CELL-FREE SYNTHESIS OF VESICULAR STOMATITIS VIRUS PROTEINS: TRANSLATION OF MEMBRANE-BOUND POLYRIBOSOMAL mRNAs

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1. Introduction

Vesicular stomatitis virus (VSV) is a negative-strand RNA containing enveloped virus [1]. The nucleocapsid contains the proteins L, N and NS and the viral RNA [1–3]. The L protein is essential for the transcription of viral RNA [4]. The viral envelope contains two virus-specific proteins M, found on the inside of the envelope and the glycoprotein G which forms the spikes and determines the antigenic property of VSV [1,2]. In the early stages of infection both G and M are found associated with host cell membranes suggesting that these two proteins may be synthesized by membrane-bound polyribosomes [5]. We have recently shown that polyribosomes obtained from VSV-infected L cells synthesize in vitro all five viral proteins. When membrane-bound polyribosomes were removed, however, the synthesis of G was no longer observed [6]. Similar results were reported using VSV-infected HeLa cell extracts [7]. In the present communication we show that only 16–19S RNA isolated from membrane-bound polyribosomes directs the synthesis of G. 12–15S and 16–19S RNAs isolated from free polyribosomes and 12–15S RNA isolated from membrane-bound polyribosomes direct the synthesis of N, NS and M.

2. Experimental

The growth of L cells, infection with VSV, isolation and characterization of VSV proteins and preparation of infected L cell extracts is as described earlier except that 2 μg/ml of actinomycin D is added 1 hr post-infection [6]. The free and membrane-bound polyribosomes are fractionated by centrifuging 1 ml of the S-4 extract on a discontinuous sucrose gradient of 2 ml of 2 M sucrose, 2.5 ml of 1.5 M sucrose and 2.5 ml of 0.5 M sucrose at 150 000 *g* for 3 hr [8]. The free polyribosomes are pelleted to the bottom and the membrane-bound polyribosomes, collected at the interphase of 1.5 M and 2 M sucrose, are recovered by centrifugation. The polyribosomal pellets are then dissolved in 0.5% SDS – 1 mM EDTA in 0.01 M Tris–HCl (pH 7.5) – 0.1 M NaCl and incubated at 37°C for 15 min. The RNA is fractionated by centrifugation on a 15–30% sucrose gradient for 18 hr at 80 000 *g* at 20°C. The pooled fractions (12–19S or 12–15S and 16–19S) are recovered by precipitation with 2.5 vol of ethanol in the presence of 0.1 M potassium acetate (pH 5.5). The wheat embryo extract is obtained by a modification of our earlier procedure using commercial wheat germ [9]. The wheat germ is ground with sand in presence of a buffer containing 0.02 M Hepes (pH 7.6), 0.001 M magnesium acetate, 0.100 M KCl, 0.002 M $CaCl_2$ and 0.006 M 2-mercaptoethanol. The extract is centrifuged at 20 000 *g* for 10 min then passed through a Sephadex G-25 column (25 × 0.9 cm) and stored at −190°C. The ascites cell-free extract (S-20) is prepared according to Mathews [10].

