

Translation of Rous Sarcoma Virus RNA in a Cell-Free System from Ascites Krebs II Cells

(*in vitro* translation/30-40S, 60-70S RNA of Rous sarcoma virus/gel electrophoresis/peptide analysis)

KLAUS VON DER HELM*† AND PETER H. DUESBERG

* Institut Suisse des Recherches Experimentales sur le Cancer, Lausanne, Switzerland; and Department of Molecular Biology and Virus Laboratory, University of California, Berkeley, Calif. 94720†

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ABSTRACT The template activities of the 60-70S RNA complex and of the 30-40S subunit RNA species of Rous sarcoma virus were tested in a cell-free protein-synthesizing system from mouse ascites Krebs II cells. Stimulation of protein synthesis over the endogenous background was about 2-fold with 30-40S viral RNA and about 1.3-fold with 60-70S viral RNA as template. Analysis by sodium dodecyl sulfate-gel electrophoresis showed that the predominant polypeptide synthesized *in vitro* in response to 30-40S RNA of Rous sarcoma virus had a molecular weight of 75,000-80,000. This polypeptide could be precipitated by antiserum against the group-specific antigens of the virus, although its molecular weight is higher than that of virion group-specific antigen proteins. Analysis of tryptic digests of the protein made *in vitro* indicates similarity to tryptic digests from authentic virion group-specific proteins. It is concluded that part of the RNA from Rous sarcoma virus is translated *in vitro* into a high-molecular-weight protein, perhaps a precursor of the virion group-specific proteins.

Because the 60-70S RNA of tumor viruses has not been shown to be infectious, and because viral replication is dependent on cellular DNA synthesis and additional cellular functions, it has not been determined whether the virion nucleic acid is in fact the viral genome (1). However, indirect evidence suggests that the RNA contains viral genetic information. Certain avian sarcoma viruses induce both tumors and the synthesis of viral proteins in some mammalian hosts, although transformed cells do not produce virus (2). This observation suggests that the viral RNA codes for these virion proteins. In addition, extensive correlations exist between structural features of tumor virus RNAs and biological properties of the virus, further suggestion of a genetic role for viral RNA (3-5).

More directly, translational evidence has suggested that RNAs of avian and murine tumor viruses can function as mRNA for viral proteins in *Escherichia coli* cell-free systems (6-8). Product analyses of proteins made under these conditions demonstrated that some of these electrophoresed along with structural proteins of the virion; had molecular weights of 10,000-45,000, the molecular weight range of the viral group-specific (gs)-proteins (7, 8); and were precipitable with antibody against the gs-antigen (6, 8). Moreover, the synthesis of large polypeptides of 120,000 and 180,000 daltons

was observed in a murine cell-free system in response to 60-70S RNA of murine leukemia virus, but no further product analysis was reported (9). None of these studies has described a biochemical analysis of the polypeptides synthesized in response to tumor virus RNA.

Because our own attempts to translate RNA of Rous sarcoma virus (RSV), both the 60-70S complex and its 30-40S subunit species (10), in an *E. coli* system have been inconclusive, we have investigated translation of viral RNA in a mammalian cell-free system prepared from mouse ascites cells (11). The predominant product has a molecular weight of about 75,000-80,000 and was precipitated by antiserum against the gs-antigen of RSV. Peptide analysis of this protein indicated that it shares peptides with the proteins of the viral gs-antigen.

MATERIALS AND METHODS

Materials

Cells and Viruses. Rous sarcoma virus (RSV) strain Prague (PR) of subgroup C was grown in C/O chick embryo fibroblasts and purified as described (10).

Viral RNA. RNA was extracted from purified RSV by the chloroform-phenol method (12) and centrifuged in a 10-25% sucrose gradient containing 0.1% sodium dodecyl sulfate (NaDodSO₄) for 45 min at 65,000 rpm in a Spinco SW65 rotor at 20°. The 60-70S peak of RNA was precipitated with 2 volumes of ethanol, and the precipitate was dissolved in standard buffer (0.05 M NaCl; 0.01 M Tris·HCl, pH 7.5, 1 mM EDTA) and used, as described, for protein synthesis *in vitro*. The 30-40S subunit species of RSV RNA (10) was prepared by heating 60-70S RNA for 3 min at 80° in standard buffer containing 0.1% NaDodSO₄, followed by sedimentation in a sucrose gradient for 90 min, and precipitation as described above. After the RNA was dissolved in standard buffer, it was used for protein synthesis *in vitro*.

