JOURNAL OF VIROLOGY, Sept. 1976, p. 950-967

Vol. 19, No. 3
Printed in U.S.A.

Cell-Free Translation of Virion RNA from Nondefective and Transformation-Defective Rous Sarcoma Viruses

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Received for publication 19 April 1976

Nondefective and transformation-defective virion subunit RNAs from two strains of Rous sarcoma virus (RSV) were translated in cell-free systems derived from Krebs IIA ascites cells, wheat germ, and L-cells. In each case the predominant viral-specific product was a polypeptide of molecular weight 76,000 that is related to the internal viral group-specific antigens, as judged by immunoprecipitation with monospecific antisera and tryptic peptide fingerprinting. No difference could be detected between the translation products of 35S RNA from nondefective and transformation-defective RSV virions, nor of 35S RNA from different strains of RSV. The 76,000-molecular-weight polypeptide synthesized in response to 35S RNA in vitro was labeled with formyl-methionine from initiator tRNA. Models for viral protein synthesis are discussed in the light of these results, and arguments positioning the group-specific antigen gene at the 5′ end of the 35S RNA are presented.

Avian RNA tumor viruses contain a 70S genomic RNA that dissociates under denaturing conditions into two or three apparently identical 35S subunits, each having a molecular weight of approximately 3.3×10^6 (6, 16, 19, 23, 42, 43). The virion subunit RNA contains genes for the internal viral group-specific (gs) antigens (*gag*), the envelope proteins (*env*), and the viral reverse transcriptase (*pol*) (6, 16, 38). The sum of the molecular weights of these proteins is about 240,000, and the total coding capacity of 35S RNA is sufficient for a polypeptide of about 300,000 molecular weight. Nondefective (nd) virion 35S RNA also contains a gene for sarcomagenic cell transformation (*sarc*), and it is possible that this also encodes a protein (16, 42). It is known that polysomal viral RNA is of the same sense as virion RNA (43), and recently a number of oncornaviral virion RNAs have been translated in various cell-free systems to investigate directly the proteins encoded by the virion RNAs (25, 27, 30, 34, 41). Rous sarcoma virus (RSV) RNA has been reported as encoding a 75,000- to 80,000-molecular-weight viral-specific protein (41), and virion RNA from murine leukemia viruses has been reported as encoding at least three viral-specific polypeptides of molecular weight 60,000, 70,000, and 180,000 in cell-free systems (18, 27).

We have translated 35S RNA from nd RSV virions to investigate what proportion of the viral genome could code for viral polypeptide(s) in vitro and to analyze the nature of the in vitro translation product. We were also interested in ascertaining whether the *sarc* gene, which represents 10 to 20% of nd 35S RNA at the 3′ end (16, 42), could encode a polypeptide in vitro and have therefore compared the polypeptides translated from the subunit RNA of nd and transformation-defective (td) RSV viruses. The td viruses are derived from nd avian sarcoma viruses, but lack the *sarc* gene (17, 24, 39).

MATERIALS AND METHODS

Materials. [^{35}S]methionine and [^{3}H]uridine were obtained from the Radiochemical Centre, at specific activities of 250 to 400 Ci/mmol and 30 Ci/mmol, respectively. Thin-layer cellulose chromatography sheets, acrylamide, bisacrylamide, and spectrograde pyridine were bought from Eastman Kodak. All other chemicals were of reagent grade. Proteins for use as molecular weight markers were obtained from Boehringer, except bovine serum albumin, which was obtained from Armour. Antisera were a gift of R. Kurth and D. P. Bolognesi. These have been extensively characterized (2). Sheep anti-rabbit antisera were obtained from Wellcome Reagents.

Growth and purification and virus. c/o chicken embryo fibroblasts (CEF) infected with nd or td RSV from cloned stocks were grown in roller bottles at 41°C in Dulbecco modified Eagle medium containing 1% heat-inactivated chicken serum, 4% calf serum, and 2 μCi of [^{3}H]uridine per ml. Medium was harvested every 2.5 h and clarified, and virus was pel-

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leted and then suspended in 0.1 M NaCl-0.01 M Tris-hydrochloride (pH 7.2)-0.001 M EDTA (STE).

Purification of viral RNA. Concentrated virus was disrupted with 0.5% lithium dodecyl sulfate and extracted three times with phenol and twice with chloroform-isoamyl alcohol (24:1), and the RNA was precipitated from the aqueous phase. RNA was pelleted by centrifugation and sedimented through a 12-ml 10 to 25% sucrose gradient at 40,000 rpm for 2 h. Gradients were fractionated, and 70S RNA was pooled and precipitated as above. A portion of 70S RNA was heat-dissociated at 80°C and subjected to electrophoresis on a 1.8% acrylamide gel as described previously (7) to identify the percentage of intact 35S RNA. The bulk 70S RNA was heated at 80°C for 45 s in 0.02 M Tris-hydrochloride (pH 7.5)-0.01 M EDTA and sedimented on a 5 to 20% sucrose gradient containing STE. 35S RNA was pooled, ethanol precipitated twice, dissolved in water at 1 mg/ml, and stored at −70°C.

Protein synthesis. Preparations and conditions for protein synthesis in the ascites cell-free system were as described (34), except that each incubation contained 50 μM spermine and 250 μM spermidine, which we and others (1) have found essential for efficient translation. A typical 25-μl protein synthesis incubation contained 1 mM ATP, 0.2 mM GTP, 2 mM dithiothreitol, 6 mM β-mercaptoethanol, 50 μM spermine, 250 μM spermidine, 10 mM creatine phosphate, 85 mM K^+, 1.7 mM Mg^{2+}, 12 mM HEPES (pH 7.0), unlabeled amino acids (200 μM each) minus methionine, 10 to 15 μCi of [^{35}S]methionine, and 10 μl of ascites S-30 extract, equivalent to 100 μg of protein. Incubations were performed at 34°C. After 90 min, 2 μl from each incubation was removed and, following the addition of 100 μl of 0.1 M KOH, incubated at 37°C for 20 min. A 3-ml portion of cold 10% trichloroacetic acid was then added; after 10 min at 0°C each sample was collected on a membrane filter (Millipore Corp.) and washed with cold 5% trichloroacetic acid; and the filter was dried and counted at approximately 60% efficiency in a liquid scintillation counter.

Immunoprecipitation. All solutions, sera, and cell-free extracts were clarified at 7,000 rpm for 10 min prior to immunoprecipitation. From 10 to 20 μl of the cell-free incubation was added to 35 μl of immunoprecipitation buffer (PBSA-bovine serum albumin [2 mg/ml]-1% Triton X-100) and reacted for 1 h at 37°C with 5 μl of the first antibody. A 50-μl portion of the second antibody (sheep anti-rabbit) was then added, and the incubation was continued at 37°C for 1 h and overnight at 4°C. The precipitate was pelleted at 2,000 rpm for 10 min and washed four times with buffer (20 mM Tris-hydrochloride [pH 7.5]-50 mM NaCl-0.5% Nonidet P-40). The pellet was then sonically treated and blended in a Vortex mixer in gel sample buffer and prepared for polyacrylamide gel electrophoresis.

Gel electrophoresis. Samples were heated at 100°C for 3 min in gel sample buffer (100 mM dithiothreitol-2% sodium dodecyl sulfate [SDS]-0.0625 M Tris-hydrochloride [pH 6.8]-10% glycerol-0.001% bromophenol blue) and analyzed on 10% and 15% discontinuous SDS-polyacrylamide gels as described (21, 35). After electrophoresis, gels were stained with Coomassie blue, destained, dried, and subjected to autoradiography with Kodak RP54 film for 1 to 7 days. The markers used were phosphorylase a (molecular weight, 90,000), phosphofructokinase (78,000), bovine serum albumin (68,000), catalase (60,000), glutamate dehydrogenase (53,000), creatine phosphokinase (40,000), and carbonic anhydrase (29,000). In some cases gels were impregnated with PPO (2,5-diphenyloxazole) and subjected to fluorography at −80°C (22).

