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Synthesis of Rauscher murine leukemia virus-specific polypeptides *in vitro*

(cell-free lysates/polyribosomes/messenger RNA/immunoprecipitation/gel electrophoresis)

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ABSTRACT The biosynthesis of specific polypeptides directed by purified viral messenger RNA from JLS-V9 cells infected with Rauscher leukemia virus has been studied in a rabbit reticulocyte lysate. The 35S viral mRNA gives rise to two major products of 65,000 and 72,000 molecular weight. The synthesis of specific polypeptides was also investigated in lysates derived from infected cells. The main products were polypeptides with molecular weights of 65,000, 76,000, and 82,000, and were preferentially made in association with membranes. The relative content of the virus-specific polypeptide of 65,000 molecular weight, synthesized in a cell-free system supplemented with purified polyribosomes, is considerably higher for membrane-bound polyribosomes.

Mature proteins of RNA tumor viruses are formed by specific cleavage of large precursor polypeptides. In avian leukosis and sarcoma-virus-infected cells the biosynthesis of a precursor [molecular weight (M_r) 76,000] of the internal virion antigens and an incompletely glycosylated precursor (M_r 70,000) of the major viral glycoprotein (gp85) has been demonstrated (1, 2). Likewise, two newly synthesized intracellular precursor polypeptides were detected in cells infected with Rauscher leukemia virus (R-MuLV) (3, 4). One polypeptide (M_r 65,000) is probably the precursor of the low-molecular-weight virion proteins, while the other (M_r 85,000) is the precursor of the viral glycoprotein (4). The virus-specific messengers involved in the synthesis of these polypeptides have been identified. The major virus-specific mRNA in productively infected cells is 35S RNA, but evidence for virus-specific RNA of smaller size (20–22 S) has been found (5–7). The latter mRNA appeared to be preferentially associated with membrane-bound polyribosomes (6, 7).

We have studied the synthesis of R-MuLV specific polypeptides in fractionated crude cell-free lysates and in cell-free systems supplemented with purified free and membrane-bound polyribosomes. Moreover, we examined the biosynthesis of virus-specific polypeptides in a rabbit reticulocyte lysate, supplemented with poly(A)-rich mRNA fractionated according to size. Evidence was obtained that virus-specific products are preferentially synthesized in association with membranes. Moreover, it could be demonstrated that addition of 35S mRNA or 20–22S mRNA to a reticulocyte lysate results in the synthesis of specific polypeptides.

MATERIALS AND METHODS

Cells and Virus. The JLS-V9 cell line derived from bone marrow cells of Balb/c mice, infected with and producing R-MuLV, was grown in Eagle's basal minimal essential medium supplemented with 10% calf serum (8). Labeling of these cells and the preparation of cell extracts for immunoprecipitation was described (3).

Preparation of Cytoplasmic Extracts and Polyribosomes. For the isolation of cytoplasmic extracts 1.5 ml of cells was lysed in 2 volumes of hypotonic buffer (10 mM Tris·HCl pH 7.5, 15 mM KCl, 1.5 mM magnesium acetate, and 2 mM dithiothreitol) by 30 strokes of a tight-fitting Dounce homogenizer. Nuclei were removed by centrifugation at 125 × *g* for 5 min. Three milliliters of the supernatant was separated into a cytoplasmic supernatant and pellet by centrifugation at 20,000 × *g* for 6 min in a Ti-50 rotor (Spinco). The membrane-rich 20,000 × *g* pellet fraction was suspended in 1 ml of hypotonic buffer. A ribosomal supernatant was prepared from the 20,000 × *g* supernatant by centrifugation at 220,000 × *g* for 2 hr in a Ti-50 rotor. Polyribosomes used in the cell-free system were isolated as described (9), except that sodium deoxycholate and Nonidet P-40 (Shell) solubilized membrane fractions were clarified by centrifugation at 12,000 × *g* for 5 min. Total polyribosomes used for the isolation of oligo(dT)-cellulose purified mRNA were prepared from cells lysed by treatment with a mixture of sodium deoxycholate and Nonidet P-40 as described (7).

