{3}H]leucine and [^{3}H]tyrosine was added at intervals after infection for 60-min pulses (20 μCi/ml). Cellular extracts were prepared by disrupting the cells with a Dounce homogenizer in a way similar to that used for RNA extraction (20). Proteins were extracted from cell fractions in precisely the same way as previously described (21). After adding one-tenth volume of glacial acetic acid, the suspensions were made 0.5 M with urea and 1% with SDS, incubated for 1 h at 37 C, and finally dialyzed at room temperature for 16 h against phosphate buffer (pH 7.2, 0.01 M) containing 0.1% SDS, 0.5 M urea, and 0.1% 2-mercaptoethanol. Viral proteins were analyzed by polyacrylamide gel electrophoresis performed as described (29), by using 7.5% acrylamide gel at 5 mA/gel for 5 h. Gels sliced into 1-mm lengths were depolymerized in 0.2 ml of hydrogen peroxide (25%) at 55 C for 4 h prior to the addition of Bray scintillation fluid. For the identification of viral proteins, complete reliance was placed on the migration of intracellular [^{3}H]proteins coelectrophoresed with authentic viral [^{14}C] proteins extracted from purified virions (21). Under these conditions it was regularly observed that intracellular [^{3}H]protein G migrates faster than the virion marker [^{14}C]protein G, as reported by Kang and Prevec (14). Radioactivity was measured in an Intertechnique SL40 spectrometer assisted by a double-labeling computer program.

RESULTS

Viral RNA synthesis at 39.2 C. A first approach to viral RNA synthesis in complementation group IV has been done by using the prototype mutant ts IV100 (20). Parallel studies of three mutants, ts IV62, ts IV111, and ts IV100, now make it possible to generalize about some properties of group IV mutants. As compared to wild type, the total amount of RNA synthesized at 39.2 C is always less than 10%. No degradation of RNA already synthesized can be detected, and the rate of synthesis appears constant without amplification throughout the cycle. Moreover, the pattern of the RNA synthesized at 39.2 C shows no change at any time after infection. It is characterized by the presence of light RNAs at the top of the gradient (less than 10*S*); in addition, among specific VSV RNA peaks, only 13 to 15*S* RNA is readily identified (Fig. 1, full-circle curves). Nevertheless, in the same experimental conditions the largest proportions of 13 to 15*S* RNA are observed with ts IV111 and the smallest with ts IV100. Therefore, a minimal and maximal residual viral RNA synthesis among the mutants studied is provided by ts IV100 and ts IV111, respectively. Consequently, further studies were carried out with these two mutants only.

Viral RNA synthesis at 39.2 C in cycloheximide-treated cells. Results stated above showed that some step(s) in transcription, especially for the synthesis of 28*S*, is (are) affected in the group IV mutants. In order to determine whether any alteration could be detected from the early stage of primary transcription defined by Huang and Manders (13), RNA synthesis at 39.2 C was studied in the presence of cycloheximide which was added just after infection (Fig. 1, open-circle curves). Whatever the mutant used, the RNA pattern obtained under these conditions differs from that obtained at 30 C in the presence of cycloheximide (Fig. 1, star curve) which shows all the characteristics of VSV primary transcription (13). The striking facts were the lack of 28*S* RNA and the similarity of the two profiles with or without cycloheximide. Despite the apparent importance of the RNA synthesis by ts IV111 under these conditions, this mutant behaves as RNA$^-$, because here we compare the RNA synthesized at 39.2 C

