Cleavage Defect in the Non-structural Polyprotein of Semliki Forest Virus has Two Separate Effects on Virus RNA Synthesis

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(Accepted 7 December 1977)

SUMMARY

When Semliki Forest virus ts-4 mutant infected cultures are grown at the permissive temperature (28 °C) and shifted to the restrictive temperature (39 °C), two different defects in RNA synthesis are manifested: (i) the synthesis of 26S RNA is stopped within 60 min (Saraste et al. 1977), and (ii) the increase in RNA synthesizing activity ceases, in contrast to cultures maintained at 28 °C, indicating that no new active RNA polymerase is formed at 39 °C. Accumulation of a non-structural precursor protein with an apparent mol. wt. of about 220000 (ns220) was demonstrated in ts-4 infected cultures shifted to 39 °C. Ns220 was labelled during short pulses given immediately after release of protein synthesis from hypertonic initiation block, suggesting that genes coding for ns220 are located near the initiation site at the 5'-end of the 42S RNA. The viral specificity of ns220 was shown by its disappearance after a shift to 28 °C and by labelling in the presence of sucrose, when no host cell protein synthesis is detectable. The two functional defects can be explained if the polypeptides responsible for the RNA polymerizing activity and that responsible for the synthesis of 26S RNA are components of the same non-structural polyprotein. A mutation in the latter polypeptide which prevents cleavage of the polyprotein would thereby prevent the further formation of active RNA polymerase. If cleavage of the polyprotein has taken place at the permissive temperature, the RNA polymerase would remain active also at 39 °C, whereas the polypeptide responsible for 26S RNA synthesis would become inactive due to the mutation.

INTRODUCTION

The alphavirus 42S RNA genome coded proteins are probably translated as two polyproteins. The messenger for the structural polyprotein p130 (mol. wt. 130000) is the 26S RNA which is a replica of the 3' third of the 42S RNA (Kennedy, 1976; Wengler & Wengler, 1976; Strauss & Strauss, 1977). The 42S RNA seems to be the messenger for at least three to four non-structural proteins which are synthesized starting from one initiation site (Cancedda et al. 1975; Glanville et al. 1976; Lachmi & Kääriäinen, 1976; Brzeski & Kennedy, 1977; Glanville & Lachmi, 1977). In Semliki Forest virus (SFV) infected cells four non-

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structural proteins ns70, ns86, ns72 and ns60 with apparent mol. wt. of 70000, 86000, 72000 and 60000 are synthesized sequentially in the above order. Tryptic peptide mapping has recently shown that two short-lived proteins with mol. wt. of 155000 (ns155) and 135000 (ns135) are precursors of the more stable non-structural proteins. The ns155 contains the peptides of ns70 and ns86 whereas the ns135 consists of ns72 and most probably also of ns60 (Glanville et al. 1978).

An RNA negative mutant, ts-4, of Semliki Forest virus has a reversible defect in the synthesis of 26S RNA as shown by shift-up experiments (Saraste et al. 1977). Here we report the detection in ts-4 infected cells of a third non-structural precursor protein, which has an apparent mol. wt. of about 220000, found only in cells shifted to the restrictive temperature. Similar size proteins have been described in Sindbis virus mutant (Bracha et al. 1976), in Sindbis virus wild type (Brzeski & Kennedy, 1977) and in SFV wild type infected cells (Clegg et al. 1976).

**METHODS**

**Cells and virus.** Secondary cultures of chicken fibroblast cells obtained from special pathogen-free animals were grown in 60 mm Petri dishes as described previously (Keränen & Kääriäinen, 1974). SFV prototype strain was cultivated as described (Kääriäinen et al. 1969), and the isolation, characterization and propagation of SFV ts-4 mutant have been described previously (Keränen & Kääriäinen, 1974).

**Growth of virus and isotope labelling.** Chicken cells were infected with ts-4 or wild type SFV (50 p.f.u./cell) at either 28 or 39 °C, as indicated. After 1 to 2 h adsorption, virus was removed and the cells were washed twice with Hanks' solution. The cells were then incubated in Eagle's minimum essential medium with or without 0.2 % bovine serum albumin (BSA) and 1 µg/ml of actinomycin D. In temperature shift experiments, fresh medium at either 28 or 39 °C was added to the cultures upon the temperature shift.

