COMPLEMENTATION IN 26 S RNA SYNTHESIS BETWEEN TEMPERATURE-SENSITIVE MUTANTS OF SEMLIKI FOREST VIRUS

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1. Introduction

In alphavirus infected cells two major virus specific RNAs are synthesized; the genome 42 S RNA and so-called 26 S RNA [1–3]. The 26 S RNA serves as the messenger for the viral structural proteins [4–8] and is a replica of about 1/3 of the 42 S genome RNA [3,9] at its 3′-end [10]. Both of these RNAs are problaby transcribed on the same template molecule, the complementary, negative strand of 42 S RNA [11]. The synthesis of 26 S RNA is specifically inhibited either by protein synthesis inhibitors early in viral replication [12] or at the restrictive temperature in cells infected with certain temperature-sensitive mutants [12–15].

Several groups of alphavirus temperature-sensitive (ts) mutants have been isolated during the last decade [14,16–19]. The mutants have been divided into different phenotypic groups on the basis of their ability to induce viral RNA synthesis at the restrictive temperature. Our Semliki Forest virus mutants form three groups: RNA positive (RNA^+), intermediate ($RNA^\pm$) and RNA negative (RNA^-) which induce 40–100%, 10–20% and $< 5\%$ of the levels of wild type RNA synthesis respectively [14].

So far complementation data are not available for Semliki Forest virus mutants, and complementation has not been detected with the Sindbis virus mutants isolated by Atkins et al. [18]. The Sindbis virus mutants, isolated from the same heat resistant wild type virus by Burge and Pfefferkorn [16] and by Strauss et al. [19] have been divided into seven complementation groups, four of which are composed of mutants defective in RNA synthesis [19,20]. The virion 42 S RNA of Semliki Forest virus is infectious [1] implying, that the enzymes needed for viral RNA synthesis are not virion components, and consequently they must be induced in the cell upon virus infection. Four virus specific non-structural proteins have been identified in Semliki Forest virus infected cells in our laboratory [21,22], and at least two of them are evidently involved in viral RNA synthesis [23].

The existence of mutants specifically deficient in 26 S RNA synthesis implies that at least one virus specific protein is needed in the synthesis of this RNA molecule. One of our Semliki Forest virus mutants, ts-1, which is phenotypically RNA positive, makes only small amounts of 26 S RNA at the restrictive temperature [14,24]. Sindbis virus mutants, phenotypically RNA negative, show deficiency in the same function, synthesis of 26 S RNA, in temperature shift up experiments [12,13,15] and a similar defect was recently found in one of our Semliki Forest virus mutants (Saraste et al., submitted for publication).

In the present study RNA negative mutants were tested for their ability to complement with ts-1 in the synthesis of 26 S RNA during mixed infection. By analyzing the labelled RNA instead of measuring the production of infectious virus, higher doses of input virus could be used without the inoculum disturbing the results as is the case in conventional complementation studies [20]. Qualitative rather than quantitative change in the RNA synthesis was measured. The RNA negative mutants were divided into two groups: those which were able to change the 42 S:26 S RNA ratio towards the normal (complementing ones) and those unable to do so (non-complementing ones). Wild type virus corrected the ratio virtually to the normal.

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2. Materials and methods

Propagation of wild type Semliki Forest virus and the mutants isolated from it were as described before [11]. Secondary specified pathogen free chick embryo fibroblasts were used as confluent monolayers in 60 mm plastic plates (Falcon plastics or Lux Scientific Corporation).

To study the RNA synthesis duplicate plates were infected with 60 PFU/cell at restrictive temperature (39°C). Actinomycin D (2 μg/ml) was present throughout. For mixed infections the two virus preparations were mixed prior to inoculation to give a final dose of 30 PFU/cell of each virus. After 1 h absorption the inoculum was removed and the cells were washed once before adding the medium. At 3 h post infection the medium was removed and replaced with 2 ml of fresh medium containing 20 μCi/plate of [^{3}H]uridine (25–29 Ci/mmol, Amersham). After 2 h incubation the cells were washed once with 0.01 M Tris–HCl pH 7.4, 0.15 M NaCl, 0.001 M EDTA-Na (TSE) and collected in 0.5 ml/plate of TSE containing 2% sodium dodecyl sulphate (SDS) as described before [14], and the acid-insoluble radioactivity was measured as before [2]. RNA sedimentation analysis was performed on 15–30% (w/w) sucrose gradients made in TSE containing 0.1% SDS as described before [25].

3. Results and discussion

Our ts-1 is very efficient in overall RNA synthesis but has a definite defect in 26 S RNA formation as can be seen in table 2. This must mean that its RNA polymerase, responsible for the synthesis of 42 S RNA is functional at the restrictive temperature. The mode of action of the postulated 'conversion protein' [12] or '26 S protein' [14] regulating the synthesis of 26 S RNA is not known, but as ts-1 shows, it is not needed for the RNA synthesis per se.