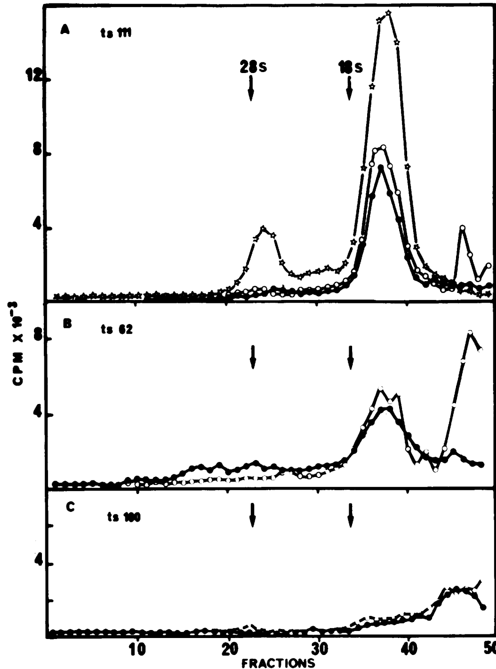

FIG. 1. *Sucrose gradient separation of RNA species made by ts group IV mutants in the presence or absence of cycloheximide. Equivalent samples of HeLa cells were infected either by ts IV100, ts IV62, or ts IV111 at the same multiplicity of infection (~ 20). After 45 min of adsorption at room temperature, actinomycin D was added to a final concentration of 10 μg/ml. Several samples (see symbols) were treated with 100 μg of cycloheximide per ml. Then all samples were labeled with 40 μCi of [³H]uridine per ml. Cells were harvested at 3 h p.i. Cytoplasmic RNAs were separated on 15 to 30% sucrose-SDS gradients, collected into 50 fractions, and acid-precipitated by 5% trichloroacetic acid. ●, RNA profile of infected cells at 39.2 C; ○, RNA profile of infected cells treated with cycloheximide at 39.2 C; ☆, RNA profile of cells infected with ts IV111 and treated with cycloheximide at 30 C. Peaks of radioactive RNA identifiable at 30 C in infections with ts IV62 and ts IV100, identical to those synthesized by ts IV111, are not shown.*

with the RNA obtained at 30 C in primary transcription which accounts for only 10% of the total RNA synthesis (13, 28).

Effect of an upward temperature shift on RNA synthesis. As was already mentioned with ts IV100 (20), a rapid change in RNA synthesis follows a temperature shift to 39.2 C after 3 h at 30 C. Hence, such experiments were simultaneously conducted with ts IV100 and ts IV111. Immediately after the upward temperature shift, RNAs were pulse labeled for 1 h and compared with the corresponding control at 30 C (Fig. 2, star curve). After transfer to 39.2 C, we observed a depression of all viral RNA peaks with both mutants (Fig. 2, full-circle curves). The single-stranded 28*S* RNA, complementary to the virion RNA synthesized at 30 C, was not recovered in the upward shift conditions. The small amount of 28*S* still detected was essentially RNase-resistant. On the other hand, the two profiles strikingly differed concerning 13 to 15*S* and 38*S* RNAs. Actually, after the upward shift, ts IV100 and ts IV111 produced both RNAs sedimenting in the 13 to 15*S* region which, like true mRNAs, hybridized completely to virion genome (98%). However, when compared to the quantity of 13 to 15*S* normally present at 30 C, the amount of 13 to 15*S* was only decreased from 10 to 50% in the case of ts IV111, whereas it was reduced less than 50% for ts IV100.

The 38*S* peak, in our upward shift conditions, was never detected in the ts IV111 profile, whereas it was always present in that of ts IV100, although in various proportions (10 to 90% of its 30-C yield).

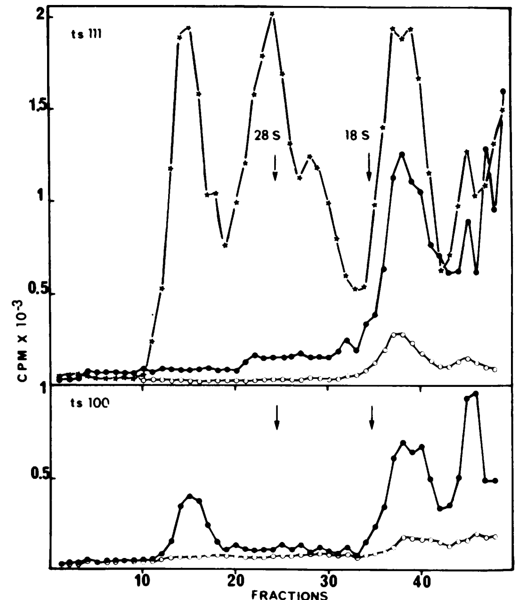

FIG. 2. *Sucrose gradient analysis of RNA species synthesized by ts IV100- and ts IV111-infected HeLa cells at 39.2 C after prior incubation at 30 C. Infected HeLa cells with ts IV100 or ts IV111 were labeled in the presence of actinomycin D with 20 μCi of [³H]uridine per ml 3 to 4 h p.i. RNAs were analyzed as in Fig. 1. ●, ts Mutant-infected cells transferred from 30 C to 39.2 C at 3 h p.i.; ○, ts mutant-infected cells at 39.2 C; ★, infected cells maintained at 30 C for 4 h. As in Fig. 1, and for the same reason, only one profile is shown.*