Isolation and Fractionation of mRNA. Poly(A)-containing mRNA of total polyribosomes was isolated by affinity chromatography on oligo(dT)-cellulose. Four mg of total polyribosomes were dissociated in buffer A [10 mM Tris·HCl pH 7.4, 500 mM NaCl, 1 mM EDTA, and 1% sodium dodecyl sulfate ($NaDodSO_4$)] at 37° for 5 min and applied to a 2-ml oligo(dT)-cellulose column in buffer A. After the oligo(dT)-cellulose was washed with buffer A and buffer B (10 mM Tris·HCl pH 7.4, 100 mM NaCl, 1 mM EDTA,and 0.2% $NaDodSO_4$) until all nonabsorbed RNA was removed, poly(A)-containing RNA was eluted with buffer C (10 mM Tris·HCl, pH 7.4).

Immunoprecipitation and Polyacrylamide Gel Electrophoresis. To the cell-free incubation mixtures immunoprecipitation buffer (10 mM phosphate pH 7.2, 0.9% NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% $NaDodSO_4$) was added and the resulting solution was clarified by centrifugation at 220,000 × *g* for 10 min in a Ti-50 rotor. Virus-specific products in the supernatant were directly or indirectly precipitated by addition of rabbit antiserum against R-MuLV as described (3). Precipitates were collected by centrifugation through a layer of 10% sucrose (weight/volume) in immunoprecipitation buffer and washed 3 times with this buffer. The resulting precipitates were dissolved in sample buffer (10), containing 6 M urea, by heating at 95° for 2 min. Immunoprecipitates were analyzed by electrophoresis on 7–18% polyacrylamide gradient slab gels with the Tris-glycine buffer system (10). After electrophoresis the

Abbreviations: R-MuLV, Rauscher leukemia virus; M_r, molecular weight; $NaDodSO_4$, sodium dodecyl sulfate.

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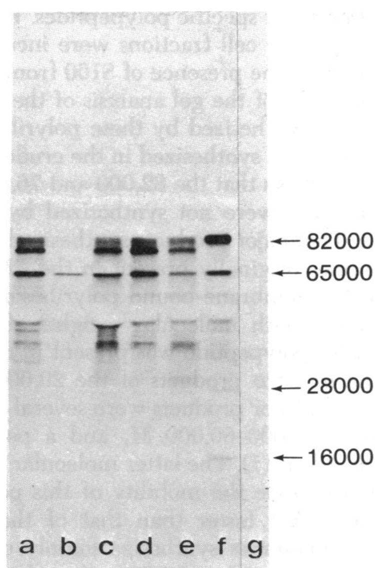

FIG. 1. NaDodSO$_4$-polyacrylamide gel electrophoresis of the virus-specific polypeptides synthesized *in vitro* in fractionated cell extracts. A standard reaction mixture contained (in a final volume of 25 μl) 15 mM Tris·HCl pH 7.5, 2.5 mM magnesium acetate, 75 mM KCl, 70 mM potassium acetate, 1 mM dithiothreitol, 4 μl of energy and amino-acid mixture [final concentrations after dilution in the complete reaction mixture: 1 mM ATP, 0.2 mM GTP, 10 mM creatine phosphate, 0.2 mg/ml of creatine kinase, 19 unlabeled amino acids in the concentrations described (13), 200 μCi/ml of [^{35}S]methionine (specific activity 170 Ci $\times$ mmol^{-1}, Radiochemical Centre, Amersham)], 3 μl (75 μg) of crude initiation factor from rabbit reticulocytes (14). Crude cell fractions, S100, and 10 μg of pH 5 enzymes (15) from infected cells were added as indicated below. Incubation was performed at 30° for 150 min. Virus-specific polypeptides were precipitated by the direct method from reaction mixtures supplemented with (a) 15 μl of 125 $\times$ *g* supernatant; (b) 15 μl of the 20,000 $\times$ *g* supernatant; (c) 5 μl of the suspended 20,000 $\times$ *g* pellet; (d) as (c) with 10 μl of S100; (e) as (c) with 3 μl of pH 5 enzymes; (f) immunoprecipitate of virus-specific polypeptides synthesized from extracts of cells pulse-labeled *in vivo* followed by processing *in vitro* (3); (g) polypeptides aspecifically precipitated (3) with antiserum against bovine serum albumin from the reaction mixture supplemented with the 125 $\times$ *g* supernatant.