For labelling with 3H-uridine (Radiochemical Centre, Amersham, specific activity 29 to 31 Ci/mmol), 20 µCi/dish was used. Labelling with 35S-methionine (Amersham, specific activity 920 Ci/mmol) was carried out in methionine-free Eagle's medium using 10 to 500 µCi/dish, as indicated in individual experiments.

**Treatment of cells with hypertonic media.** Infected chicken cells were exposed to either 335 mM-NaCl (Saborio et al. 1974; Nuss et al. 1975) or 0.1 to 0.5 M-sucrose for 40 to 60 min in 3 ml of medium. The medium was removed and new medium containing 35S-methionine was added. 35S-Methionine labelling was carried out in methionine-free Eagle's medium supplemented with 0.2 % BSA and, as indicated in the text, also with 0.1 to 0.5 M-sucrose. At the end of the pulse, the medium was removed and sucrose-free Eagle's medium containing a 20-fold excess of the normal concentration of methionine was added for variable times. The cells were washed with 0.1 M-NaCl, 0.01 M-tris-HCl pH 7.4, and collected in 2 % aqueous SDS as described previously (Lachmi & Kääriäinen, 1976).

**Polyacrylamide gel electrophoresis.** Polyacrylamide slab gels with 10 % main gel and 3 to 5 % spacer gels were made according to the method of Neville (1971) and modified as described by Lachmi et al. (1975). The gels were stained with 0.25 % Coomassie blue in 50 % trichloroacetic acid (TCA) for 45 min and destained in 8 % acetic acid. In most cases, the stained gels were treated with dimethylsulphoxide and PPO and dried for fluorography as described by Bonner & Laskey (1974). Myosin was a generous gift from Rockefeller University, given by Dr Polly Etkind.

**RNA analysis.** RNA was isolated from cells disrupted by 2 % sodium dodecyl sulphate (SDS) and was analysed using 15 to 30 % (w/w) sucrose gradients made in 0.14 M-NaCl,
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Fig. 1. Rate of RNA synthesis in ts-4 infected chicken cells maintained at 28 °C or shifted to 39 °C at hourly intervals. Chicken cells infected with ts-4 at 28 °C were maintained at 28 °C and were either exposed to 3H-uridine (20 μCi/dish) for 60 min at 28 °C or were shifted to 39 °C and pulsed similarly at 10 h post-infection. The cells were taken into 2% SDS and TCA precipitated radioactivity was determined. ●—●, Pulsed at 28 °C; ○——○, shifted to 39 °C and pulsed between 10 and 11 h post-infection.

RESULTS

RNA synthesis

The rate of RNA synthesis in ts-4 infected chicken cell cultures was studied at the permissive temperature (28 °C) as well as in cultures which had been shifted to the restrictive temperature (39 °C). Two cultures were taken at hourly intervals: one was labelled with 3H-uridine for 60 min at 28 °C and the other shifted to 39 °C. All the shifted cultures were labelled simultaneously for 60 min with 3H-uridine at 10 h post-infection.

After 4 h incubation at 28 °C the rate of RNA synthesis increased rapidly (Fig. 1), similar to that of the wild type SFV. The RNA synthesis continued in all cultures which had
been shifted to the restrictive temperature. Interestingly, there was no increase in the rate of RNA synthesis in any of the cultures shifted to 39 °C. There was rather a decrease, maximally of about 50%, compared to the RNA synthesizing activity observed at 28 °C at the time of shift (Fig. 1). These results suggest that after the shift to the restrictive temperature no new active polymerase is formed, and that the RNA synthesizing activity detected at 39 °C as long as 8 h after the shift was due to RNA polymerase which was formed during the incubation at 28 °C. The same result was obtained when wild type SFV infected cultures were shifted to 39 °C in the presence of cycloheximide (100 μg/ml).

Analysis of the RNAs formed at 28 °C and after the shift to 39 °C confirmed our previous findings that virtually no 26S RNA is synthesized at the restrictive temperature (Fig. 2; Saraste et al. 1977; Sawicki et al. 1978). Thus, two different steps are affected when ts-4 infected cultures are shifted to the restrictive temperature: (i) the synthesis of 26S RNA is shut off rapidly, and (ii) the formation of new polymerase is prevented.