The rate of RNA synthesis declined after shift to 39.2 C as shown by the gradual decay of RNA labeling, without specific alteration of any one peak during the second and third hour after the shift (not shown). Finally, the effect of prior incubation at 30 C disappeared, because 3 h after a shift the profile became the same as if it was obtained from infected cells maintained at 39.2 C throughout the experimental time course (Fig. 2, open-circle curves).

It can be seen from the control curve in Fig. 2 that even at 30 C all the mutants in group IV induce a remarkable RNA profile, because 38*S* is generally the highest peak, and 28*S* is especially broad. In this important and heterogeneous 28*S* region, additional RNA categories appeared. One of them, 23*S* RNA, was previously mentioned (20) but was not regularly observed either at 30 C or at 39.2 C depending on unknown contingencies.

Combined effect of cycloheximide treatment and upward temperature shift. In upward temperature shift experiments, viral proteins synthesized during the first 3 h at 30 C may interfere with or mask some consequences of the mutation taking place at 39.2 C. Therefore, to interpret the former results, intervention of these proteins must be estimated by preventing further protein synthesis after the shift. Thus, cycloheximide was added at the time of the upward temperature shift (3 h postinfection [p.i.]) in cells infected respectively by the ts IV100 and ts IV111 mutants (Fig. 3), whereas a sample was maintained in parallel at 30 C as a control (Fig. 3, square curve). The transfer to 39.2 C led to a decrease of both classes of mRNAs, which was especially drastic at the level of 28*S*.

We verified with wild-type virus that none of these facts was the result either of the direct action of cycloheximide or of its combined effect with the upward temperature shift. First, as did other authors (19, 30), we found that addition of cycloheximide at any time during an infectious cycle at 30 C was conducive to a shutoff of 38*S* RNA synthesis (visible on the control curve), whereas transcription of 28*S* and 13 to 15*S* RNA was unaffected or even enhanced as compared with cells without the drug. Second, the combined effect of a temperature change and cycloheximide treatment was shown to merely promote an extra amplification of transcription.

Now comparison of the two profiles obtained for each mutant after an upward temperature shift in the presence or absence of cycloheximide pointed out the role of newly synthesized proteins at 39.2 C. Although both types of profiles have already been reported, but from nonsimultaneous analyses, we preferred to compare new curves resulting from another experiment. De novo protein synthesis at 39.2 C resulted for both mutants in a dramatic reduction of the amount of 13 to 15*S* and almost a disappearance of 28*S* RNA (Fig. 4). Moreover, 38*S* RNA appeared when cycloheximide was omitted, but only with ts IV100.

Protein synthesis by mutants ts IV100 and ts IV111. The synthesis of viral proteins has been investigated in conditions similar to those used for the RNA synthesis. In our hands, CEF supporting a much more active and rapid protein synthesis than HeLa cells, without exhibiting consistent differences, were preferentially used.

Figure 5 shows the electrophoretic profiles of the viral proteins extracted from CEF infected with the mutants ts IV100 and ts IV111 at 30 C, 39.2 C, and in upward temperature shift conditions. All five viral proteins were present in normal amounts in cells infected at 30 C with the two mutants and were labeled from 2 to 3 h p.i. At nonpermissive temperature and during the same labeling period, the level of synthesis was markedly reduced, espeically with the mu-