Extracts of Ascites Krebs II Cells and Translation of RNA *in Vitro*. S 30 cell-free extract from ascites Krebs II cells was prepared according to Mathews and Korner (11), except Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Calbiochem, Los Angeles, Calif.) was used instead of Tris buffer and the pre-incubation of the S 30 supernatant was for 30 min at 32° instead of 37°. It was used immediately or after storage at -70°. A typical incubation experiment *in vitro* contained per 20- μ l incubation volume: 30 mM Hepes (pH 7.5), 85 mM KCl, 3.0 mM MgOAc₂, 1 mM ATP, 0.2 mM

Abbreviations: RSV, Rous sarcoma virus; gs-antigen, group-specific antigen; NaDodSO₄, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

† To whom requests for reprints should be addressed.

‡ Where the initial part of this work was performed.

GTP, 10 mM creatinephosphate, 0.15 mg/ml of creatinephosphate kinase, 6 mM 2-mercaptoethanol, 2 mM dithiothreitol, 19 amino acids at 50 μ M, 1–4 μ Ci of [³⁵S]methionine (at 50–200 Ci/mmol; New England Nuclear Corp. or kindly provided by B. Hirt), and 15 μ l of cell-free extract. After addition of 2 μ g of viral RNA the mixture was incubated for 10 min at 32°; subsequently one more μ g of viral RNA was added and incubation was continued for 50 min. Better yields of protein were obtained by stepwise rather than by simultaneous addition of RNA. Trichloroacetic acid-precipitable radioactivity was determined from aliquots. The remaining reaction mixture was analyzed by NaDodSO₄-polyacrylamide gel electrophoresis (see below) or precipitated by antiserum against the viral gs-antigens (see below).

Product analysis

NaDodSO₄-Polyacrylamide Gel Electrophoresis. *In vitro* translation samples (20 μ l) were incubated with RNase (20 μ g/ml) and 10 mM EDTA, pH 7.5, for 10 min at 37°, boiled for 3 min in 2% NaDodSO₄, 3% mercaptoethanol, 15 mM Tris·HCl (pH 6.8), and 10% glycerol, then subjected to electrophoresis (80 V, 30 mA, 3 hr) on a slab gel containing 12.5% or 15% acrylamide as described (15). Gels were stained with Coomassie brilliant blue, destained, dried, and exposed to x-ray film for 1–4 days.

Tryptic Digest and Exchange Column. The high-molecular-weight polypeptide made *in vitro* was cut out of the gel (see Fig. 1); the peptide was eluted (1 day at room temperature) from the gel with 0.1% NaDodSO₄ in 0.1 M NaHCO₃ buffer (pH 8.4). It was then precipitated in the presence of 100 μ g of bovine serum albumin carrier protein with 10% trichloroacetic acid to remove the NaDodSO₄ and washed three times with ethanol-ether (1:1) and ether. The precipitate was taken up and kept for 1 hr at 4° in performic acid (1.9 ml of concentrated formic acid and 0.1 ml of H₂O₂ reacted for 1 hr at room temperature). Subsequently the solution was twice lyophilized and digested at 37° by two consecutive additions of 3 μ g of trypsin (TPCK treated; Worthington, Inc.) in 50 mM NaHCO₃ and lyophilized.

Preparation of virion gs-proteins with sedimentation coefficients of 1–2 S from Triton X-100 disrupted virus followed published procedures (13). The viral gs-proteins were precipitated with trichloroacetic acid and digested as described for the polypeptides made *in vitro*. For exchange chromatography, the tryptic peptides were taken up in 0.2 ml of buffer A containing 280 ml of acetic acid and 3 ml of pyridine per liter of solution (pH 2.5), and applied to a cation exchange chromatography column (Chromobeads, Technicon) of 1 × 17-cm dimension. The column was eluted at 50° with an exponential gradient of 2 × 120 ml of buffer A and 1 × 120 ml of buffer B containing 189 ml of acetic acid and 108 ml of pyridine per liter of solution (pH 4.5), at a flow rate of 15 ml/hr. Fractions of 3 ml were taken and the buffer was evaporated at 100°. The remaining peptides were taken up in 0.2 ml of 10 mM HCl and 2.5 ml of Aquasol (New England Nuclear) and their radioactivity was determined in a scintillation counter. Corrections were made for spillover of ¹⁴C into the ³H channel.