**Protein synthesis**

Duplicate ts-4 infected cultures maintained for 6 h at 28 °C were either shifted to 39 °C or maintained at 28 °C and labelled with 35S-methionine for 15 min with or without pretreatment with 335 mM-NaCl or 0.5 M-sucrose. The hypertonic media were used to reduce the host protein synthesis which, during the early period of infection, makes the detection of virus proteins difficult (Lachmi & Kääriäinen, 1977). The labelled proteins resolved on a discontinuous polyacrylamide slab gel are shown in Fig. 3.

At 39 °C two large proteins can clearly be seen in ts-4 infected cultures treated with either
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Fig. 3. Fluorogram of 10% polyacrylamide slab gel of ts-4 induced, $^{35}$S-methionine-labelled proteins at 39 °C (A, B, C) and at 28 °C (E, F, G). Ts-4 infected cells maintained for 6 h at 28 °C were either shifted to 39 °C or kept at 28 °C and were exposed to 0.5 M sucrose (B, F) or to 335 mM NaCl (C, G), or were kept as controls (A, E) for 40 min and were thereafter labelled with $^{35}$S-methionine for 15 min at 100 μCi/dish and subsequently chased for 45 min before harvest. Ts-1 marker containing the previously identified non-structural proteins is shown in lane D, and the proteins of $^{35}$S-methionine-labelled SFV is shown in lane H. Position of capsid (C) and envelope proteins E1 and E2 as well as the precursor of E2 and E3, p62, and the non-structural proteins ns70 (70), ns72 (72), ns86 (86), ns135 (135) and ns155 (155) are indicated, as is the new ts-4 specific protein (220).

salt or sucrose. The smaller of these proteins migrated with the previously identified non-structural precursor protein ns155 (mol. wt. 155000) (Lachmi & Kaariainen, 1976, 1977). The larger protein, which is not detected in cultures maintained and labelled at 28 °C, migrated with myosin and thus has a mol. wt. close to 220000.

The possibility that the 220000 mol. wt. protein is virus-specific, and perhaps a new non-structural precursor protein like the large protein found in Sindbis virus infected cells (Bracha et al. 1976; Brzeski & Kennedy, 1977), was investigated as follows: ts-4 infected cultures incubated at 28 °C for 6 h were shifted to 39 °C and treated with 335 mM NaCl for 40 min. This treatment specifically blocks the initiation of protein synthesis but allows elongation to take place (Saborio et al. 1974). When the hypertonic block of initiation is removed by restoring the isotonicity of the medium, synchronous initiation of protein
synthesis takes place. Using different pulse lengths, it is possible to determine the gene order in polyproteins (Saborio et al. 1974; Clegg, 1975). We have used this method to determine the gene order in the non-structural polyproteins of Semliki Forest virus to be ns70, ns86, ns72 and ns60 (Lachmi & Kääriäinen, 1976).

Duplicate cultures of ts-4 infected cells were released from the hypertonic block of initiation and labelled for 2, 6 and 18 min at 39 °C to label the ns70, ns70 plus ns86, and finally all the non-structural proteins (Lachmi & Kääriäinen, 1976). After the pulse, unlabelled methionine was added and incubation was continued either at the restrictive or permissive temperature for a further 60 min. The fluorogram of proteins separated in a polyacrylamide slab gel is shown in Fig. 4. After a 2 min pulse at 39 °C followed by a 60 min chase at 28 °C only ns70 of the non-structural proteins is labelled (Fig. 4, lane A). It seems to consist of a double band under these conditions. Of the structural proteins, the N-terminal capsid protein is labelled but in this polyacrylamide gel it has been run out from the gel to increase the resolution of the large proteins. Lane B in Fig. 4 shows the proteins labelled in cultures pulsed for 2 min at 39 °C followed by a 60 min chase at 39 °C. Essentially

Fig. 4. Fluorogram of 10% polyacrylamide gel showing the sequential labelling of ts-4 induced proteins at 39 °C followed by a chase at 28 °C (A, C, E) or 39 °C (B, D, F). Ts-4 infected cultures maintained at 28 °C for 6 h were shifted to 39 °C and exposed to 335 mM NaCl for 40 min at 39 °C. They were labelled thereafter with 35S-methionine for 2 min (A, B) with 300 μCi/dish, for 6 min (C, D) with 100 μCi/dish, or for 18 min (E, F) with 33 μCi/dish. The cultures were chased after the pulse for 60 min. The designation of the proteins is the same as in Fig. 3.
only two proteins are labelled, namely ns155 and the 220000 dalton protein shown in Fig. 3. This result would indicate that both these proteins are derived from the N-terminal part of the non-structural polyprotein and thus contain ns70.