3. Results and discussion

Gel electrophoretic analysis of the products of in

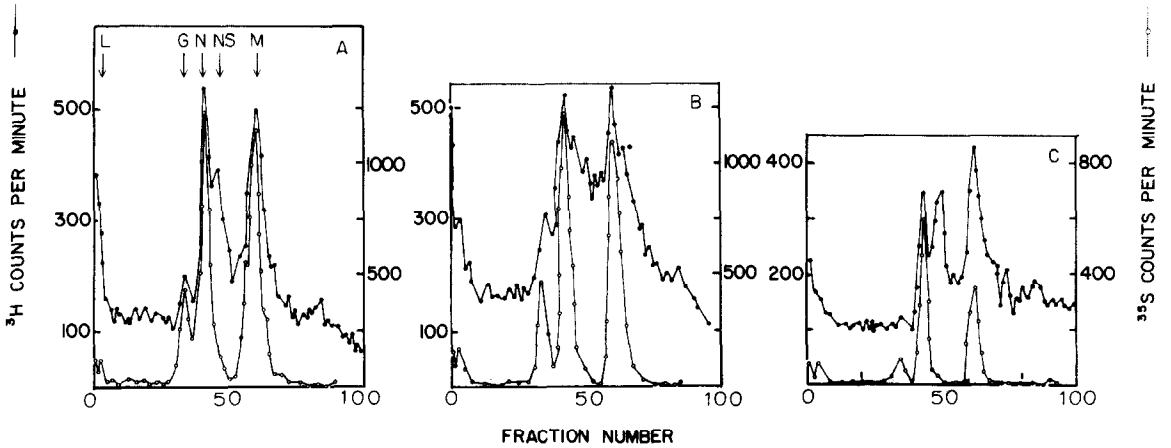

Fig.1. Electrophoretic profiles of [^{3}H]valine labeled proteins synthesized in vitro by unfractionated, free and membrane-bound polyribosome extracts. In the case of membrane-bound and free polyribosomes, S-150 supernatant fractions (1 mg/ml) from infected cells were also present. [^{35}S]Methionine labeled VSV proteins were used for internal calibration. Reaction mixtures containing [^{3}H]valine labeled proteins were treated with NaOH, precipitated with 10% Cl_3CCOOH containing 6% casamino acids washed with 5% Cl_3CCOOH, and acetone and redissolved in 2% SDS-0.2% mercaptoethanol and heated at 100°C for 2 min. Approx. 30 000 cpm [^{3}H]-labeled proteins synthesized in vitro were co-electrophoresed with 10 000 cpm of [^{35}S]-labeled VSV marker proteins on a 10 cm 7.5% polyacrylamide gel containing 0.1% SDS. A) Unfractionated extract (S-4) B) Membrane-bound polyribosomes and C) Free polyribosomes.

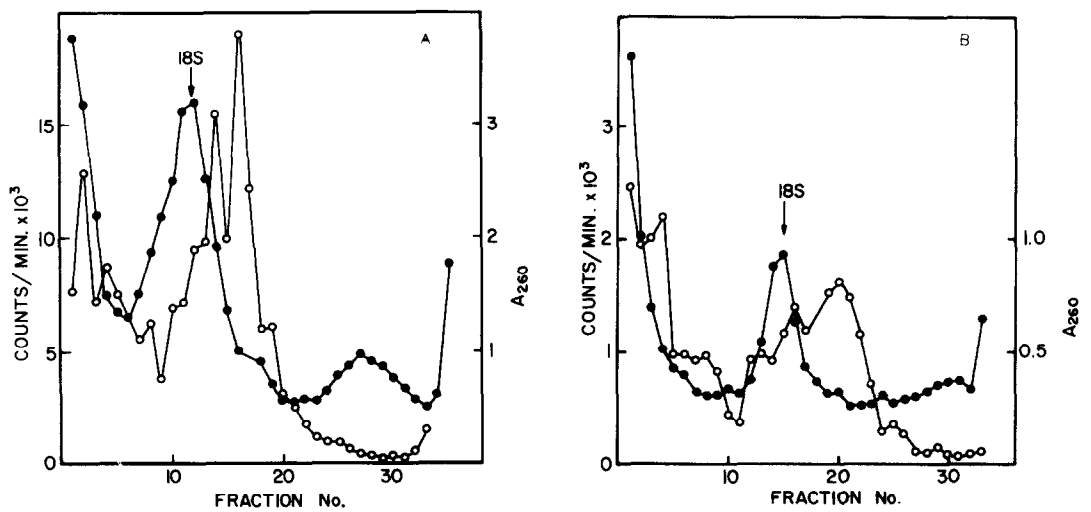

Fig.2. Sedimentation analysis of polysomal RNAs from free and membrane-bound polysomes on sucrose gradients. One hour post-infection of 2 × 10^8 L cells with VSV (4 × 10^9 p.f.u.), 2 μg/ml of actinomycin D was added and after 30 min 2.7 μCi/ml of [^{3}H]uridine (46.2 Ci/mmole) was added. Infected cells were harvested at 4 hr post-infection and membrane-bound and free polysomes were isolated. The labeled RNAs were extracted and analyzed on 15–30% SDS-sucrose gradients. Centrifugation was carried out at 220 000g for 9½ hours at 25°C. 0.3 ml fractions were collected and 5% Cl_3CCOOH precipitable radioactivity and the A_{260} profile were analyzed. A) RNA from free polysomes, B) RNA from membrane-bound polysomes. (●–●–●) A_{260}; (○–○–○) counts/minute of [^{3}H]uridine incorporated.

vitro protein synthesis show that both unfractionated and membrane-bound polyribosomes synthesize proteins which comigrate with viral proteins G, N, NS and M (figs. 1A and 1B). In the case of membrane-bound polyribosomes the peak corresponding to G in the product moved slightly faster than the viral marker G (fig. 1B). The product obtained from free polyribosomes showed peaks corresponding to N, NS and M but no significant peak migrating with or close to G was observed (fig. 1C).