Analysis of tryptic peptides. Proteins for fingerprinting were separated by gel electrophoresis, after which the wet gel was covered with Saran Wrap and exposed to X-ray film. The relevant band was excised from the gel, and the protein was eluted with shaking from the gel slice into 5 ml of elution buffer (20 mM sodium bicarbonate-0.1% SDS-2 mM phenylmethylsulphonylfluoride) containing 50 μg of bovine serum albumin at 37°C for 20 h. Proteins were precipitated, oxidized, and digested with L-1-tosylamide-2-phenylethylchloromethyl ketone-treated trypsin as described (35). The digested peptides were lyophilized three times and stored at −20°C. Small portions were applied to precoated thin-layer cellulose plates in water and subjected to electrophoresis at pH 2.1, 3.5, or 6.5. Electrophoresis was for 75 min at 600 V using a water-cooled (15°C) Shandon flat-plate electrophoresis unit. Cyanol FF, orange G, and crystal violet were used as visual markers. Ascending chromatography in the second dimension using *n*-butanol-acetic acid-water-pyridine (30:6:24:20) was continued until the solvent front reached the top of the sheet (approximately 4 h).

Preparation of [^{35}S]methionine-labeled RSV. A 90-mm dish of CEFs infected with RSV was incubated with Dulbecco modified Eagle medium minus methionine containing 5% dialyzed calf serum and 100 μCi of [^{35}S]methionine per ml for 3 h. The medium was removed, and complete medium was added for another 3 h. The pooled harvests were clarified, and virus was sedimented through 20% sucrose onto a 60% sucrose pad at 25,000 rpm for 1 1/2 h. The banded virus was then twice sedimented to equilibrium on 5-ml, 20 to 65% sucrose gradients. Virus was analyzed for purity by electrophoresis on a polyacrylamide gel.

Preparation of f-[^{35}S]Met-tRNA$_f^{met}$. tRNA$_f^{met}$ from purified ascites or wheat germ tRNA was charged with [^{35}S]methionine, which was then chemically formylated, by a modification of a previously published method (35). Each 25-μl incubation was as above, except that 3 μg of exogenous tRNA containing f-[^{35}S]Met-tRNA$_f^{met}$, with a specific activity of 3.6×10^5 cpm/μg of tRNA, and 5 μM nonradioactive methionine were added instead of [^{35}S]methionine.

RESULTS

Conditions of cell-free protein synthesis with RSV 35S RNA. The integrity of 35S RNA subunits from purified preparations of virion

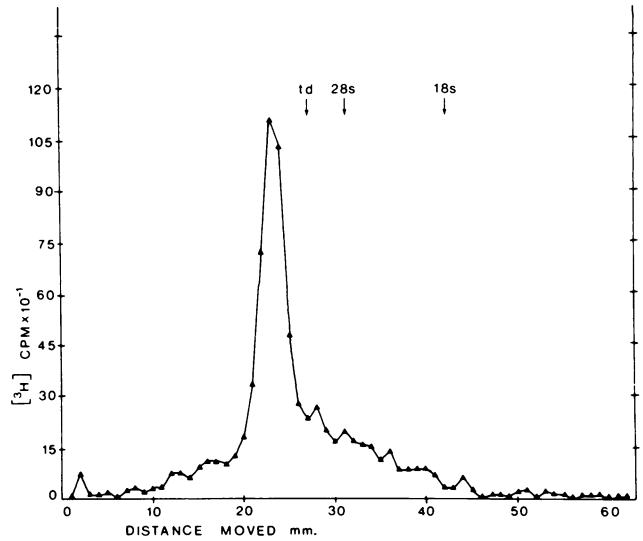

FIG. 1. *Electrophoresis of B77 RSV 35S RNA used for cell-free translation. Radioactively labeled 70S RNA, extracted from virus harvested at 2.5-h intervals from infected cells, was heated in electrophoresis sample buffer and subjected to electrophoresis in 1.8% polyacrylamide gels as described (4). The relative positions of td 35S RNA and 18S and 28S rRNA markers are indicated.*

70S RNA from B77-RSV of subgroup C was analyzed by gel electrophoresis. 35S RNA was prepared by sucrose gradient centrifugation following heat denaturation of preparations of virion 70S RNA thus shown to be composed of intact nondefective 35S RNA (Fig. 1). Purified 35S RNA was tested for its ability to direct the synthesis of proteins in a cell-free system from mouse Krebs IIA ascites tumor cells. The concentration of 35S RNA that gave maximal stimulation of protein synthesis in the ascites system was 60 μg/ml, which is comparable with other viral mRNA's tested, such as encephalomyocarditis (EMC) and polyoma viral mRNA's. The optimum concentrations of Mg^{2+} and K^+ for the in vitro translation of RSV 35S RNA were established as 1.7 mM and 85 mM, respectively. These values are identical to those obtained for total c/o CEF cellular mRNA. Varying the K^+ and Mg^{2+} concentrations did not qualitatively alter the translation products directed from the 35S RNA.

The maximum stimulation of protein synthesis over background was 2.5-fold for 35S RNA, but only very slight stimulation was seen with intact 70S RNA (Table 1). Compared with other viral RNAs of similar size such as EMC and poliovirus, RSV 35S RNA is a relatively poor mRNA in the ascites cell-free system.

Gel analysis of the in vitro translation product. The polypeptides synthesized in vitro were analyzed by electrophoresis on SDS-polyacrylamide gels. Samples were subjected to electrophoresis in parallel with markers of known molecular weight that were visualized by staining with Coomassie blue. Figure 2 shows that, relative to the background of endogenous protein synthesis in the ascites cell-free system, RSV 35S RNA directs the synthesis of only one major protein, which has an apparent molecular weight of 76,000 on both 10% and 15% polyacrylamide gels (Fig. 3). This band frequently appears as a doublet. 70S RNA also directs the synthesis of the 76,000-molecular-weight protein (see Fig. 7), but to a much smaller extent,

TABLE 1. *[^{35}S]methionine incorporation in an ascites cell-free system in response to RSV RNA*[a]

RNA added	[^{35}S]methionine incorporated into acid-insoluble material (cpm)
None	44,000
nd B77 35S RNA	104,100
td B77 35S RNA	103,200
nd PrC 35S RNA	98,600
nd B77 70S RNA	47,300
EMC RNA	265,400

[a] In each case except the control, 1 μg of the relevant RNA was added to a 25-μl incubation. At the end of each incubation, 2 μl was withdrawn and assayed for incorporation of [^{35}S]methionine into polypeptide as described in Materials and Methods. EMC RNA was used for comparison since it is a viral mRNA of the same size as RSV 35S RNA.

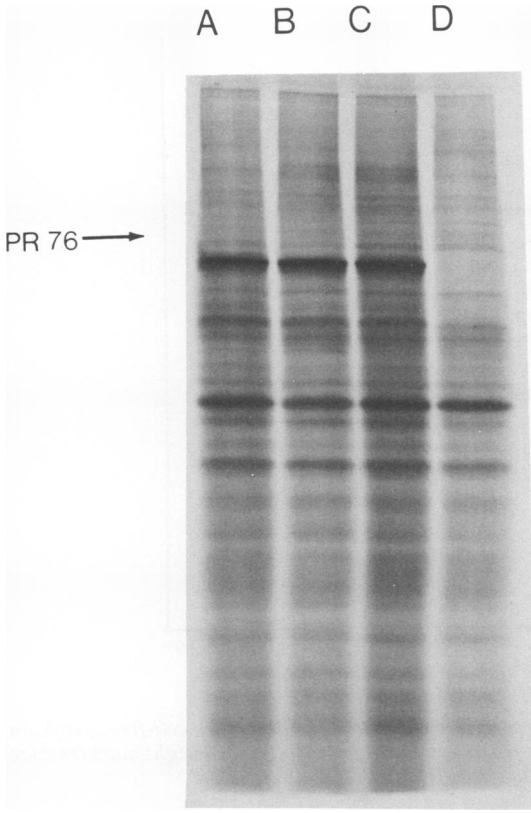

FIG. 2. *Electrophoretic analysis of the ascites cell-free system products. Slot A, Plus td B77 RSV 35S RNA; slots B and C, plus nd B77 RSV 35S RNA; slot D, no additions. A 20-μl portion of each 25-μl incubation was analyzed by electrophoresis on a 15% polyacrylamide gel. 35S RNA was added to a concentration of 60 μg/ml. The viral-specific product is indicated as PR 76.*

and this may arise from spontaneous dissociation of the 70S RNA into active subunits. A similar protein is made in the L-cell and wheat germ cell-free systems in response to 35S RNA, although less efficiently than in the ascites system (data not shown), and also in the reticulocyte lysate system (H. Pelham, A. J. Pawson, and T. Hunt, unpublished observation).