Labelling for 6 min at 39 °C followed by a 60 min chase at 28 °C shows the presence of ns70, now clearly as a double band, and also of ns86 (Fig. 4, lane C). This was as expected from our previous results using the ts-1 mutant of SFV (Lachmi & Kääriäinen, 1976). The pattern of labelled proteins becomes, however, more complicated due to the start of host protein synthesis. Again, cultures labelled at 39 °C and chased at 39 °C show the ns155 and the 220000 dalton protein but almost no ns86 or ns70, suggesting that both ns70 and ns86 are constituents of the two large proteins. When the labelling period is extended to 18 min, the ns72 protein also becomes labelled in cultures shifted to 28 °C for the chase (Fig. 4, lane E). Only small amounts of ns155 can be seen and the presence of the 220000 dalton
protein is difficult to exclude due to the heavy labelling of host proteins in this region. The cultures labelled and chased at 39 °C show ns155 as heavily labelled, as is the ns135, which appears only after 10 min or longer labelling periods (Fig. 4, lane F). There seems to be some processing of the non-structural polyprotein even at 39 °C, since ns86 and ns72 can be seen when large amounts of radioactivity are applied to the gel.

These results suggest that the new protein with mol. wt. of about 220000 found in ts-4 infected cells pulsed at 39 °C is a virus-specific non-structural precursor protein derived from the N-terminal part of the non-structural polyprotein.

Shut-off of host protein synthesis by hypertonic media

Specific labelling of virus proteins after the hypertonic block of initiation is limited to only a short period of time, since host cell protein synthesis starts to recover within a few minutes after the release of the block (Sabario et al. 1974) as can clearly be seen also from Fig. 4. To overcome this difficulty we determined conditions to specifically label the virus proteins in the presence of hypertonic media. SFV and mock infected cells were grown for 3 h at 39 °C and then incubation was continued in the presence of different concentrations of sucrose for 60 min, followed by a 15 min pulse with 35S-methionine in the same media (Fig. 5a). The protein synthesis in the mock infected cultures is almost completely shut off when incubated and labelled in the presence of 0.2 M-sucrose. Under these conditions protein synthesis in SFV infected cells was reduced to about 20% of the level in the presence of isotonic medium.

We took advantage of these results to label the cells after hypertonic block of initiation induced by 335 mM-NaCl. As can be seen, there is some recovery of host cell protein synthesis during the 30 min pulse with 35S-methionine when mock infected cells are transferred to isotonic medium (Fig. 5b). If the mock infected cells are labelled in 0.1 M or higher sucrose, no recovery of protein synthesis is seen. However, SFV infected cultures showed reduced but detectable protein synthesis in media containing up to 0.3 M-sucrose.
The polyacrylamide slab gel in Fig. 6 shows the effect of different concentrations of sucrose on the labelling of the large proteins in ts-4 and mock infected cells after release from the hypertonic block of initiation. The cells were labelled for 30 min and chased for 105 min before analysis of the proteins. In the mock infected cells, a large protein migrating more slowly than the 22000 dalton ts-4 protein is clearly seen if the labelling is carried out in the absence of sucrose (Fig. 6, lane C), but is not detectable when 0.1 M or higher sucrose concentrations are used during the labelling (Fig. 6, lanes E, G and I). The same protein is also seen in the ts-4 infected cells labelled without sucrose but not in those labelled in the presence of sucrose. The most heavily labelled protein is the ns155, but the 220000 dalton protein is seen with all the sucrose concentrations used during the labelling.

These results support the idea that the 220000 dalton protein in ts-4 infected cells is clearly a virus-specific one. The pulse-chase experiments suggested that it is most probably a precursor of the non-structural proteins, and therefore we designate it as ns220. The results also show that unequal labelling of some host proteins after the release of hypertonic block of initiation can cause severe difficulties in the interpretation of the results.