The VSV specific polysomal RNAs from infected L cells were labelled with [^{3}H]uridine and the labelled RNA fractions from the membrane-bound and free polysomes were analyzed. Results (fig.2) show that two distinct species of RNAs sedimenting around 16–17S and 13–14S are present in the free polysomes while the membrane-bound polysomes appear to contain an additional and partially resolved species of RNA sedimenting at 18–19S region. Since G protein has a mol. wt of 69 000, the 18–19S RNA fraction present in the membrane-bound polysomes could code for G. The VSV specific 28S mRNA moved into the bottom of the gradient. In order to test the mRNA activities of the polysomal RNA fractions, we isolated and fractionated RNAs from membrane-bound and free polysomes. The A_{260} profiles did not show any peaks at 18–19S, 16–17S and 13–14S regions because of the presence of excess 18S rRNA. We, therefore, pooled RNA fractions sedimenting at 12–15S and 16–19S. Translation of these RNA fractions in ascites and wheat embryo extracts shows that maximum stimulation was obtained in the wheat embryo system (table 1). The polypeptide products directed by 12–15S and 16–19S RNA fractions were analyzed by electrophoresis on SDS (sodium dodecyl sulfate) polyacrylamide gels (figs. 3 and 4). The 12–15S RNA fractions directed only the synthesis of N, NS and M (fig.3). The 16–19S RNA fractions also directed the synthesis of N, NS and M (fig.4). The 16–19S RNA fraction from membrane-bound polyribosomes, in addition, showed the synthesis of a protein which moved slightly faster than authentic G protein (fig. 4B). Preliminary data shows that the [^{35}S]methionine containing tryptic peptides obtained from the slightly faster moving peaks of in vitro synthesized G have similar electrophoretic mobilities as the tryptic peptides obtained from viral G. The slightly faster electrophoretic mobility of the synthesized G protein thus might be due to non-glycosylation of G in vitro. Morrison et al. [11], also observed an increased mobility of in vitro synthesized G protein.

The results presented in this communication show that the glycoprotein G is synthesized exclusively on the membrane-bound polyribosomes. The specific

Table 1
Stimulation by mRNA fractions from free polyribosomes and membrane-bound polyribosomes in wheat embryo and ascites extracts

mRNA		[^{35}S]Methionine incorporated (cpm)	
		Wheat embryo extract	Ascites extract
-mRNA		16 800	12 500
Free	12–19	202 500	52 600
Polyribosomal	12–15	160 300	–
RNA	16–19	79 700	–
Membrane-bound	12–19	310 800	30 700
Polyribosomal	12–15	112 800	–
RNA	16–19	73 600	–

Reaction mixtures (0.025 ml) contained mRNA fractions, [^{35}S] methionine (specific activity 90 Ci/mmol) and either ascites or wheat embryo extracts. The optimum Mg^{2+} and K^+ concentrations were found to be 3.5 mM and 90 mM for wheat embryo extracts and 3.5 mM and 100 mM for ascites extracts. Aliquots (0.015 ml) were counted for hot Cl_3CCOOH insoluble material.

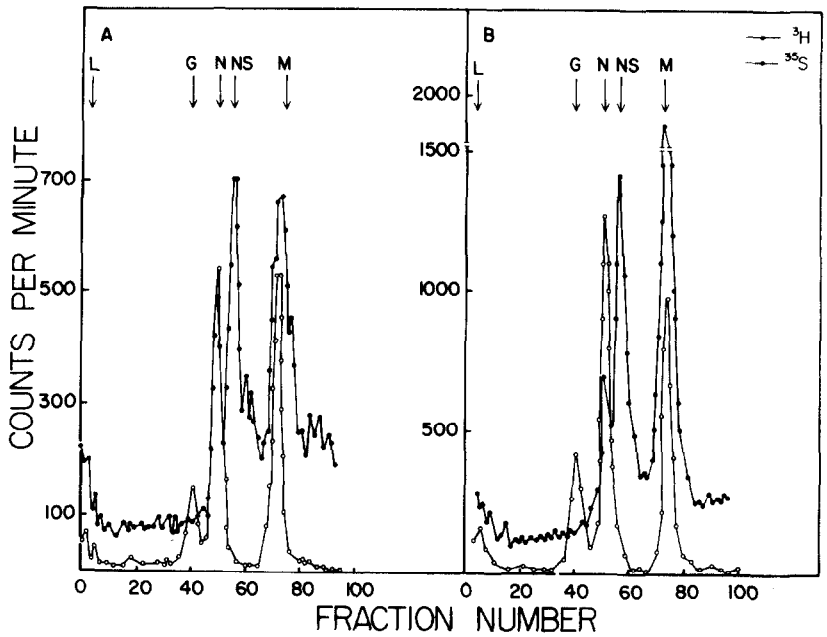

Fig.3. Polyacrylamide gel patterns of translation products in wheat embryo system of 12–15S RNA from free and membrane-bound polyribosomes. After incubation at 30°C for 60 min the reaction was stopped by adding 0.2 mg/ml of pancreatic RNase. The [^{3}H]leucine labeled proteins synthesized in vitro were precipitated with Cl_3CCOOH and redissolved in 2% SDS and 0.2% mercaptoethanol. Approximately 80 000 cpm of [^{3}H]-labeled proteins were co-electrophoresed with 20 000 cpm of marker [^{35}S]-labeled VSV proteins. A) Products directed by 12–15S RNA from free polyribosomes. B) Products coded by membrane-bound polyribosomal 12–15S RNA.