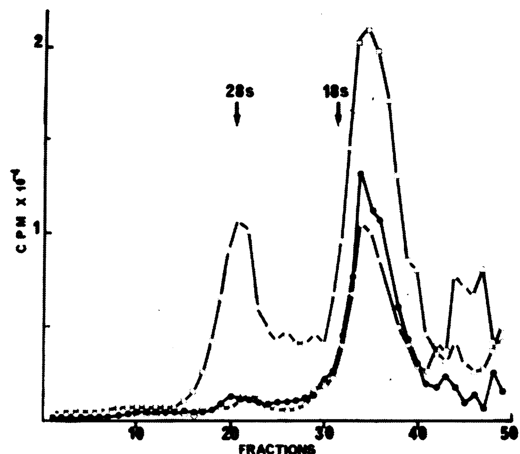

FIG. 3. *Sucrose gradient pattern of RNA synthesized by ts IV100 and ts IV111 after addition of cycloheximide in upward shift experiments. Infected and actinomycin D-treated HeLa cells were incubated 3 h at 30 C. At 3 h p.i., cycloheximide was added (100 µg/ml). Fifteen minutes later, cells were labeled with 20 µCi of [³H]uridine per ml for 1 h and analyzed as in Fig. 1.* ●, *ts IV111-Infected cells transferred from 30 C to 39.2 C at 3 h p.i. time of addition of the antibiotic;* ○, *ts IV100-infected cells treated as ts IV111-infected cells;* □, *control infected cells maintained at 30 C after addition of the drug.*

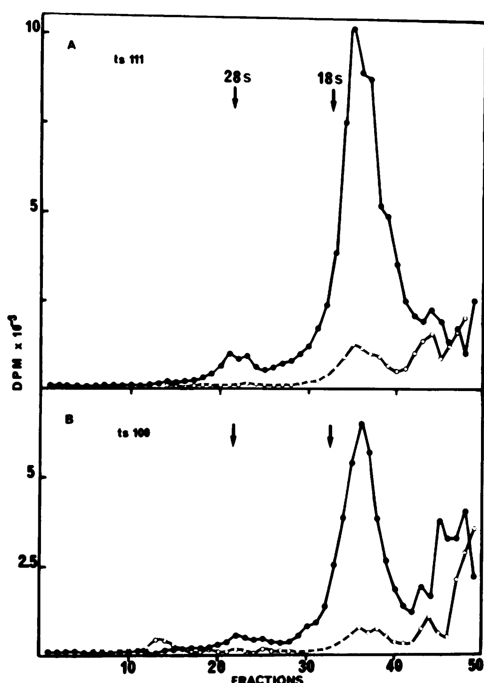

FIG. 4. *Sucrose gradient centrifugation of viral RNA made by ts IV100- or ts IV111-infected cells, in the presence or absence of cycloheximide in upward temperature shift experiments. After infection with ts IV100 or ts IV111, actinomycin D-treated cells were incubated 3 h at 30 C. At 3 h p.i., cells were transferred to 39.2 C, and one of the duplicates received cycloheximide (100 μg/ml). At 3.15 h, all cells were labeled with 20 μCi of [³H]uridine per ml for 1 h.* ●, *Infected cells with cycloheximide added at the time of the shift;* O, *infected cells without cycloheximide.*

tant ts IV100; in the last case, only the proteins N and NS emerged from the background. At 39.2 C, the ts IV111-infected cells gave a better-defined profile, but the N protein peak appeared low as compared with the other ones. With both mutants, the L protein was virtually absent. The pattern of protein synthesis obtained with the two mutants in the upward shift condition are shown in panel B. The main difference between the two mutants was in the behavior of the N protein. During the 1-h pulse at 39.2 C which followed the 2-h pulse at 30 C, the N protein peak decayed drastically in cells infected with ts IV111, but was not affected in the case of the mutant ts IV100. This result has been regularly observed in repeated experiments. The drop of the N protein was due to an absence of synthesis rather than to a rapid destruction of newly synthesized polypeptides, because in a chase experiment of labeled proteins at 30 C no drastic effect was observed at 39.2 C at the level of any viral protein (unpublished data). The L protein synthesis was reduced in a similar way for both mutants.

A peculiar effect of the upward shift was observed at the level of the G protein; its synthesis was markedly increased in cells infected with ts IV100 and ts IV111. This effect was not transitory and continued to be observed during successive 1-h pulses.

The transcription process in cells infected with mutant ts IV100 or ts IV111 was increased when upward shift was combined with a treatment of cycloheximide (Fig. 4). To observe the effect on protein synthesis, HeLa cells or CEF both infected with ts IV111 have been treated by cycloheximide (100 μg/ml) at the time of the upward shift to 39.2 C and extensively washed before the 1-h amino acid pulse. Compared with Fig. 5, Fig. 6 shows that the same profile of proteins is obtained, but the protein peaks are higher; this may result from the presence of more mRNA in that condition. Parenthetically, the electrophoretic profiles were qualitatively similar with proteins extracted from CEF or HeLa cells.