Immunoprecipitation. Monospecific rat antiserum against gs-antigen was kindly provided by R. Nowinski (McArdle Laboratory, Univ. of Wisconsin). Each *in vitro* incubation

TABLE 1. Stimulation of [³⁵S]methionine incorporation by 30–40S and 60–70S RNA of RSV in a mouse ascites cell-free system*

Experiment	RNA added	[³⁵ S]Methionine incorporated in acid-precipitable cpm
1	—	16,790
	30–40S	34,360
	60–70S	24,230
2	—	25,200
	30–40S	52,020
	60–70S	32,540
3	—	18,520
	30–40S	42,350
	60–70S	23,620

* Reaction mixtures were as described in *Materials and Methods*.

sample (20 μ l) was treated with 5 μ l of antiserum, and Nonidet P40 (Shell Co.) was added to a final concentration of 0.5%. The mixture was incubated for 10 min at room temperature and overnight at 4°. Fifty microliters of antibody against rat serum made in rabbit (Microbiol. Assoc.) was then added to each sample, incubated for 10 min at room temperature and overnight at 4°. The visible precipitate was centrifuged, washed twice in standard buffer containing 0.5% Nonidet P40, and then centrifuged through a 1-ml cushion of 20% sucrose in standard buffer and 0.5% Nonidet P40 (20 min, 9000 rpm, in an SS 34 Sorvall rotor). The precipitate was dissolved by boiling in NaDodSO₄ and mercaptoethanol-containing buffer, as described above for gel electrophoresis.

RESULTS

Effects of 60–70S and 30–40S RNA of RSV on protein synthesis *in vitro*

Table 1 shows the results of typical translation experiments *in vitro* in a cell-free system from ascites Krebs II cells. 30–40S RNA of RSV stimulated polypeptide synthesis *in vitro* about 2-fold and 60–70S RNA of RSV about 1.2- to 1.4-fold over the background of controls to which no RNA was added, as calculated from the incorporation of [³⁵S]methionine into trichloroacetic acid-precipitable proteins. The kinetics of [³⁵S]methionine incorporation in response to 30–40S RNA of RSV were linear for 20–30 min and reached a plateau at 45–60 min. Optimal Mg⁺⁺ concentration was 3.0–3.2 mM at a K⁺ concentration of 85 mM. Maximum amino-acid incorporation was obtained with 30–40S RNA of RSV at a final concentration of 150 μ g/ml (not shown).

Analysis of the product

NaDodSO₄-Polyacrylamide Gel Electrophoresis. The [³⁵S]-methionine-labeled products of the *in vitro* translation directed by 30–40S RNA of RSV as well as by 60–70S RNA of RSV were analyzed by NaDodSO₄-polyacrylamide gel electrophoresis. In the reaction that was directed by 30–40S RNA of RSV, a polypeptide double band was seen (Fig. 1b) in the molecular weight range of 75,000–80,000 (marked PR in Fig. 1) which is not present in the control reaction carried out in a parallel experiment without viral RNA (Fig. 1c). The material appearing as a double band in Fig. 1b migrated as a single band in other experiments (not shown). An analysis of the

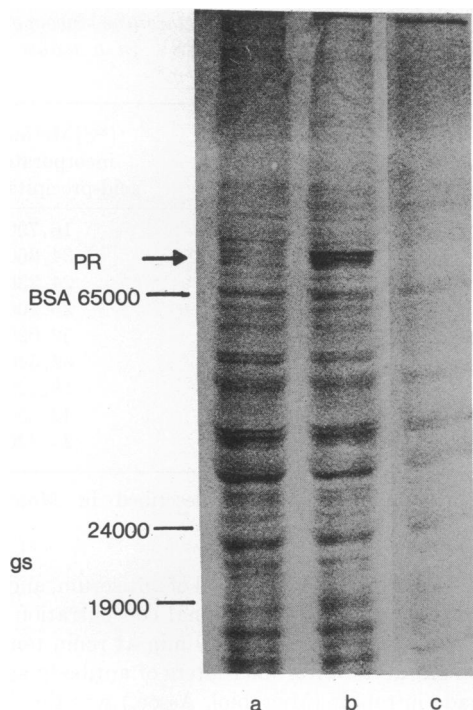


FIG. 1. Autoradiogram of NaDodSO₄-polyacrylamide (12%) gel electrophoresis of [³⁵S]methionine-labeled polypeptides synthesized *in vitro* in response to (a) 60–70S RNA of RSV, (b) 30–40S RNA of RSV, (c) no added RNA. Unlabeled bovine serum albumin (BSA, 65,000 daltons) and RSV proteins, including the gs-proteins of 24,000 and 19,000 daltons, were coelectrophoresed in the same gel to serve as markers. The marker proteins were located in the gel by staining with Coomassie blue.

chemical basis of the double band will have to await a preparative isolation of each component. In the reaction that contained 60–70S RSV RNA, only faint bands can be seen in the 75,000–80,000 molecular weight range (Fig. 1a). In addition a slight nonspecific stimulation of endogenous protein synthesis was observed (see Fig. 1a and c and Table 1). In neither the 30–40S nor the 60–70S RNA-directed synthesis could [³⁵S]-methionine-labeled polypeptides be detected in the molecular weight range of the virion gs-antigen peptides (10,000–30,000, marked gs in Fig. 1) which were not also found in the control experiment (compare Fig. 1a, b, and c).