The rationale behind the present study was that different RNA negative mutants might be defective in different proteins needed for viral RNA synthesis. Since ts-1 is deficient only in the formation of 26 S RNA, those RNA negative mutants in which the 'conversion protein' is functional, can presumably supply this function in mixed infection with ts-1. The resultant complementation in 26 S RNA synthesis was studied by measuring the amount of 42 S and 26 S RNA synthesized in infected cells, as illustrated in figs 1 and 2. Under these experimental conditions, using a long pulse in the middle of the growth cycle, the amount of replicative intermediates is very low [2,14] and thus the radioactivity sedimenting at 26 S region can be used as a measure of the amount of the 26 S RNA.

In wild type infected cells the ratio of 42 S:26 S RNA is about 1.4, whereas for ts-1 it is markedly higher (table 1, see also reference [14]). When the cultures are simultaneously infected with both viruses, the ratio returns to that of the wild type virus (fig.1, table 2). This would mean that either the 42 S RNA synthesis of ts-1 is inhibited or the 26 S RNA synthesis is enhanced. If the latter is true,

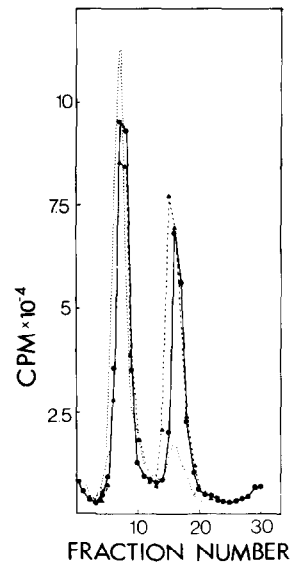

Fig.1. Sucrose gradient analysis of virus specific RNAs from infected cells. Cells were infected either with ts-1 or wild type Semliki Forest virus or simultaneously with both viruses at 39°C in the presence of actinomycin D (2 μg/ml). A [^{3}H]uridine pulse (20 μCi/plate) was given 3 to 5 h postinfection after which the cells were lyzed in 2% SDS and RNAs were analyzed on 15 to 30% sucrose gradients. Centrifugation was for 12 h at 24 000 rev./min at 22°C in Spinco SW27.1 rotor. Acid-insoluble radioactivity in the gradient fractions was measured. Sedimentation from right to left. (○. . . .○) ts-1; (▲- - -▲) wild type Semliki Forest virus; (●——●) ts-1 × wild type Semliki Forest virus.

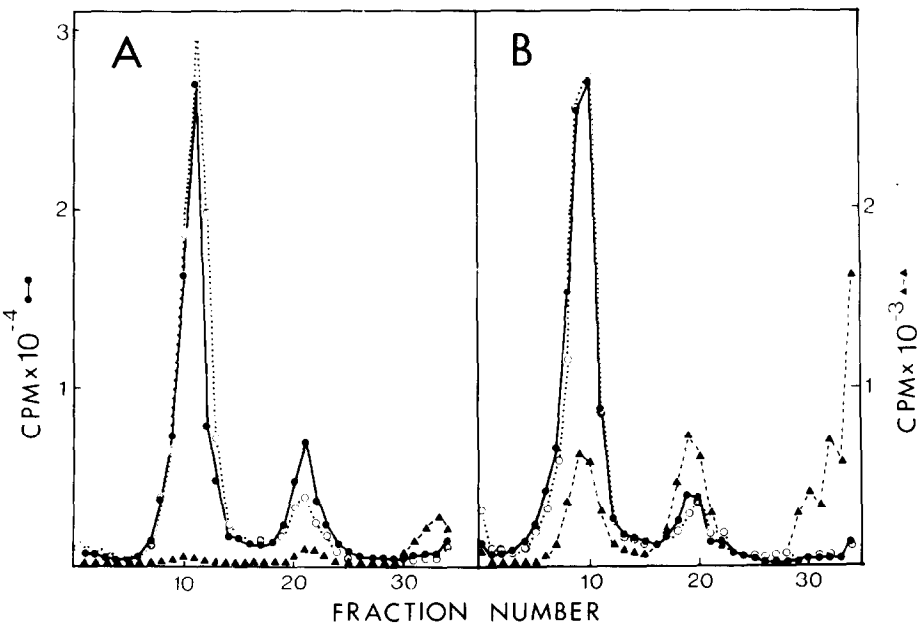

Fig. 2. Sucrose gradient analysis of virus specific RNAs from infected cells. Experimental conditions as for fig. 1. Notice the difference in the scale for the triagles. A: (○. . . .○) ts-1; (▲- - -▲) ts-8; (●——●) ts-1 × ts-8. B: (○. . . .○) ts-1; (▲- - -▲) ts-14; (●——●) ts-1 × ts-14.

the component needed for 26 S RNA synthesis is provided by the wild type virus to the replication complex of ts-1, suggesting that this component is free to diffuse inside of the cell.