Immunoprecipitation of the in vitro translation product. To further investigate the nature of the 76,000-molecular-weight polypeptide encoded by RSV 35S RNA, antisera raised against viral proteins were used in immunoprecipitation assays of the in vitro product (Fig. 4a). The 76,000-molecular-weight protein is specifically immunoprecipitated by antiserum raised against detergent-disrupted virus, which is known to react with the internal gs antigens, and by monospecific antiserum to P27, the major viral gs antigen. It is also immunoprecipitated by monospecific antiserum raised against the gs antigen P12 (Fig. 4b). The 76,000-molecular-weight protein is present in very low amounts in the immunoprecipitation with control rabbit serum. Relative to control serum, the 76,000-molecular-weight protein is not precipitated by neutralizing antiserum against whole B77(c)-RSV, which reacts only with the viral glycoproteins, nor by monospecific antiserum raised against purified Gp85 (the major viral glycoprotein) of subgroup C.

These findings imply that the 76,000-molecular-weight protein contains the gs antigens P27 and P12. It is known that in whole cells the gs antigens (P27, P19, P15 and P12) of avian RNA tumor viruses are translated as a 76,000-molecular-weight precursor (Pr76) that is subsequently cleaved to the separate gs antigens (41). The 76,000-molecular-weight protein translated in vitro was therefore analyzed further by comparing its tryptic peptide map with those of whole virus and of the individual gs antigens.

Tryptic peptide analysis. B77-RSV was labeled with [^{35}S]methionine and purified as described above. Labeled virus was applied to a preparative polyacrylamide gel, and the gs antigens P27, P19, and P15 were eluted from the gel after electrophoresis. P12 could not be detected and probably contains relatively little methionine. The 76,000-molecular-weight protein synthesized in vitro in response to 35S RNA in a large-scale incubation was eluted from preparative gels in the same way. The individual gs antigens, the in vitro 76,000-molecular-weight protein, and whole, disrupted virus were then each oxidized and digested with trypsin. Two-dimensional tryptic peptide maps of the digested proteins were then prepared using thin-layer cellulose plates (Fig. 5).

All of the peptides from the 76,000-molecular-weight in vitro product correspond to peptides from whole virus and from the isolated gs antigens. Analysis of the individual gs antigens demonstrates that the 76,000-molecular-weight protein has peptides in common with each of the gs antigens examined. A peptide map of actin, a major cellular protein made in vitro, eluted, and digested in parallel with the other proteins, had no peptides in common with the gs antigens. The tryptic fingerprints shown employed electrophoresis at pH 6.5 in the first dimension. The similarity of the peptides has been further demonstrated by using electrophoresis at pH 2.1 and pH 3.5 in the first dimension.

35S RNA from B77 RSV would therefore ap-

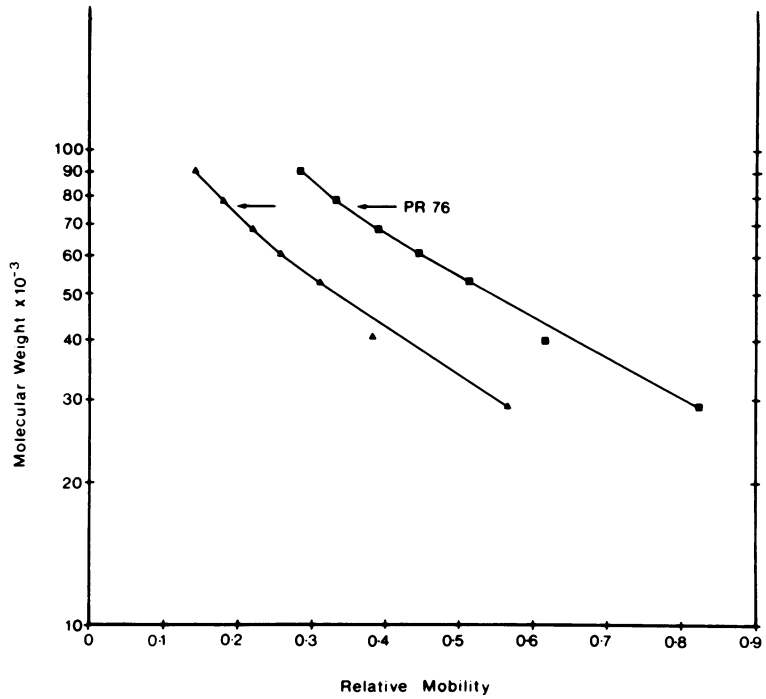

FIG. 3. *Analysis of the molecular weight of the polypeptide synthesized in the ascites cell-free system in response to B77 RSV 35S RNA on 15% (▲) and 10% (■) polyacrylamide gels. Molecular weight markers were as described. The position of the viral-specific product is indicated as PR 76.*

pear to code in vitro for a protein that, by analysis of its molecular weight, antigenic activity, and peptide composition, is similar if not identical to the primary translation product of viral gs antigen-specific mRNA sequences in whole cells.

Formyl-methionine labeling. To exclude the possibility of an extensive unstable sequence at the N-terminal end of the 76,000-molecular-weight protein made in the ascites system in response to 35S RNA, in vitro incubations were performed using chemically formylated [^{35}S]Met-tRNA$_f^{met}$ as the radioactive label. In eukaryotic cells, methionine from Met-tRNA$_f^{met}$ is donated only into N-terminal positions. The methionine used in our experiments is formylated, although not physiologically, to ensure that it is not cleaved from the N-terminus of the nascent protein (35). Incubations contained 5 μM nonradioactive methionine to reduce the possibility of [^{35}S]methionine being transferred to Met-tRNA$_m^{met}$ via a transacylation reaction and thereafter incorporated into internal positions. Figure 6 shows that the 76,000-molecular-weight product is labeled under these conditions. Relative to the amount of f-Met label incorporated into the 76,000 gs antigen band, little is incorporated into the endogenous ascites proteins. This implies that most of these endogenous proteins have already been initiated and are only undergoing chain elongation in vitro. The 76,000-molecular-weight protein thus labeled by f-Met can be immunoprecipitated by antiserum against whole disrupted virus. These observations suggest that Pr 76 synthesized in vitro can be labeled with f-Met. To prove more rigorously that the 76,000-molecular-weight polypeptide was labeled in the N-terminal position, f-Met-labeled product was eluted from the gel and digested with Pronase. The resulting peptides were subjected to electrophoresis in parallel with a number of synthetic f-[^{35}S]Met-containing dipeptides. Preliminary results show that the label is present in an f-Met dipeptide, but so far it has not been possible to positively identify the second amino acid. The finding that the Pr 76 made in vitro from 35S RNA is labeled N-terminally by methionine from initiator tRNA excludes the possibility of a precursor to Pr 76 with an extended N-terminal sequence.

Translation of viral RNA from other strains of RSV and td viruses. In addition to 35S RNA from RSV strain B77(c), 35S RNA from strains

Prague B and Prague C was also tested in the ascites cell-free protein synthesizing system. In each case the translation product was identical with that observed with B77-RSV 35S RNA, both on the criteria of gel mobility (Fig. 7) and, in the case of Pr-C, tryptic peptide analysis (Fig. 5). This was not unexpected, since the gs antigens are thought to show little variation between the different strains of RSV (13), although antigenic differences can be detected between subgroups.

td viruses of RSV have been isolated. The virion subunit RNA of these td viruses has lost the transforming gene, which constitutes approximately 15% near to the 3' end of the nd viral 35S RNA (16, 42). When 35S RNA from td viruses of B77 and Pr-C was tested in the ascites system, the major translation product was a band identical to the 76,000-molecular-weight protein seen with nd 35S RNA (Fig. 2 and 7), and this protein was translated with equal efficiency from the nd and td viral 35S RNAs.