DISCUSSION

Analysis of viral RNAs made at 39.2 C after infection with any one of the group IV mutants demonstrates that both the expected transcription and replication cannot take place. Discussing our previous data (20), we proposed that transcription was the step directly concerned by the mutation. In the present paper, shorter labeling pulses and upward temperature shift experiments conducted to amplify the effects of the mutation confirm that transcription is undoubtedly affected. This appears from the early phase of primary transcription during which are observed the alterations in RNA synthesis characteristic of group IV mutants, i.e., lack of 28*S* and a lower degree of 13 to 15*S* transcription (Fig. 1). In a recent study, Unger and Reichmann (28) reported the deficient RNA synthesis of one mutant of group IV at nonpermissive temperature. They argued about the special alteration of the long mRNA, but there is no real discrepancy between their observation and our own, because in conditions similar to theirs (5 h at 39.2 C) we actually found labeling in the 28*S* region, although it was not recovered as one conspicuous peak (20). In addition, when they examined the primary transcription of their group IV mutant, they noticed the same quantitative change at nonpermissive temperature as we related. Because, by definition, among viral

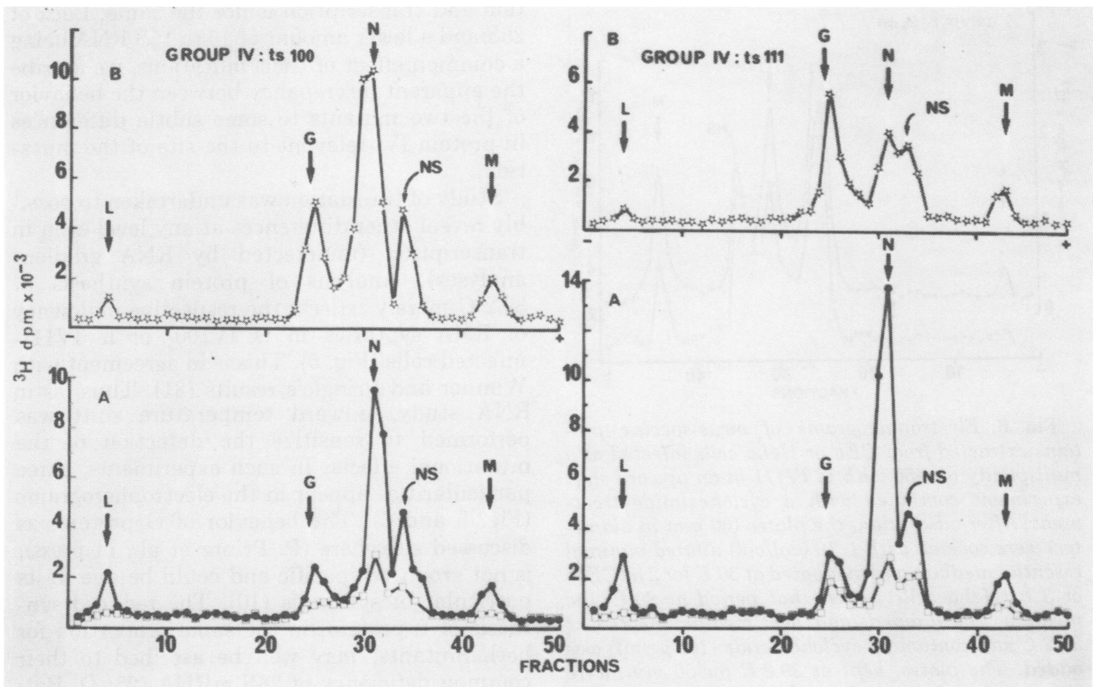

FIG. 5. *Electropherograms of virus-specific proteins extracted from CEF infected at a multiplicity of ~ 50 with ts IV100 or ts IV111 under permissive, nonpermissive, or upward shift conditions. After an adsorption period of 40 min at room temperature, three plates (60 mm in diameter) for each mutant were covered with 2 ml of 1:10 (vol/vol)-diluted minimal essential medium and incubated at 30 C (two plates) or 39.2 (one plate). Two hours later, [^{3}H]leucine and [^{3}H]tyrosine (10 μCi/ml each) were added to one culture at 30 C and to the other at 39.2 C; cells were harvested after 1 h of labeling (2 to 3 h p.i.). The remaining plate at 30 C was brought to 39.2 C at 2.30 h p.i. and labeled from 3 to 4 h p.i. Proteins were extracted from cytoplasmic contents of cells, disrupted with a Dounce homogenizer, with 10% acetic acid, 0.5 M urea, 1% SDS, and 0.1% 2-mercaptoethanol. Extracted [^{3}H]proteins were subjected to electrophoresis in 7.5% neutral SDS acrylamide gels along with marker [^{14}C]proteins extracted from purified virions. Vertical arrows show the peak positions of the four marker [^{14}C]proteins. Left hand side, ts IV100; right hand side, ts IV111. Panels A: ●, permissive temperature; □ nonpermissive temperature. Panels B: upward shift conditions.*

