Volume 232, number 1, 35–38 FEB 05823 May 1988

Small cytoplasmic RNA from mouse cells covalently linked to a protein

Andrey B. Vartapetian[+], Tatyana N. Makarova[+], Eugene V. Koonin*, Vadim I. Agol[+]* and Alexey A. Bogdanov[+]

[+] *A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 119899* and **Institute of Poliomyelitis and Viral Encephalitides of the USSR Academy of Medical Sciences, Moscow Region 142782, USSR*

Received 29 February 1988

A low-molecular-mass RNA from the cytoplasm of mouse Krebs II cells was found to be covalently linked to a protein with an apparent molecular mass of 13 kDa. The protein appears to be attached to the 5′-terminus of the RNA molecule, which is approx. 20 nucleotides long.

small cytoplasmic RNA; RNA-protein complex; Viral protein VPg

1. INTRODUCTION

Genomic RNAs of a number of eukaryotic viruses are known to possess at their 5′-termini a covalently linked low-molecular-mass viral protein termed VPg (reviews [1,2]). Of considerable interest is the question whether RNAs with covalently bound proteins are present in uninfected cells. This question is all the more legitimate as enzymatic activity capable of selective hydrolysis of a phosphodiester bond between viral RNA and VPg has been found in uninfected cells [3].

In this paper we demonstrate that an RNA species, approx. 20 nucleotides long, is present in the cytoplasm of mouse Krebs II cells in the form of a covalent complex with a protein of apparent molecular mass 13 kDa. The protein appears to be attached to the 5′-terminus of the RNA.

2. MATERIALS AND METHODS

RNA from mouse Krebs II ascites carcinoma cells was isolated essentially as described in [4]. Briefly: cells were suspended in sodium acetate buffer, pH 5, in the presence of 1 mM PMSF, lysed with 0.5% SDS and treated with phenol at 60°C. The nucleic acids were precipitated from the aqueous phase with ethanol, treated with DNase I (50 μg/ml, Worthington), and the RNA was further purified by four phenol deproteinizations in the presence of 1% SDS.

The resultant RNA preparation (100 μg) was labelled with the ^{125}I Bolton and Hunter reagent (0.5 mCi, Amersham) in 0.1 M sodium borate, pH 8.5, 1 mM EDTA, 0.1% lithium dodecyl sulfate for 2–12 h at 4°C. After labelling, the RNA was purified by phenol extraction and ethanol precipitation.

Digestion of RNA with RNase T_2 (10 U/ml, Calbiochem) was performed in 10 mM ammonium acetate, pH 4.5, and 1 mM EDTA for 1 h at 37°C. Incubation with proteinase K (100 μg/ml, Merck) was in 10 mM Tris-HCl, pH 7.5, 0.1% SDS for 1 h at 37°C.

RNAs and proteins were fractionated by size in the standard electrophoresis gel systems described by Maxam and Gilbert [5] and Laemmli [6], respectively.

Correspondence address: A.B. Vartapetian, A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 119899, USSR

3. RESULTS

3.1. *Identification of an RNA covalently linked to a protein*

The search for putative covalent RNA-protein complexes in the mouse Krebs II cells was based on the following approach. The cellular RNA was separated from the non-covalently bound proteins by five phenol-SDS extractions. The RNA

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

preparation thus purified was radiolabelled with the ^{125}I Bolton and Hunter reagent, which is known to label proteins, but not RNA. Then RNA was fractionated by size by PAGE in 8% gel with 7 M urea (fig.1). One major radioactive band was readily observed, which migrated slightly faster than tRNA. The detected band was sensitive to proteinase K treatment (fig.1, lane 2), as well as to RNase T_2 treatment (fig.1, lane 3), indicating the existence of both RNA and protein components in the material under study. It should be mentioned, however, that the RNA component was highly resistant to hydrolysis with RNases T_1 and U_2.

The RNA-protein linkage proved to be quite stable under the following rigorous denaturing conditions: phenol extraction with 1% SDS; treatment with 1% SDS and 3 M urea at 100°C for 5 min; 2 M LiCl and 3 M urea; 0.2 N HCl at 37°C for 1.5 h, and partially resistant to treatment with 1 N NaOH at 37°C for 1 h. This extraordinary stability has enabled us to conclude that the protein is attached to the RNA covalently.

To localize this covalent RNA-protein complex within the cell, we have separately analyzed nuclear and cytoplasmic RNA preparations. A major part of the complex was found to be present in the cytoplasm, while only a negligible portion of it was detectable in the nuclear RNA preparation (not shown).

3.2. *Characterization of the RNA-linked protein*

In order to characterize the protein component of the complex, ^{125}I-labelled RNA-protein was analyzed by SDS-PAGE (fig.2). In this gel system, as well as in that shown in fig.1, the investigated material appeared to be electrophoretically homogeneous, with a mobility slightly slower than that of the cytochrome *c* marker (M_r 12300). Hydrolysis of the protein component with proteinase K resulted in the disappearance of the initial ^{125}I-containing band (fig.2, lane 2). Treatment of the complex with RNase T_2, which should give rise to RNA-free protein, exerted no effect on the mobility of the ^{125}I-labelled component (fig.2, lane 3), though the RNA moiety of the complex was degraded (fig.1, lane 3). It should be mentioned in this context that destruction of short RNA fragments cross-linked to proteins may have no effect on the electrophoretic mobilities of such complexes in SDS-PAGE [7].