In particular, no reproducible differences were seen in the translation products of the nd and td viral 35S RNAs. This implies that if a protein is encoded by the *sarc* gene at the 3' end of the 35S RNA, it is either translated in vitro at low efficiency or is translated in vivo from a separate mRNA. This argument also applies to the envelope and polymerase functions of the 35S RNA.

We have therefore modified the cell-free system in several ways in an attempt to translate proteins other than the gs antigens from the 35S RNA. For example, RSV virions contain a number of tRNA species, both free in the virion and associated with the 70S RNA (8, 31). It is possible that the free tRNA's, which are derived from the host cell, are necessary for viral protein synthesis. tRNA was therefore purified from virions and from RSV-transformed CEFs, assayed for integrity by charging with [^{35}S]methionine, and added to the ascites cell-free system. A small stimulation in total protein synthesis was observed, but no qualitative difference in the translation products was detected.

Furthermore, it is known from electron microscope studies that DNA binding proteins have the property of unwinding the tightly coiled secondary structure of the RSV 35S and 70S RNAs (23). Preincubation of 35S RNA with fd DNA binding protein had no effect on its translation in the ascites system. In the wheat germ cell-free system, on the other hand, 35S RNA is a poor messenger, and preincubation of 35S RNA with fd DNA binding protein leads to a specific stimulation of synthesis of the 76,000-molecular-weight protein by up to fivefold. However, no additional proteins can be seen in the wheat germ system, either with or without fd DNA binding protein (A. J. Pawson and W. F. Mangel, unpublished observation).

The presence of a capped, methylated 5'-terminus of the type m^{7}GpppN has been shown to be necessary for the efficient translation of several viral mRNA's (3). However, addition of *S*-adenosyl methionine, which is the methyl donor into such blocked ends, has no effect on the translation of 35S RNA. This is consistent with recent findings that virion 35S RNA already has a blocked, methylated 5'-terminal structure (10).

DISCUSSION

We have tested nd and td 35S RNA from two different strains of RSV in cell-free protein synthesizing systems from several sources. One major viral-specific polypeptide has been identified as being translated in response to these RNAs. This has a molecular weight of 76,000 and is comprised of the gs antigens P27, P19, P15 and P12. The presence of P27, P19 and P15 in this polypeptide has been established by analysis of [^{35}S]methionine-containing tryptic peptides, and that of P12, which contains little methionine and therefore cannot be analyzed in this way, by immunoprecipitation with a monospecific antiserum. This is in agreement with the findings of von der Helm and Duesberg (41). The gs antigens are synthesized in vivo as a precursor, which has been identified by immunoprecipitation of extracts from pulse-labeled infected cells and has an apparent molecular weight of 76,000 (40). No larger precursor to the gs antigens in vivo has been detected despite rigorous investigation (40). The major translation product encoded in vitro by 35S RNA and the gs antigen precursor in infected cells would therefore seem to be very closely related. The 76,000-molecular-weight polypeptide translated in vitro can be labeled in the N-terminal position by formyl-methionine derived from the initiator tRNA. This suggests that there are no extensive unstable polypeptide sequences at the N-terminal end of the nascent protein. One of the major objectives of this work was to ascertain whether 35S RNA codes for proteins other than Pr 76 in vitro. To examine this we have studied the translation of several strains and defective viruses of RSV. 35S RNA contains genes coding for reverse transcriptase and the protein constituent of the envelope glycoproteins in addition to that for the gs antigens. It is possible that the proteins other than the gs antigens encoded by 35S RNA are translated

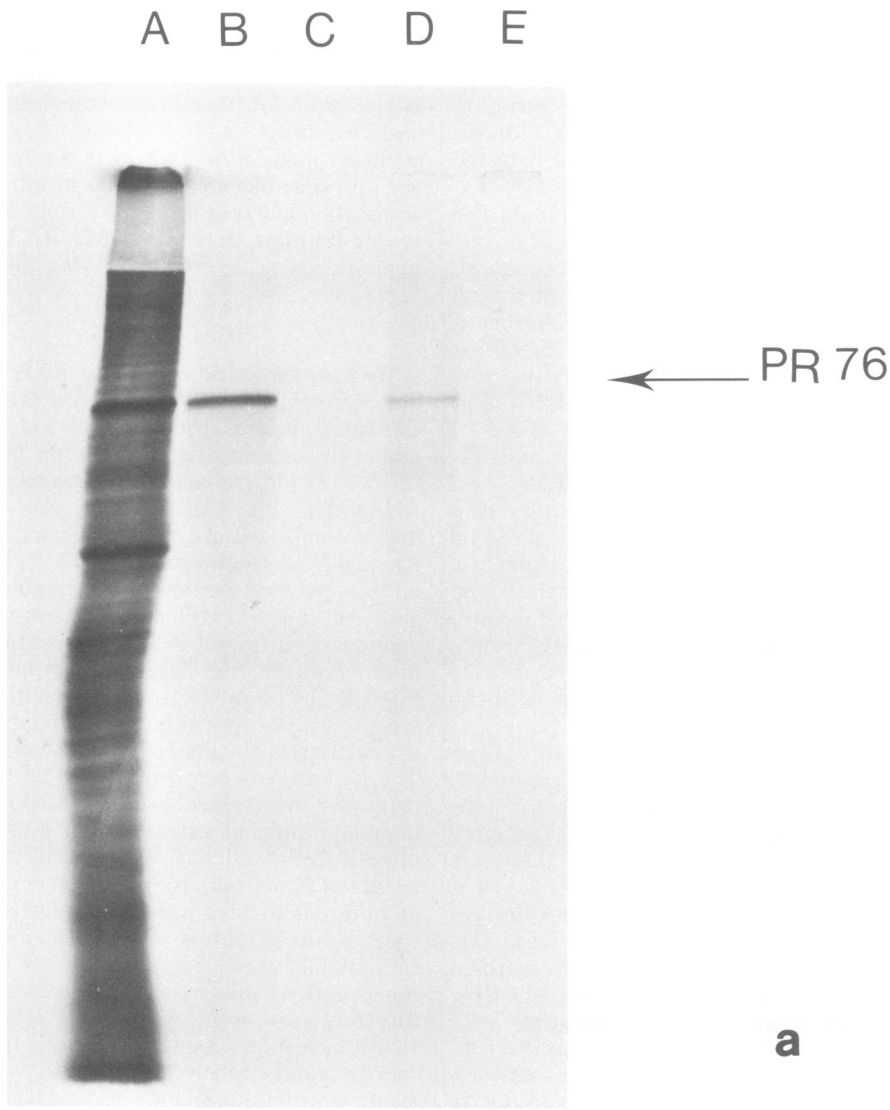

FIG. 4. *Immunoprecipitation of the ascites cell-free system products synthesized in response to nd B77(c) 35S RNA. All incubations were primed by 35S RNA. (a) Slot A, no immunoprecipitation; slot B, immunoprecipitation with antiserum raised against whole disrupted virus; slot C, immunoprecipitation with anti-gp 85(c) serum; slot D, immunoprecipitation with anti-P27 serum; slot E, immunoprecipitation with rabbit normal control serum. Immunoprecipitates were analyzed on a 15% polyacrylamide gel. (b) Slot A, immunoprecipitation with antiserum raised against whole disrupted virus; slot B, immunoprecipitation with anti-P12 serum. The polyacrylamide gel was impregnated with PPO and subjected to fluorography as described (19).*