Immunoprecipitation. To test whether the protein synthesized in response to 30–40S RNA of RSV included virion structural proteins, it was subjected to indirect immunoprecipitation with rat antiserum against the gs-antigens of RSV. Rat antiserum was added to the cell-free reaction mixture, and after incubation, anti-immunoglobulin against rat antibody was added to insure complete precipitation (*Materials and Methods*). The immune precipitate was then washed and analyzed by NaDodSO₄-polyacrylamide gel electrophoresis (*Materials and Methods*). The precipitate showed a band in the molecular weight range of 75,000–80,000 which could not be seen in a parallel immunoprecipitation of the endogenous reaction (Fig. 2). However, the immunoprecipitate of the 30–40S RNA-stimulated and of the control reaction contained some radioactivity that may have been derived from nonspecifically precipitated radioactive material and that did not show distinct bands on the gel. We conclude

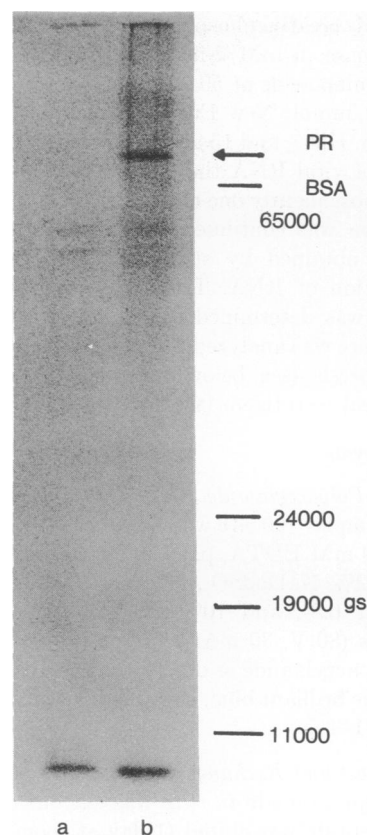


FIG. 2. Autoradiogram of NaDodSO₄-polyacrylamide (15%) gel electrophoresis of [³⁵S]methionine-labeled polypeptides made in response to (a) no added RNA, (b) 30–40S RNA of RSV. The polypeptides of a reaction mixture *in vitro* were immunoprecipitated before electrophoresis (*Materials and Methods*). Protein markers were the same as those described for Fig. 1 except that the viral gs-protein of 11,000 daltons was also present in the gel.

that a protein of 75,000–80,000 daltons which is serologically related to the virion gs-antigen was made *in vitro* in response to 30–40S RNA of RSV.

Tryptic Fingerprints. To determine more directly whether the 75,000- to 80,000-dalton protein synthesized *in vitro* in response to 30–40S RSV RNA shares common sequences with authentic virion gs-proteins, their tryptic fingerprint patterns were compared. The 75,000- to 80,000-dalton double band was cut out of a dried polyacrylamide slab gel (Fig. 1b); the protein was eluted and digested with trypsin (*Materials and Methods*). The [³⁵S]methionine-labeled tryptic peptides were cochromatographed on a cation exchange (Chromobeads) column with tryptic peptides from gs-antigen of [³H]-methionine-labeled virus (*Materials and Methods*). Fig. 3 shows a typical elution pattern of peptides synthesized *in vivo* and *in vitro*. Of 13 elution peaks obtained from the 75,000- to 80,000-dalton protein, 11 peaks chromatographed with peptides of authentic virion gs-antigen. Two peaks (x and y , Fig. 3) had no direct counterparts in the digest of viral gs-antigen. The ratio of ³⁵S/³H was not constant in all peaks, suggesting that relative concentrations of peptides in the 75,000- to 80,000-dalton protein synthesized *in vitro* and of peptides derived from virion gs proteins are not the same. This could be due to our use of a nonequimolar mixture of

viral [^3H]gs-proteins to prepare tryptic peptides since (i) virion gs-proteins may not be assembled at equimolar ratios in the virus (10), and since (ii) our procedure for isolation of viral gs-proteins (*Materials and Methods*) may have been selective for the predominant gs-protein species.

We conclude that the 75,000- to 80,000-dalton polypeptide made in our *in vitro* system shares tryptic peptides with the gs-antigens of the virion.