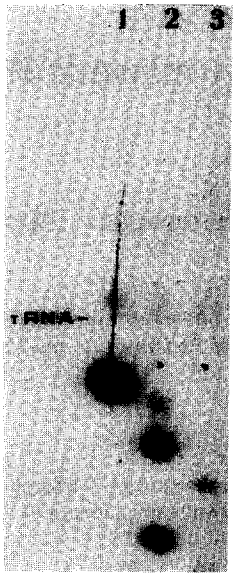

Fig.1. Identification of the protein-linked RNA. RNA from mouse cells was labelled with the ^{125}I Bolton and Hunter reagent and electrophoresed in 8% PAGE: 1, untreated; 2, treated with proteinase K; 3, treated with RNase T_2.

Thus, the RNA-linked protein has a low apparent molecular mass of 13 kDa. Consistent with this estimate, the tryptic peptide map of the protein shows about ten ^{125}I-peptides (not shown).

3.3. *The protein-linked RNA is 'small'*

The treatment of ^{125}I-labelled protein covalently linked to RNA, with proteinase K, has resulted in the formation of a number of free (not linked to RNA) peptides and of RNA covalently linked to the residual peptide, which still contained some radioactive iodine. Such ^{125}I-RNA-peptide was identified by PAGE (fig.3). This material was susceptible to RNase T_2 digestion (fig.3, lane 3). Partial hydrolysis of its RNA component with RNase T_2 resulted in the formation of a 'ladder' (fig.3, lane 4). This has enabled us to estimate the size of RNA molecule to be about 20 nucleotides long.

The treatment of ^{125}I-RNA-peptide with snake venom phosphodiesterase gave rise to the product, which co-migrated with the fastest band of the RNase ladder (not shown). This observation, together with the resistance of the RNA component to 5′-terminal labelling by polynucleotide

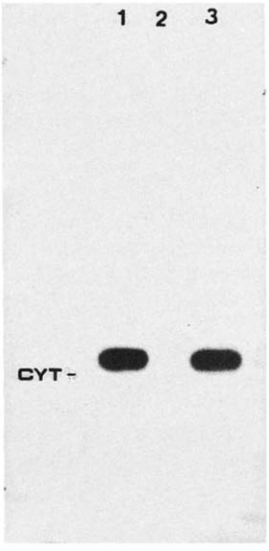

Fig.2. Characterization of the RNA-linked protein. Electrophoresis of the ^{125}I RNA-protein in 20% SDS-PAGE: 1, untreated; 2, treated with proteinase K; 3, treated with RNase T_2.

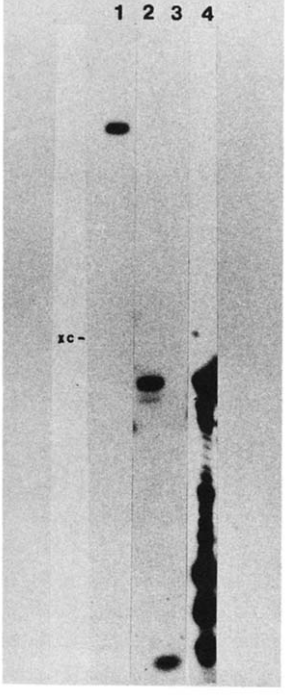

Fig.3. Electrophoresis of the ^{125}I-RNA-peptide in 20% PAGE with 7 M urea: (1) RNA-protein; (2) RNA-peptide derived by the proteinase K treatment of RNA-protein; complete (3) and partial (4) hydrolysis of the RNA-peptide with RNase T_2.

kinase, suggests that the protein is attached to the 5′-terminus of the RNA.

On the basis of the size of the RNA and protein components of the complex and of the specific radioactivity of proteins iodinated with the ^{125}I Bolton and Hunter reagent, it could be estimated that the protein-bound RNA comprises approx. 0.01% of the total cellular RNA content, which is roughly equivalent to 10^5 copies of the complex per cell.

4. DISCUSSION

The fact that 5′-termini of RNA genomes of many animal and plant viruses are covalently bound to proteins has become known during the last decade [1,2]. The present study demonstrates that the existence of RNAs with covalently linked proteins is not restricted to the viral world. An RNA species, approx. 20 nucleotides long, covalently linked, apparently at its 5′-terminus, to a protein with a molecular mass of 13 kDa has been discovered in the cytoplasm of mouse cells. The RNA-protein linkage is extremely stable and, judging by its hydrolytic stability, is likely to be a phosphodiester bond.

At present, we cannot exclude that the formation of such a small RNA component of the complex is due to non-specific degradation of protein-linked RNA of a greater length. However, its homogeneity, as well as the presence of undegraded high-molecular-mass RNAs in our preparations argues against this supposition. Preliminary evidence indicates that very similar RNA-protein complexes can be found in a variety of cells (unpublished).

Several cellular and viral proteins were shown to be associated with some ribonucleotide material [7–10]. It is unclear whether they are related to the covalent RNA-protein complex described here.

Evidently, the small size and the high copy number of the discovered protein-linked RNA pose questions about its function and the mechanism of the complex formation. Whether the protein is attached to the already synthesized RNA molecule, or the protein priming of RNA synthesis occurs, as discussed for the covalent viral RNA-protein complexes [1,2], remains to be seen. One may wonder whether this RNA-protein complex could be the genuine substrate for a cellular

unlinking activity capable of detaching VPg from viral RNAs [3].

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