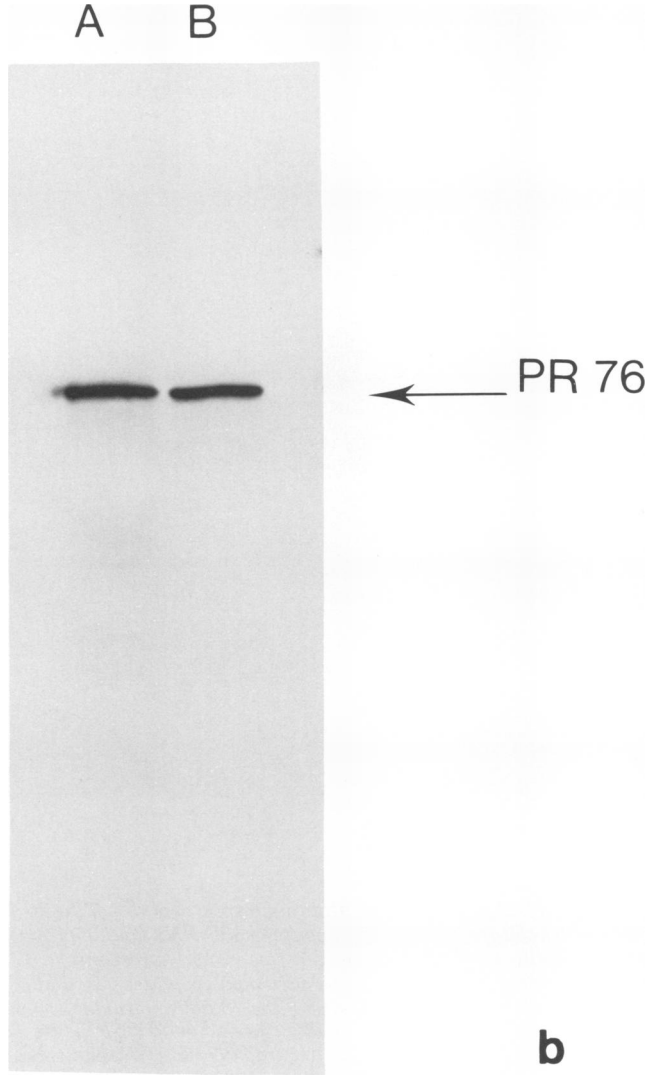

FIG. 4B.

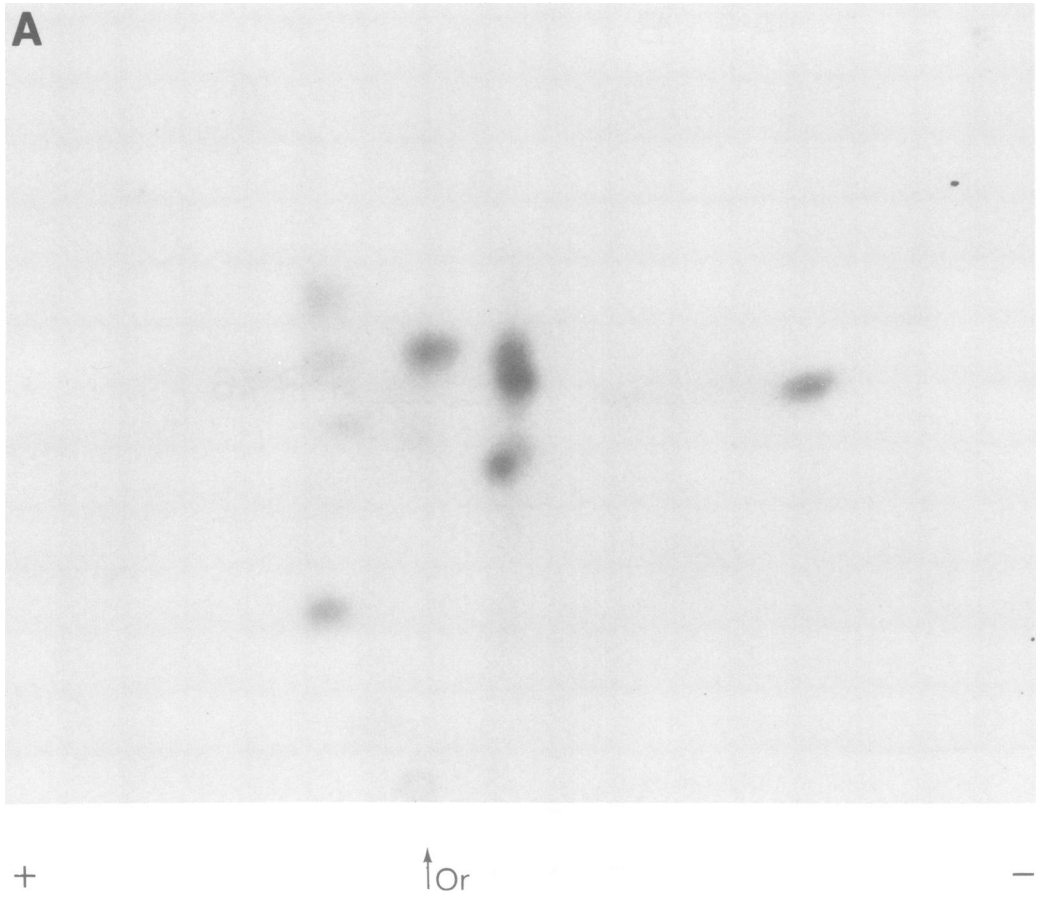

FIG. 5. *Fingerprint analysis of [^{35}S]methionine-containing tryptic peptides of the 76,000-molecular-weight product synthesized in the ascites cell-free system in response to 35S RNA and of virion proteins. (A) and (B) 76,000-molecular-weight polypeptide synthesized in the cell-free system in response to B77 RSV 35S RNA and Prague-C RSV 35S RNA, respectively; (C) total B77 RSV virion polypeptides; (D) and (E) P27 and P15 virion polypeptides, respectively; (F) map of the tryptic peptides of the 76,000-molecular-weight polypeptide synthesized in vitro showing which peptides are attributable to P27 (◍) and which to P15 (⊜). Electrophoresis was at pH 6.5 with the anode to the left. Chromatography was towards the top of the sheet. Samples were applied at the bottom and in the middle of the thin-layer cellulose sheets as indicated (Or). Samples for electrophoresis contained approximately 100,000 cpm.*

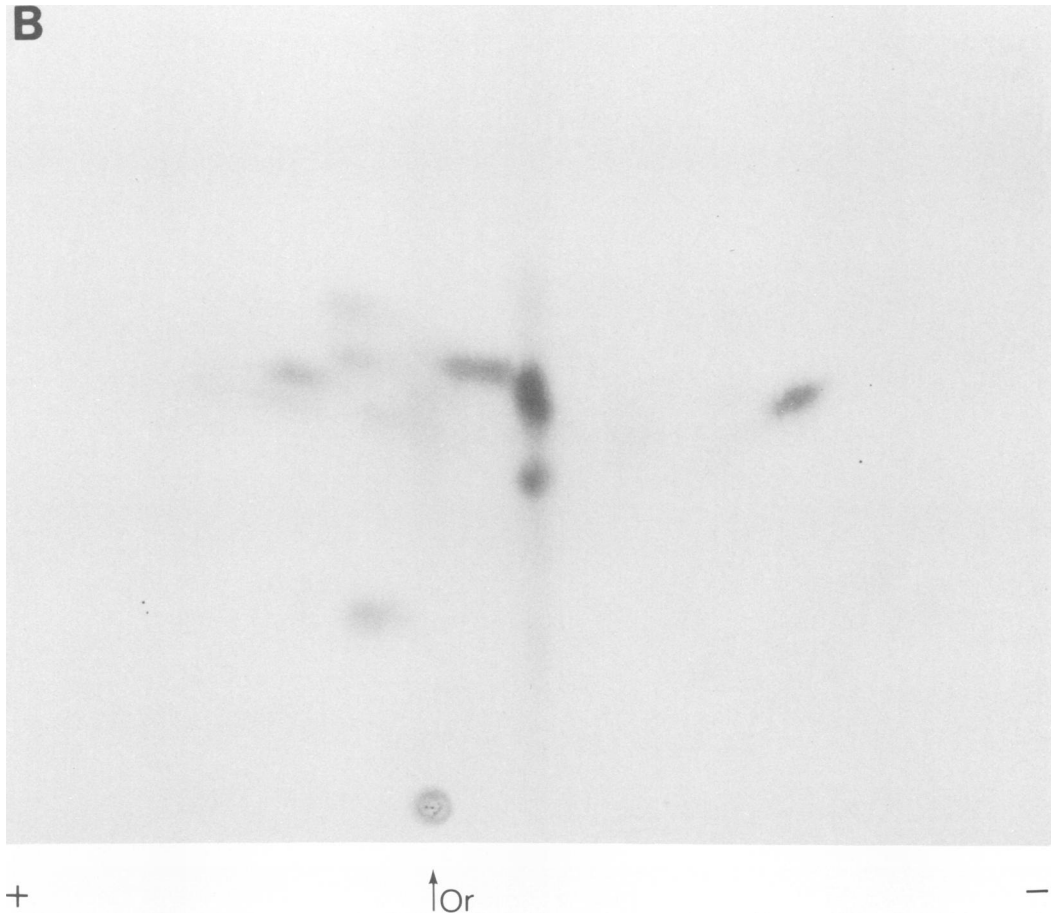

FIG. 5*B*.