DISCUSSION

Our experiments indicate that 30–40S RNA of RSV is translated in a cell-free mouse ascites system, predominantly into a 75,000- to 80,000-dalton protein. On the basis of its reactivity with antiserum against gs-proteins and by its fingerprint pattern, this protein appears to be structurally related to authentic viral gs-proteins.

It would appear from our experiments and from the preliminary observations of Naso *et al.* (9) that large polypeptides are synthesized in cell-free mammalian systems in response to tumor virus RNA, whereas small (<45,000 daltons) polypeptides are made in the *E. coli* system (6–8). We can only speculate on the basis of this difference. It is conceivable, for example, that under our conditions translation of 30–40S RNA closely resembles translation in infected cells. In virus-infected cells a 76,000-dalton precursor protein was detected which is subsequently cleaved into gs-proteins of 11,000–24,000 daltons (15, 16). Our 75,000- to 80,000-dalton polypeptide made *in vitro* is similar to this natural precursor because the two proteins have approximately the same molecular weight and have a similar tryptic peptide composition if analyzed by cation exchange chromatography (unpublished observation). By contrast, translation in the *E. coli* system may involve different mechanisms of initiation and chain termination and may, therefore, lead to peptides of the same or similar sizes as those of authentic virion gs-antigens.

The extent of sequence homology between the protein made *in vitro* and viral gs-proteins cannot be fully determined from these experiments, because only about 13 peaks, which correspond to at least 13 tryptic peptides, were resolved (Fig. 3). A protein of 75,000–80,000 daltons may be expected to yield as many as 75 to 80 tryptic peptides. However, many of these peptides may lack methionine (14) and would, therefore, not be detected in our system. Further work using other radioactive amino acids than methionine to label viral peptides will be required to determine the extent of sequence homology between our protein made *in vitro* and viral gs-antigens.

The notion that the 30–40S subunit species of 60–70S RNA of RSV is a better template for protein synthesis than the 60–70S RNA complex of RSV is compatible with preliminary observations by others (7). It is also consistent with earlier observations that only 30–40S and not 60–70S RNA of RSV exists and presumably functions as mRNA in the cytoplasm of infected cells (17–21) and that virus harvested at very short intervals from infected cells contains only 30–40S RNA and little or no 60–70S RNA (22). Thus it appears that the 30–40S subunit species rather than the 60–70S complex of RSV RNA functions as a template for translation. Several reasons may account for the relatively low activity of the 60–70S RNA complex of RSV in stimulating protein synthesis: (i) 60–70S RNA contains partially double-stranded regions (23), which may directly inhibit protein synthesis in

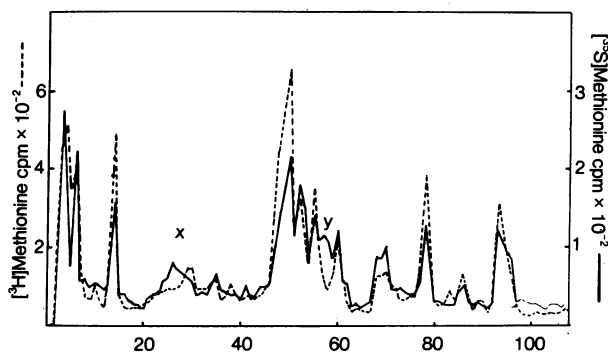


FIG. 3. Tryptic peptides of polypeptides labeled with [^{35}S]methionine *in vitro* (—) and virion gs-antigens labeled with [^3H]methionine *in vivo* (---) after elution from a cation exchange column in a pH range of 2.5–4.5 (*Materials and Methods*). For explanation of peaks marked x and y, see text.

our system (24); (ii) 60–70S RNA has a complex superstructure consisting of at least two 30–40S RNA subunits linked at several sites in a network-type structure (25). This structure may interfere with binding and movement of ribosomes.

Given a complexity of about 3.5×10^6 daltons for RSV RNA (26–28), the 75,000- to 80,000-dalton polypeptide synthesized in our system represents about one-third of the potential genetic information of RSV RNA. As yet no further virus-specific polypeptides have been detected by electrophoretic analysis of our protein synthesized *in vitro* that were not also present in the control reaction. Nevertheless it is conceivable that other products are made *in vitro* that contain no, or very little, methionine and thus would not have been detected. Further, it may be possible that under different ionic conditions, or in other cell free systems, different sequences of viral RNA become available for translation.

It will be interesting to test whether the RNAs of viruses with different genetic properties, such as replication- or transformation-defectiveness, also direct the synthesis of different polypeptides in *in vitro* systems.

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