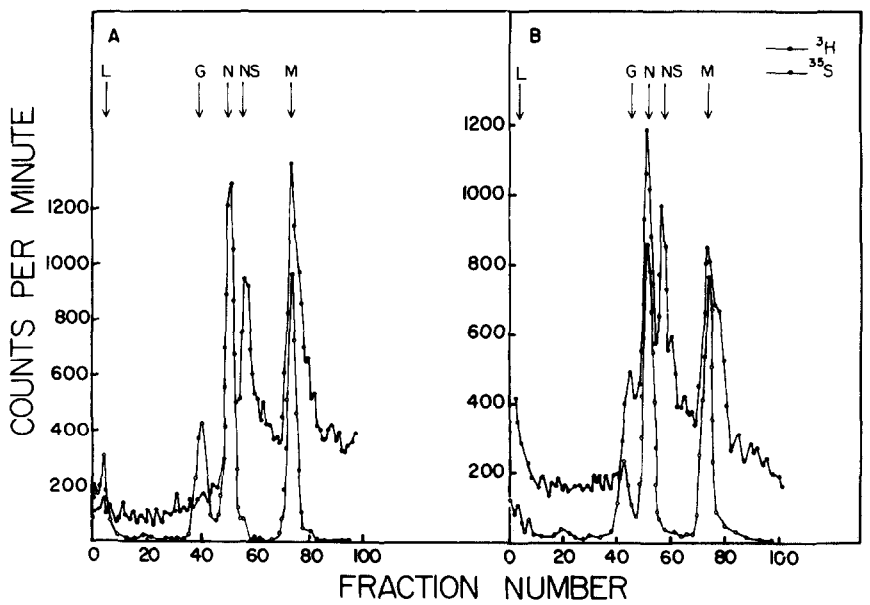

Fig.4. Polyacrylamide gel patterns of proteins coded by 16–19S RNA from free and membrane-bound polyribosomes. A) 16–19S RNA from free polyribosomes. B) 16–19S RNA from membrane-bound polyribosomes.

stimulation of the synthesis of G protein by the 16–19S RNA fraction from membrane-bound polysomes and the presence of an RNA species sedimenting at 18–19S only in the membrane-bound polysomes suggests that the 18–19S RNA peak may contain the mRNA for G protein. The in vitro synthesized G is, however, made in low amounts and appears not to be glycosylated. Glycosylation, a process which is believed to occur on membranes [12], may, therefore, be essential for efficient translation of the mRNA for G. The glycosylation of the G protein and its subsequent transfer across the cell membranes to form the spikes of the virion [13] could explain the observed requirement of membranes for its synthesis.

We have recently learned that Lodish and co-workers have observed that mRNA isolated from membrane-bound polyribosomes can direct the synthesis of G protein (H. F. Lodish, personal communication).

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References

[1] Howatson, A. F. (1970) Adv. Virus.Res. 16, 165–256.
[2] Wagner, R. R., Prevec, L., Brown, F., Summers, D. F., Sokol, F. and MacLeod, R. (1972) J. Virol. 10, 1228–1230.
[3] Emerson, S. U. and Wagner, R. R. (1972) J. Virol. 10, 297–309.
[4] Emerson, S. U. and Wagner, R. R. (1973) J. Virol. 12, 1325–1335.
[5] Wagner, R. R., Snyder, R. M. and Yamazaki, S. (1970) J. Virol. 5, 548–558.
[6] Ghosh, H. P., Toneguzzo, F. and Wells, S. (1973) Biochem. Biophys. Res. Commun. 54, 228–233.
[7] Grubman, M. J. and Summers, D. F. (1973) J. Virol. 12, 265–274.
[8] Murthy, M. R. V. (1972) J. Biol. Chem. 247, 1936–1943.
[9] Ghosh, K. and Ghosh, H. P. (1972) J. Biol. Chem. 247, 3369–3375.
[10] Mathews, M. B. (1972) Biochim. Biophys. Acta. 272, 108–118.
[11] Morrison, T., Stampfer, M., Baltimore, D. and Lodish, H. F. (1974) J. Virol. 13, 62–72.
[12] Schachter, H., Jabbal, I., Hugdin, R. L., Pinteric, L., McGuire, E. J. and Roseman, S. (1970) J. Biol. Chem. 245, 1090–1100.
[13] Eiserling, F. A. and Dickson, R. C. (1972) Ann. Rev. Biochem. 41, 467–502.