Based on this supposition the RNA negative mutants were tested for their ability to enhance the synthesis of 26 S RNA in mixed infections with ts-1. The overall RNA synthesis in the mixed infections was about the same as in ts-1 controls, or slightly higher (table 2). However, two mutants, ts-4 and ts-11, interfered with the RNA synthesis of ts-1 and are therefore not included in this study. Interference induced by ts-11 seems to be caused by a structural component of the parental virus and has been described elsewhere [25].

Two of the non-interfering mutants, ts-8 and ts-12, could apparently enhance the synthesis of 26 S RNA but neither of them could change the 42 S:26 S RNA ratio to that of the wild type virus (table 2). This could be the case if the 42 S RNA of these mutants

Table 1
42 S : 26 S RNA ratio in infected cells[a]

	Number of determinations	Mean ratio of 42 S : 26 S RNA	Range
Wild type Semliki Forest virus	19	1.4	1.1–2.4
ts-1	13	5.9	4.3–8.2

[a]Data were collected from several experiments in which the cells were infected with 20–50 PFU/cell at 39°C in the presence of actinomycin D. RNA, labelled from 3 to 5 h postinfection was analyzed on 15 to 30% sucrose gradients (as for fig. 1) and the acid-insoluble radioactivity in 42 S and 26 S RNA was determined

Table 2
RNA synthesis in mixed infections with Semliki Forest virus mutants[a]

Cells infected with	Number of determinations	Total RNA % of ts-1 control	% of total in		42 S : 26 S RNA ratio	Complementation in 26 S RNA synthesis
			42 S RNA	26 S RNA		
ts-1	3	100	71	14	5.1	
Wild type virus	2	124	49	38	1.3	
ts-1 × wild type virus	2	107	52	33	1.6	+
ts-1 × ts-6	2	132	74	13	5.7	–
ts-1 × ts-8	3	90	68	18	3.8	+
ts-1 × ts-9	2	118	73	12	6.1	–
ts-1 × ts-12	3	93	67	19	3.5	+
ts-1 × ts-14	3	109	73	14	5.2	–

[a]Experimental conditions as for fig.1. Acid-insoluble radioactivity in sedimenting form (> 10 S) was used to calculate the incorporation

is not replicated and translated efficiently under these conditions and the amount of the 'conversion protein' remains therefore low. This would mean, that ts-1 is not able to supply the RNA polymerase function to the RNA negative counterpart. Different efficiencies of the replication between the two genomes could be expected for example, if the components of the RNA polymerase complex are not free to diffuse but rather form a complex at or near the site of their synthesis and start more often the replication of the very same RNA molecule on which they were translated.

The complementation levels obtained with alphavirus mutants are generally fairly low [19,20] and as discussed by Strauss et al. [19] the RNA negative mutants are especially difficult in these studies. Therefore I suggest that the slight enhancement in the synthesis of 26 S RNA obtained with ts-8 and ts-12 could be taken as a sign of complementation.

Small amount of viral RNA is synthesized even by the RNA negative mutants at the restrictive temperature. When this RNA was analyzed (table 3) it turned out that two of the non-complementing mutants, ts-6 and ts-9, were specifically deficient in 26 S RNA synthesis, so that most of the residual RNA synthesis resulted in 42 S RNA production. The complementing mutants made relatively higher amounts of 26 S RNA. The results fit well to the complementation pattern, except in the case of ts-14, in which the 42 S:26 S RNA ratio was more or less normal, but which did not complement. The reason for this remains open.

Although several alphavirus mutants show a deficiency in 26 S RNA synthesis it does not mean that they all are defective in the postulated 'conversion protein'. Since the nonstructural proteins appear to be translated as a polyprotein [22] it is possible that the following cleavage process is affected by the mutations. Thus polypeptides with no defect in their

Table 3
RNA synthesis by the RNA$^-$ mutants at the restrictive temperature[a]

Cells infected with	Number of determinations	Total RNA % of ts-1 control	42 S : 26 S RNA ratio
ts-6	2	5.1	3.0
ts-8	3	2.0	0.6
ts-9	2	2.5	3.5
ts-12	3	2.6	0.9
ts-14	3	4.2	1.0

[a]Experimental conditions as for table 2

structure would become inactive since they remain in the precursor form. Similar defects have been detected in the structural proteins of Sindbis virus [26] and Semliki Forest virus [27].

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