proteins only those associated with the virion are involved in primary transcription (13), it follows that one of these structural proteins, essential particularly for 28*S* RNA synthesis, is altered in group IV mutants. Moreover, in vitro transcriptase activity at 39.2 C has been demonstrated with this group of mutants (20, 26), whereas it failed for group I mutants; in the latter group the lesion seems to be more likely in the enzyme than in the template (P. Printz et al., *in* R. D. Barry and B. W. J. Mahy, (ed.), Negative strand viruses, in press; R. R. Wagner, personal communication). Therefore, besides eventual host factors (19), we must emphasize that another viral structural protein, which for convenience will be termed protein IV, is necessary to obtain a faithful in vivo transcription. The ts event renders it rapidly inactive at nonpermissive temperature. This is observed for protein IV of the incoming virions in primary transcription (Fig. 1), as well as for the new molecules made at 30 C and shifted to 39.2 C when additional protein synthesis was prevented by cycloheximide (Fig. 3). However, when cells infected either by ts IV100 or ts IV111 were kept 3 h at the nonpermissive temperature and then shifted downward to the permissive one in the presence of cycloheximide, transcription was completely recovered (not shown). Therefore, the ts event does not seem to be irreversible, and protein IV is not degraded at high temperature.

Upward temperature shift experiments, with and without cycloheximide addition, posed the problem of the effect of the mutation in the group IV mutants at the level of replication. First, there is a persistant synthesis of 38*S* with ts IV100 after an upward shift without cyclohex-

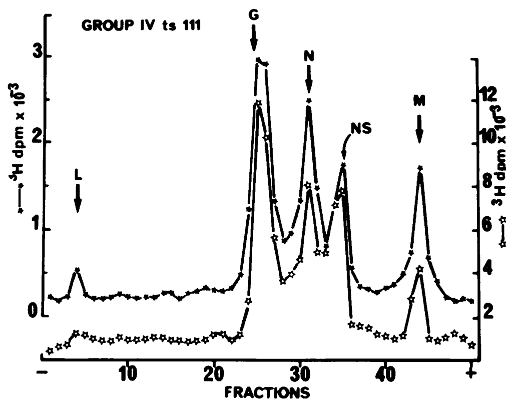

FIG. 6. *Electropherograms of virus-specific proteins extracted from CEF or HeLa cells infected at a multiplicity of ~50 with ts IV111 in an upward shift experiment combined with a cycloheximide treatment. After adsorption, the plates (60 mm in diameter) were covered with 1:10 (vol/vol) diluted minimal essential medium and incubated at 30 C for 2 h (CEF) or 3 h (HeLa cells). After that period at 30 C, the medium was drained, and new medium heated at 39.2 C and containing cycloheximide (100 μg/ml) was added. The plates, kept at 39.2 C for 50 min, were then washed extensively and covered with 2 ml of medium devoid of cycloheximide, but heated at 39.2 C. Cells, maintained at 39.2 C, were pulse labeled with [^{3}H]leucine and [^{3}H]tyrosine (10 μCi/ml each) from 3 to 4 h p.i. (CEF) or from 4 to 5 h p.i. (HeLa cells). Cytoplasmic extracts were treated as described in Fig. 5. Vertical arrows show the peak positions of the four marker [^{14}C]proteins.* ☆, *CE fibroblasts;* ★, *HeLa cells.*