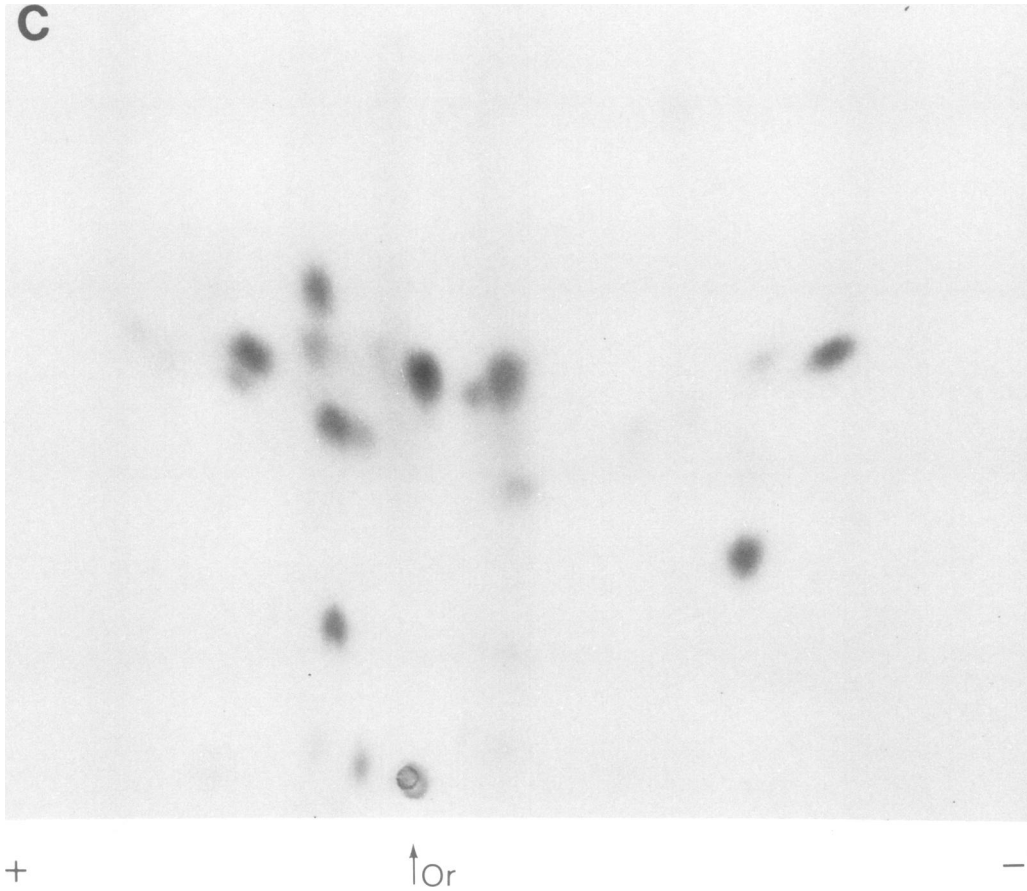

FIG. 5C.

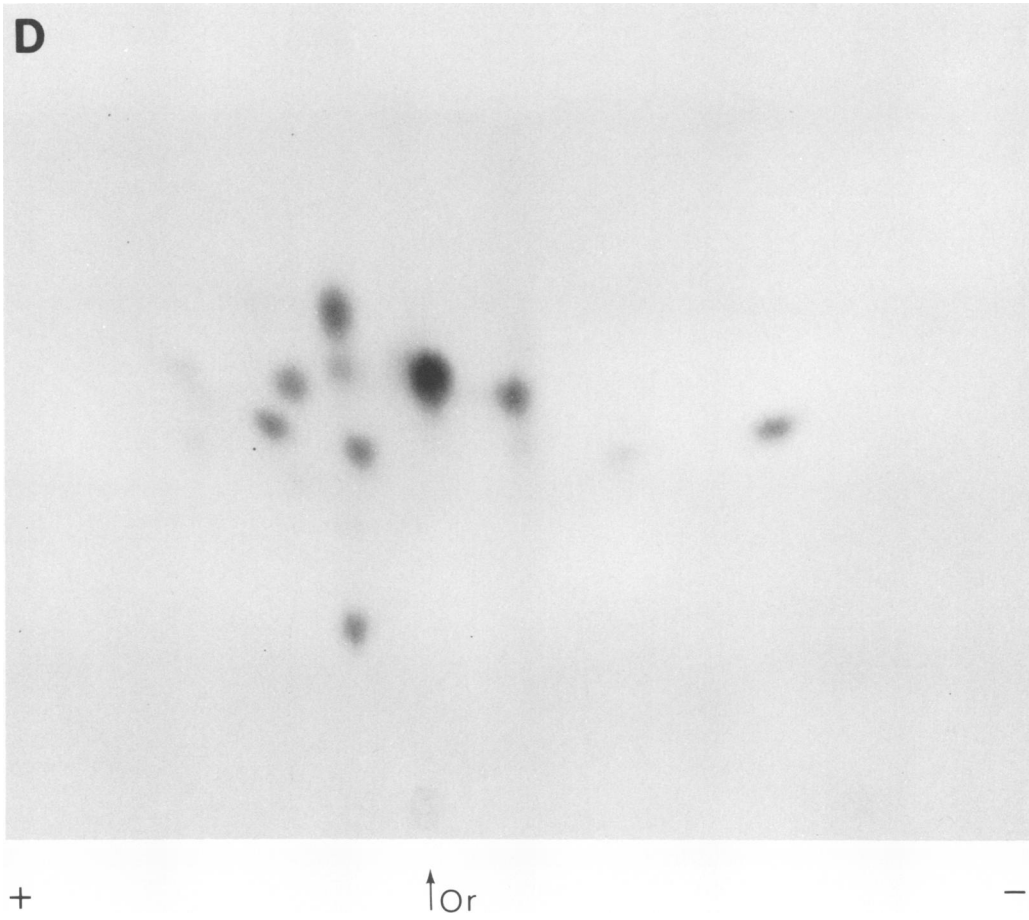

FIG. 5*D*.