DISCUSSION

We have studied the RNA and protein synthesis of a temperature sensitive mutant, ts-4, of Semliki Forest virus. Ts-4 is phenotypically an RNA negative mutant, which cannot synthesize significant amounts of virus RNA when infection is initiated at the restrictive temperature (Kerätänen & Kääriäinen, 1974). When infection is started at the permissive temperature, it is able to continue RNA synthesis for long periods of time, showing that the RNA polymerase function of this mutant is intact.

As we have previously reported, the relative amounts of the RNAs synthesized are affected by the shift to the restrictive temperature: synthesis of 26S RNA stops rapidly whereas that of 42S RNA continues (Saraste et al. 1977; Sawicki et al. 1978). Thus the mutation apparently affects a protein which regulates the synthesis of 26S RNA (Sawicki et al. 1978). The functional defect is reversible since 26S RNA synthesis starts again when the cultures are shifted back to the permissive temperature, it is able to continue RNA synthesis for long periods of time, showing that the RNA polymerase function of this mutant is intact.

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The fact that there is no increase in the RNA synthesizing activity after the ts-4 infected cultures were shifted to 39 °C suggests that no new active RNA polymerase was formed at this temperature. The same result was obtained for SFV wild type infected cultures exposed to cycloheximide (see also Wengler & Wengler, 1975; Clegg et al. 1976).

These results can be simply explained by the accumulation of a large 220000 dalton protein in the ts-4 infected cultures labelled after shift to 39 °C. This protein seems to be virus-specific and a non-structural protein precursor since it is labelled under conditions when practically no host proteins are synthesized and is processed in cultures labelled at 39 °C and chased at 28 °C, to yield previously identified non-structural proteins. The labelling of ns220 and ns155 at 39 °C during a 2 min pulse after release of the cells from hypertonic block of initiation would suggest that both these proteins are translated from the 5'-end of the 42S RNA, which is the messenger RNA for the non-structural proteins of Semliki Forest virus (Glanville & Lachmi, 1977).

The functional defects of ts-4 can be understood if we assume that the mutated protein regulating the synthesis of 26S RNA is one of the non-structural proteins, which are apparently cleaved from a giant polyprotein (Cancedda et al. 1975; Lachmi & Kääriäinen, 1976; Glanville et al. 1976). A mutation in one of the non-structural proteins apparently causes a cleavage defect in the polyprotein and results in accumulation of ns220 and ns155.

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The resultant large proteins contain polypeptides of the RNA polymerase which cannot function in the uncleaved form. This would explain why no increase in RNA synthesis takes place after the ts-4 infected cultures have been shifted to the restrictive temperature.

In wild type SFV infected cells a short-lived protein of comparable size to our ns22o has been described by Clegg et al. (1976). Pulse-chase experiments suggested that this protein (p200) is a precursor of the RNA polymerase components pol 63 and pol 90 (Clewley & Kennedy, 1976). Two non-structural precursor proteins with apparent mol. wt. of 230000 and 215000 accumulate in Sindbis virus wild type infected cells in the presence of zinc ions (Brzeski & Kennedy, 1977). Taken together, these results suggest that our 220 may represent accumulation of an intermediate in the cleavage of the large non-structural polyprotein detectable in the wild type infected cells as well.

More than one mutation in the non-structural proteins of ts-4 cannot be ruled out at present. Since the synthesis of 42S RNA continues after the shift to 39 °C this mutation is not manifested at 39 °C when the protein has been synthesized at the permissive temperature. A second mutation would presumably only affect the cleavage defect(s) resulting in the accumulation of ns220 and ns155. It is of interest that in the structural polyprotein of alphaviruses, mutation of the capsid protein is known to cause accumulation of the whole polyprotein in uncleaved form indicating that several cleavages can be inhibited by a single mutation (Burge & Pfefferkorn, 1966; Scheele & Pfefferkorn, 1970; Schlesinger & Schlesinger, 1973; Keriinen & Kääriäinen, 1975; Lachmi et al. 1975).

This investigation was supported by the Finnish Academy, Sigrid Juselius Foundation and Finnish Science Society. We are grateful for the technical assistance of Ms Raija Lahdensivu. Actinomycin D was a kind gift from Merck, Sharp & Dohme.

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(Received 11 October 1977)