imide. Second, neither 38*S* nor any RNA species noncomplementary to virion genome are detected with ts IV111 under the same conditions. Recent reports (19, 30; P. Printz et al., in press) gave evidence of an equilibrium between transcription and replication and of a shift of this equilibrium towards transcription in the absence of replication by devotion of the whole RNA polymerase activities to mRNA production. After an upward temperature shift, only the addition of cycloheximide allowed this increased transcriptase activity in both mutants (Fig. 4). Accordingly, even with ts IV111 in the absence of cycloheximide, transcription was still restricted as if equilibrium was preserved, arguing for a potentially present replicase function although unable to finally lead to 38*S* or possible precursors (15). Whereas the recoverable transcriptase activity is nearly identical for both mutants in the presence of cycloheximide (Fig. 3), in upward shift experiments without the drug (Fig. 2) the balance between replication and transcription is not the same. Lack of 28*S* and a lower amount of 13 to 15*S* RNA being a common effect of their mutations, we ascribe the apparent discrepancy between the behavior of the two mutants to some subtle differences in protein IV, relevant to the site of the mutation.

Study of translation was undertaken to possibly reveal other differences at any level even in transcription (undetected by RNA gradient analyses). Analysis of protein synthesis at 39.2 C merely reflects the respective deficiency of RNA synthesis in ts IV100- or ts IV111-infected cells (Fig. 5). This is in agreement with Wunner and Pringle's results (31). Thus, as in RNA study, upward temperature shift was performed to sensitize the detection of the mutational effects. In such experiments, three particularities appear in the electropherograms (Fig. 5 and 6). The behavior of G protein, as discussed elsewhere (P. Printz et al., in press), is not group IV-specific and could be due to its particular biosynthesis (10). The reduced synthesis of L protein, in the same proportion for both mutants, may well be ascribed to their common deficiency of 28*S* mRNA (25; D. Baltimore et al., *in* R. D. Barry and B. W. J. Mahy, (ed.), Negative strand viruses, in press). If L protein is responsible for the transcriptase activity detected in vitro (7), absence of new molecules of L transcriptase, along with failure of replication at 39.2 C without prior incubation at 30 C, would explain that no amplification occurs in RNA synthesis with group IV mutants. Thus, we conciliate two explanations suggested by Unger and Reichmann, namely, lack of replication and block in additional synthesis of proteins required for transcription (28). Finally, the major difference in protein synthesis between the two mutants investigated concerns the synthesis of N protein; they also differ in their 38*S* production (Fig. 2). As a new protein synthesis is required for 38*S* RNA formation (19, 30; Fig. 3), a causal relationship can be drawn from these two observations. If the recovery of intracellular virion RNA was packaged primarily with N protein, unstabilization of 38*S* RNA might result from the lack of this protein. This would partially explain protein requirement for replication, because other viral proteins also appear to be involved in the 38*S* RNA synthesis (manuscript in preparation).

Another explanation for the different behavior, with regard to the replication process, between the two mutants in the same complementation group could be given if protein IV, differently affected by the various ts point

mutations, is involved in several functions. Our results make it conceivable that protein IV is concerned with some aspects of signal recognition and, thus, could intervene both in transcription and replication. Effectively, transcription is supposed to set on the putative start or stop signals (18, 23) along the template. The presence of intracellular 38*S* RNA complementary to virion RNA (D. Baltimore et al., in press; S. M. Perlman and A. S. Huang, submitted for publication) suggested that replication might also function by opening one initiation site, with all others remaining masked. Interacting with either the template, the transcriptase, the putative replicase, or with any complex between them, protein IV should be devoted to signals switch-on and -off or simply to their recognition, giving quantitative and qualitative efficiency to transcription or replication or both. This hypothesis would account for the observation that in group IV mutants RNA synthesis in vivo at 39.2 C occurs as if transcriptase alone was at work, because as in the in vitro reaction (3) (i) input genomes of group IV mutants are completely transcribed (8), and (ii) these mutants give mostly RNA species of short sizes (4, 12, 13, 20, 30). In addition, we demonstrated that transcription of 28*S* RNA and 13 to 15*S* RNA may occur independently, which is consistent with some sort of sequential transcription of the VSV RNA (22).

Protein IV cannot be identified by merely studying in vivo protein synthesis, because, besides the transcriptase L protein and genome RNA, the complete VSV transcription apparatus comprises in vitro (5, 6, 27), as well as in vivo (1), protein N, NS, and eventual minor proteins; protein IV could be any one of them. Its proposed functions may be modulated by some chemical transformation (24).

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