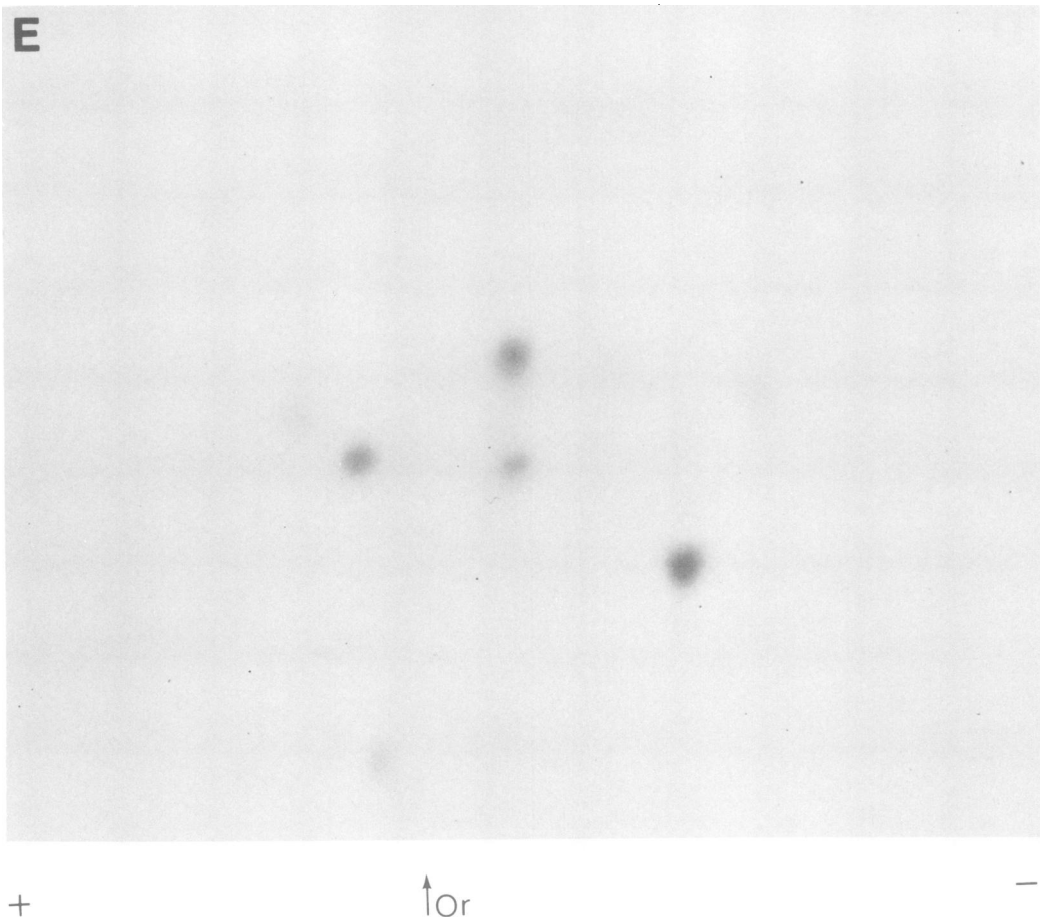

FIG. 5*E*.

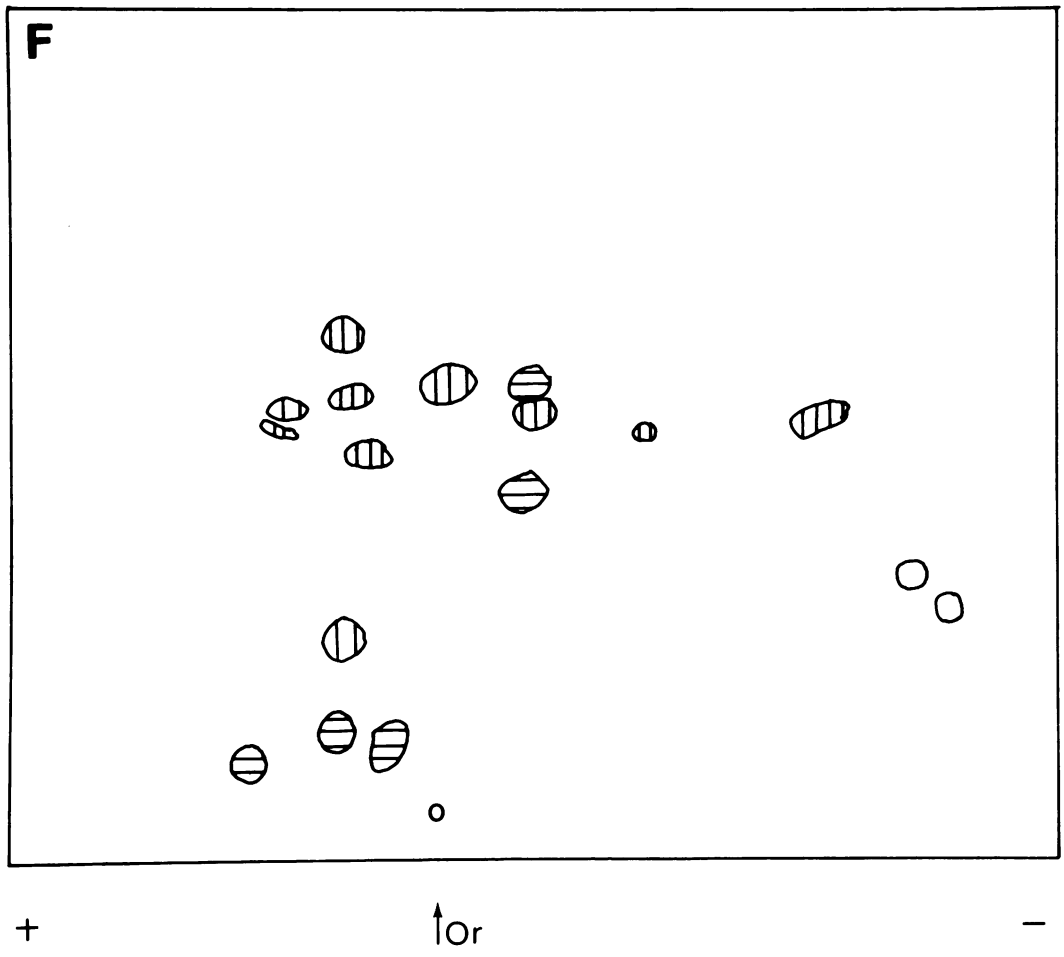

FIG. 5F.

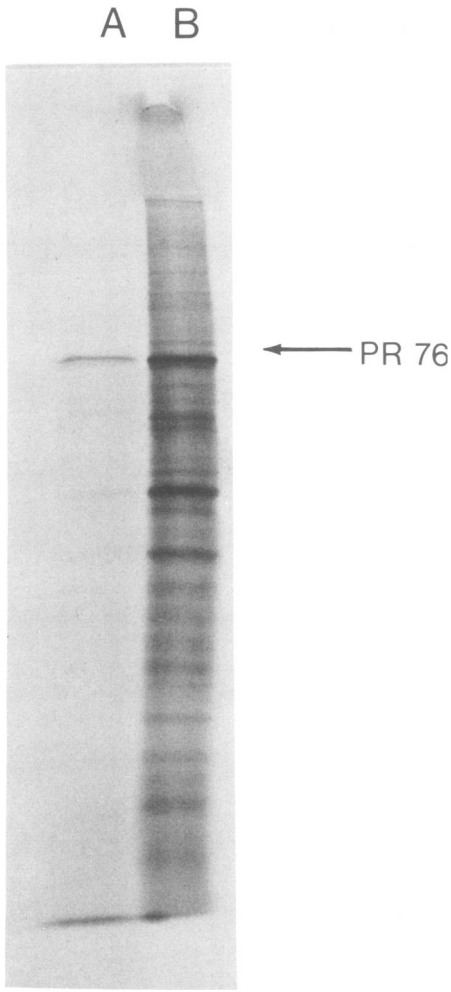

FIG. 6. *Electrophoretic analysis of the f-Met-labeled polypeptides synthesized in response to B77 RSV 35S RNA. Slot A, cell-free products labeled with f-Met in response to 35S RNA; slot B, cell-free products labeled with [^{35}S]methionine in response to 35S RNA. The position of PR 76 is indicated.*

from the 35S RNA in vivo at low efficiency, and our finding that 35S RNA only codes in vitro for the gs antigens may be due to inability to detect these low levels of synthesis. It is also possible that the *sarc* gene may encode a protein, although this has never been identified. Our evidence from the in vitro translation of virion RNA from td viruses compared with that from nd viruses suggests that if the *sarc* gene does encode a protein this is not translated efficiently in vitro from 35S RNA.

