SEMLIKI FOREST VIRUS-SPECIFIC NONSTRUCTURAL PROTEIN IS ASSOCIATED WITH RIBOSOMES

Marjut RANKI, Ismo ULMANEN and Leevi KÄÄRIÄINEN
Department of Virology, University of Helsinki, Haartmaninkatu 3, SF-00290 Helsinki 29, Finland

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1. Introduction

Semliki Forest virus (SFV) 42 S RNA genome codes probably for eight different polypeptides. Four of these are nonstructural proteins with mol. wt 70 000 (ns70), 86 000 (ns86), 72 000 (ns72) and 60 000 (ns60). They are translated as a giant polyprotein in the above order [1,2]. At least three of them have been found in association with the RNA replication complex [3]. The structural proteins, capsid protein and envelope glycoproteins E1, E2 and E3, are translated from a subgenomic 26 S mRNA as a 130 000 mol. wt polyprotein [4,5]. The amino-terminal capsid protein is cleaved from the growing polyprotein before the translation of the envelope proteins starts [5,6]. The nascent envelope proteins penetrate the ER membrane, become glycosylated and are transported to the plasma membrane [5,6].

After cleavage the capsid protein remains associated with the polysomal ribosomes until it is transferred to the nucleocapsid (which consists of 42 S RNA and capsid proteins, [4,5]) or is released to the monosome pool after the translation of the polyprotein has been completed [7–9].

Here we report that one of the nonstructural proteins, ns86, is also associated with ribosomes and can be crosslinked with the ribosomal RNAs by ultraviolet irradiation.

2. Materials and methods

The origin and cultivation of the SFV prototype strain in HeLa and BHK21 cells have been described [3,8]. For labeling of ribosomal RNAs the cells were exposed to [^{3}H]uridine (Amersham) for 18 h prior to infection with SFV as in [7,8]. Labeling of virus-specific proteins with [^{35}S]methionine (after high salt shock, 15 μCi/10^6 cells) was done as in [3] for 30 min followed by a 5 min chase.

For analysis of the cytoplasm the cells were broken by Dounce homogenization after swelling in hypotonic buffer (0.01 M NaCl, 0.01 M Tris–HCl (pH 7.4), 0.0015 M $MgCl_2$). The mitochondrial pellet (P15) was obtained from the postnuclear supernatant by centrifugation at 15 000 × *g* for 20 min. The respective supernatant is designated as S15. Sucrose gradient centrifugation of cellular fractions and analysis of RNAs was as in [3,7,8]. CsCl density gradient centrifugation and salt treatment of the ribosomes was done as in [8]. The proteins were analyzed by discontinuous sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and quantitated as in [2].

For ultraviolet irradiation the samples (300 μl) were layered on parafilm on ice and exposed to a 15 W Hanovia lamp operating at 254 nm at a distance of 11 cm. The surface energy density after 10 min exposure was 0.25 J/cm^2.

Immunoprecipitation with anticapsid IgG was done as in [7].

3. Results and discussion

When the postnuclear supernatant from SFV-infected cells is further fractionated by centrifugation at 15 000 × *g* for 20 min the viral envelope glycoproteins and RNA replication complex are sedimented almost quantitatively [3] leaving ~ 50% of the viral nucleocapsids in the supernatant, S15. If the S15 is

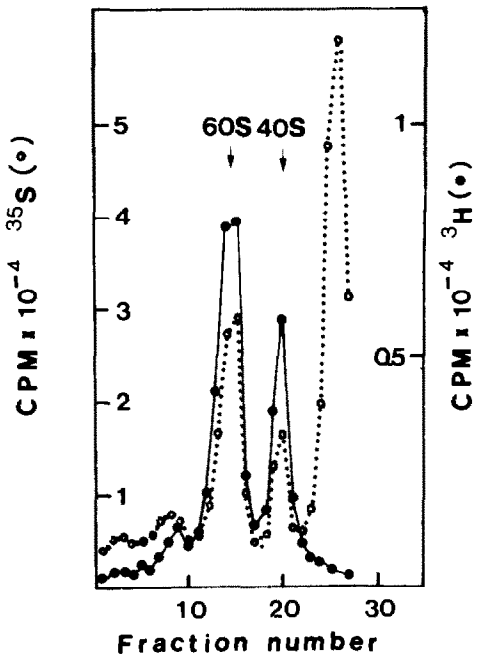

Fig.1. Sucrose gradient analysis of ribosomal subunits from S15. Infected HeLa cells, labeled with [^{3}H]uridine prior to infection, were exposed to [^{35}S]methionine for 30 min 4.5 h after infection. S15 was treated with EDTA (20 mM) and layered on top of a 15–30% (w/w) sucrose gradient made in 0.14 M NaCl, 0.01 M Tris–HCl (pH 7.4) and 0.001 M EDTA. Centrifugation was in a SW 27.1 rotor at 25 000 rev./min for 17 h at 4°C. Acid-insoluble radioactivity was determined from aliquots of the fractions.

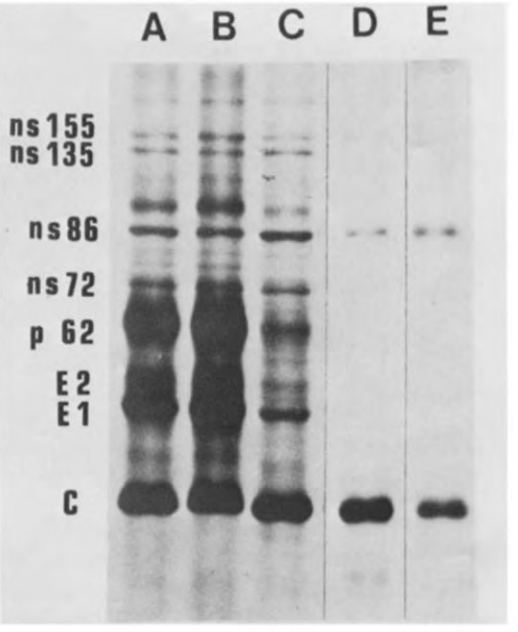

Fig.2. Fluorogram of 7.5% polyacrylamide slab gel of the postnuclear supernatant (A), P15 (B), S15 (C) and 60 S (D) and 40 S (E) ribosomal subunits isolated from S15. The 60 S and 40 S peaks were pooled and aliquots treated with 0.5 M NaCl at 0°C, followed by resedimentation at 24 000 rev./min for 19 h in SW 27 rotor. The resedimented subunits were pelleted, the pellets resuspended in the gel sample buffer and electrophoresed for 4.5 h at 15 mA.

treated with 20 mM EDTA to dissociate the polyribosomes and monosomes some virus-specific proteins sediment with the ribosomal subunits (fig.1). The identification of the 60 S and 40 S peaks as ribosomal subunits is based on their density in CsCl as well as their content of 28 S and 18 S prelabeled RNA, respectively.

Resedimentation of the 60 S and 40 S ribosomal subunits after treatment with 0.5 M NaCl was carried out with 52% and 34% recoveries of the [^{35}S]-methionine label, respectively. Polyacrylamide gel electrophoresis analysis of the labeled proteins of the resedimented subunits is shown in fig.2. Only two virus-specific proteins, the capsid protein and the nonstructural protein ns86 can be seen. The association of capsid protein with the ribosomes has been established and studied in more detail in [7–9], whereas the association of ns86 with ribosomes is a new finding.

Quantitation of ribosome-associated capsid and ns86 proteins was carried out for both S15 and P15 (table 1). After EDTA treatment most of the capsid protein is associated with the large subunit whereas the distribution of ns86 is almost equal in both subunits.

Table 1
Distribution of [^{35}S]methionine-labeled capsid-and ns86-protein among ribosomes derived from P15 and S15

Cell fraction		cpm × 10^{-3} of [^{35}S]methionine	
		Capsid	ns86
S15:	60 S	1419	96
	40 S	306	79
P15:	60 S	264	67
	40 S	32	62

Table 2
Immunoprecipitation of ribosomal subunits from S15 with anticapsid IgG

Material	Treatment	Relative radioactivity ($^{35}S/^{3}H \times 100$)[a]	
		Capsid	ns86
40 S	None	137	35
40 S	Anti-capsid IgG	757	35
60 S	None	228	15
60 S	Anti-capsid IgG	773	17

[a] Capsid and ns86 bands from polyacrylamide gels were excised and radioactivity determined. The ^{35}S radioactivity was divided by the [^{3}H]uridine activity of the sample to standardize in respect to the amount of ribosomal RNA

When the isolated ribosomal subunits were subjected to immunoprecipitation with anti-capsid IgG, the precipitate showed enrichment of only capsid but not ns86-protein. This suggests that the two viral proteins are not prevalently linked to the same ribosomes (table 2).