There are a number of mechanisms whereby viral proteins could be translated in cells. 35S RNA has been reported to be present in the polyribosomes of RNA tumor virus-infected cells (9, 37), although this does not necessarily mean that it is a functional mRNA in vivo. If 35S RNA were the sole mRNA species coding in vivo for viral proteins, there are at least two ways in which this could happen. The entire viral genome could be translated as a single protein of 250,000 to 300,000 molecular weight that is subsequently cleaved to the separate viral proteins. The RNAs of the picornaviruses EMC and poliovirus are translated in this way (4, 14). The very different amounts in which the RNA tumor virus proteins are observed in the cell (28) means that this would involve very different turnover rates for the viral proteins. Our finding that 35S RNA only codes in vitro for the 76,000-molecular-weight gs antigen precursor might be thought to militate against translation of the entire genome into a single polyprotein, but this could be an in vitro artefact such as that known to occur during the synthesis of other viral capsid and envelope proteins. For example, Semliki forest virus 26S RNA encodes both capsid and envelope proteins, and these are synthesized as a polyprotein, which in whole cells is subsequently cleaved to yield equimolar amounts of the two proteins. In vitro, however, for reasons that are not yet understood, the capsid is synthesized with much greater efficiency than the envelope proteins (N. Glanville, M. Ranki, J. Morser, L. Kääriäinen, and A. E. Smith, Proc. Natl. Acad. Sci. U.S.A., in press).

An alternative mechanism for the translation of 35S RNA from one initiation site would be that the majority of ribosomes terminate at the end of the gs antigen cistron on the 35S RNA, but some continue into the next cistron. Such a model has been proposed to explain the unequal synthesis of viral proteins present in cells infected with mengovirus (29). Recent results reported for the murine RNA tumor viruses suggest that a larger precursor may be made at low levels in vivo and that the virion RNA may code for this in vitro (18, 26, 27).

Rather than the single initiation site model described above, 35S RNA might contain functional internal initiation sites for protein synthesis, which are not identified by labeling with formyl-methionine in vitro. Jacobson and Baltimore have proposed that a single eukaryotic mRNA can only code for one polypeptide chain (15), and indeed most mRNA's have been found to contain only one ribosome binding site. Recently, however, RNAs have been isolated which contain internal initiation sites for protein synthesis (i.e., polyoma virus [36], Semliki forest virus [Glanville et al., in press], brome mosaic virus [33], tobacco mosaic virus [20], and Sindbis virus [5]). However, in all these

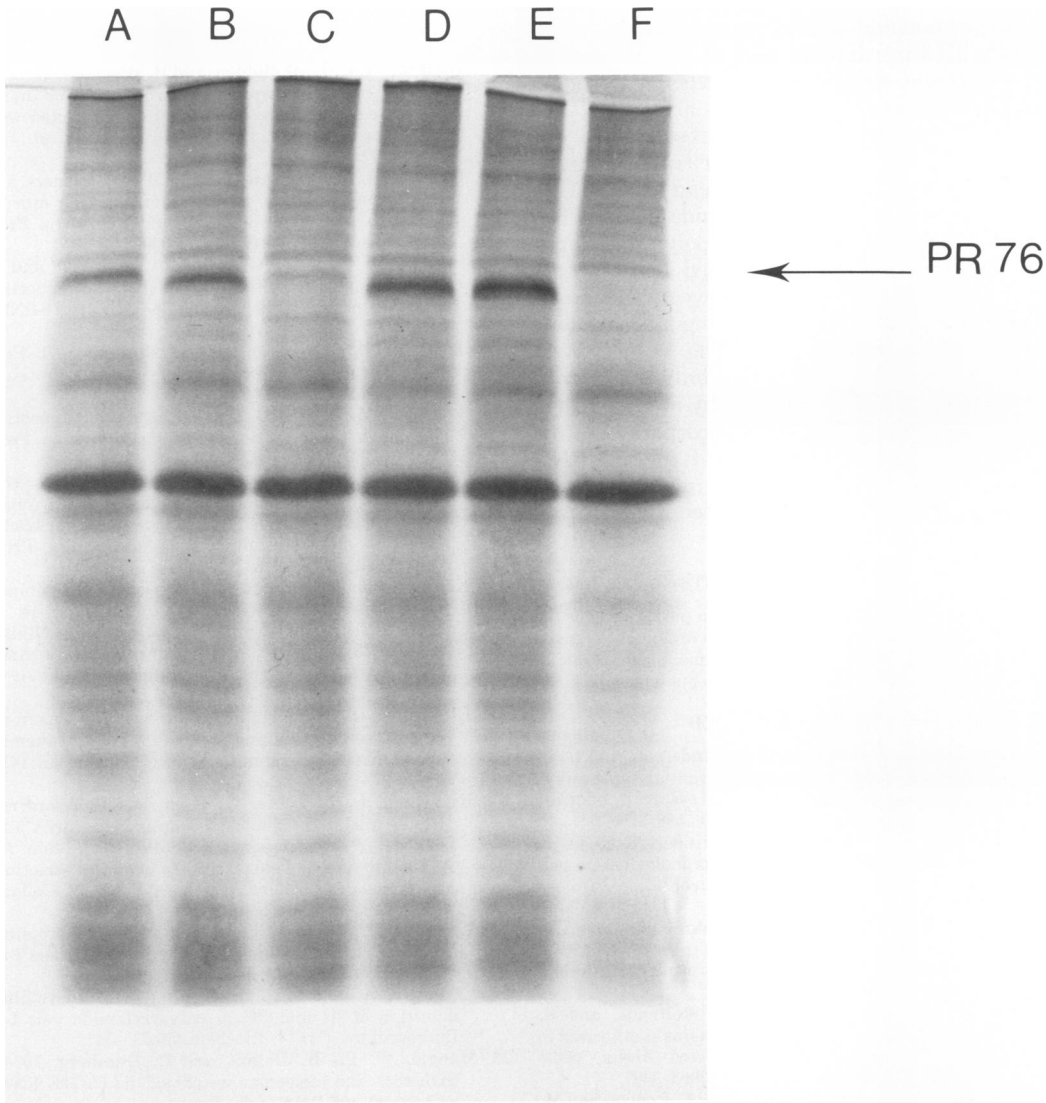

FIG. 7. *Comparison of the cell-free products made in response to different viral RNAs. Slot A, plus nd Prague-C RSV 35S RNA; slot B, plus td Prague-C RSV 35S RNA; slot C, plus nd B77 RSV 70S RNA; slot D, plus nd B77 RSV 35S RNA; slot E, plus td B77 RSV 35S RNA; slot F, no additions. The position of PR 76 is indicated.*

cases the internal sites are relatively inactive, and smaller more active messenger species are made with these sites at the 5′ end. Polyoma virus late 19*S* mRNA, for example, in addition to an active cistron for VP2, contains an internal initiation site and coding information for VP1. The latter is not efficiently expressed until the 19*S* RNA is processed into a smaller 16*S* RNA species (36). The possibility of active internal initiation sites would not therefore seem likely for 35*S* RNA, but suggests an alternative mechanism for viral protein synthesis: the viral proteins may be made from smaller mRNA's, each corresponding to a separate cistron on the 35*S* RNA.

There is evidence that, in cells infected with RNA tumor viruses, viral-specific RNAs smaller than 35*S* are present (9, 11). It is not clear whether these can act as functional mRNA's. The size of mRNA from RSV-infected cells that can code in vitro for viral proteins is currently under investigation. Recent findings with Rauscher murine leukemia virus-infected cells indicate that viral mRNA's smaller than 35*S* are able to code for proteins in vitro (12). However, none of the in vitro translation prod-

ucts has been adequately characterized.

Our finding that nd and td 35S RNA from two different strains of RSV codes in vitro predominantly for the gs antigens argues that the gene for these proteins lies nearest to the 5′ end of the 35S RNA. The gene for transformation has been positioned at the 3′ end and that for the envelope proteins in the middle of the 35S RNA (16, 42). Thus, either the polymerase or gs antigen function must be located at the 5′ end. For the reasons presented above it is thought that eukaryotic ribosome binding sites are only active when they are near the 5′ end of an mRNA. The observations that only the gs antigen precursor can be detected by in vitro translation and that f-Met can be incorporated at its N-terminus argue that unless RSV 35S RNA is translated by some unique mechanism the gene for the gs antigens is located nearest its 5′ end.

ACKNOWLEDGMENTS

We thank R. MacCormick for help in purification of the viral RNA and R. Harvey for assistance in cell-free translation. We thank Ian M. Kerr and Hans Bloemendal for allowing us to see manuscripts prior to publication.

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