Ultraviolet light induces covalent crosslinkage between nucleic acids and proteins [10] and it has turned out to be a useful technique in studying nucleic acid and protein interactions [11]. Here, the ribosomal subunits from infected cells were exposed to ultraviolet light to find out whether the virus-specific proteins could be crosslinked to ribosomal RNAs. The experimental conditions were such that after irradiation (10 min exposure) the ribosomal subunits could be reisolated without detectable change in sedimentation rate. Furthermore, no nonspecific crosslinking of a soluble protein ([^{14}C]ovalbumin, 0.2 mg/ml) to the ribosomal RNAs occurred under the conditions used.

Figure 3 shows that virus specific protein label in both 40 S and 60 S subunits became crosslinked to RNA and that crosslinking was proportional to the ultraviolet dose. After 10 min irradiation ~11% of the [^{35}S]methionine label in both cases was found cosedimenting with the ribosomal RNA. To analyze the proteins that evidently were crosslinked, the RNA pools in fig.3 (10 min irradiation) were precipitated with ethanol, then treated with ribonucleases T1, T2 and A and the released proteins subjected to analysis by polyacrylamide gel electrophoresis. Both ns86

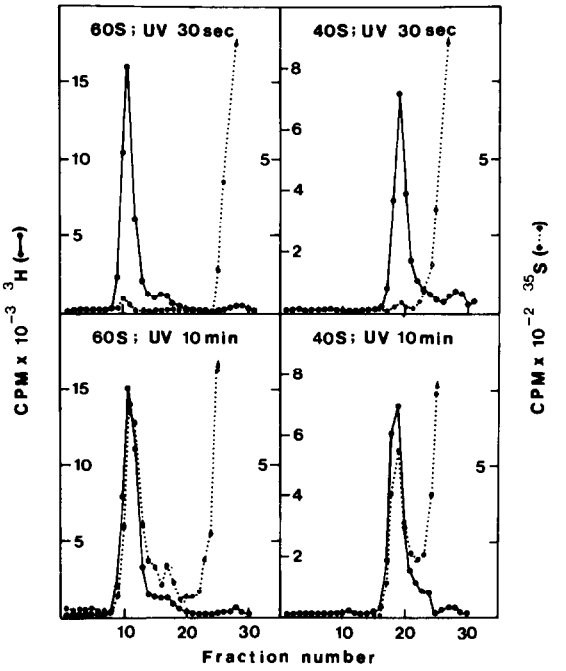

Fig.3. Ultraviolet light-induced crosslinking of viral proteins to ribosomal RNA. The ribosomal subunits (fig.1) were exposed to ultraviolet irradiation for 30 s and 10 min giving a surface energy density of 0.012 and 0.25 J/cm^2, respectively. After treatment the ribosomes were dissociated with SDS and the RNAs isolated by centrifugation in 15–30% sucrose gradients (SW 27.1 rotor, 25 000 rev./min, 17 h 22°C).

and capsid protein were detected in the gel. The recovery of the proteins after analysis was, however, too small to allow precise quantitation. If ribonuclease treatment was omitted no proteins entered the gel confirming that covalent crosslinkage to 28 S and 18 S RNA had occurred.

From the eight polypeptides coded by SFV two are specifically associated with ribosomes. In the case of capsid protein this association seems to serve an important function in the assembly of the nucleocapsid [7]. The ns86 protein has been identified as a putative component of the viral RNA replication complex [3], but its exact function is unknown and so is the significance of its association with the ribosomes. Anyhow, it is the only one of the three viral nonstructural proteins involved in the RNA synthesis, which has affinity to ribosomes.

Quantitations presented here and in [7] imply

that only a small proportion of the ribosomes carry ns86. Thus it is improbable that ns86 could be responsible for, e.g., the host protein synthesis inhibition in the α-virus-infected cells [4,5].

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