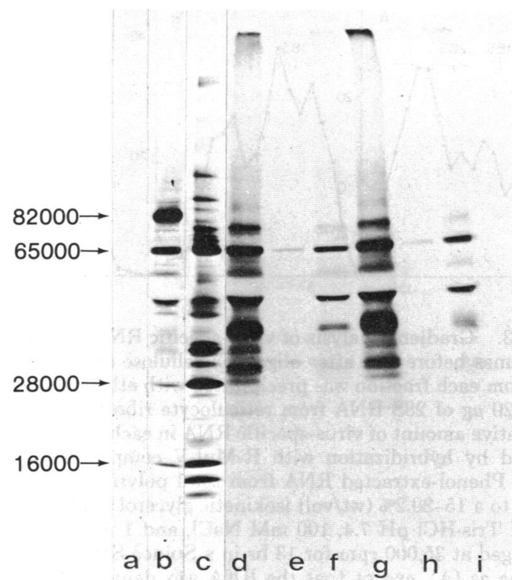

FIG. 2. Analysis of the virus-specific polypeptides synthesized by isolated polyribosomal fractions. The cell-free system contained (in a final volume of 25 μl) 12 μl of S100 from rabbit reticulocytes, 0.6 $A_{260\,nm}$ unit of polyribosomes, 4 μl of energy and amino-acid mixture as described in the legend of Fig. 1, and 20 mM *N'*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) pH 7.5, 2 mM magnesium acetate, 60 mM KCl, 80 mM potassium acetate, and 1 mM dithiothreitol. Cell-free incubation was performed at 26° for 150 min. Indirect immunoprecipitate of polypeptides synthesized *in vivo* (a) using antiserum against bovine serum albumin; (b) using antiserum against R-MuLV; (c) R-MuLV labeled with [^{14}C]aminoacids (3). Indirect immunoprecipitation of virus-specific polypeptides synthesized in the cell-free system supplemented with (d) polyribosomes associated with the 125 $\times$ *g* pellet; (e) free polyribosomes; (f) membrane-bound polyribosomes; (g), (h), and (i) same as (d), (e), and (f) except that 200 μM amino-acid analogues (*p*-fluorophenylalanine, canavanine, and azetidine-2-carboxylic acid) were present in the incubation mixture.

gel was stained, treated with 22.2% 2,5-diphenyloxazole (PPO) in dimethyl sulfoxide (weight/volume) (11), and dried on Whatman 3 MM filter paper; the radioactive bands were visualized by autoradiography.

RESULTS

Virus-specific Polypeptides Synthesized in Fractionated Cell Extracts. Hybridization experiments had demonstrated that the relative amount of virus-specific mRNA is four to six times higher in membrane-bound polyribosomes than in free polyribosomes (7, 12). In accordance with these observations we expected and actually found a similar distribution of newly synthesized virus-specific polypeptides between a cytoplasmic 20,000 $\times$ *g* pellet and supernatant fraction after incubation of these fractions *in vitro*. Cells were lysed in hypotonic buffer and nuclei were removed by low speed centrifugation. The supernatant was fractionated by centrifugation at 20,000 $\times$ *g* into a pellet (membrane fraction) and a supernatant fluid (membrane-free fraction). These crude fractions were tested for their protein-synthesizing activity, and the virus-specific products were directly precipitated with antiserum against R-MuLV. Immunoprecipitation was always performed on equal amounts of [^{35}S]methionine incorporated into polypeptides.

Analysis of the immunoprecipitates by polyacrylamide gel electrophoresis showed that three major virus-specific polypeptides were synthesized in the membrane-rich 125 $\times$ *g* supernatant (Fig. 1a) and the 20,000 $\times$ *g* pellet fractions (Fig. 1c). The molecular weights of these R-MuLV specific polypeptides were 65,000, 76,000, and 82,000. *In vivo* only two major precursor polypeptides were newly synthesized, pp65 and pp82 (Fig. 1f) (molecular weights of 65,000 and 82,000, respectively). Incubation of the 20,000 $\times$ *g* pellet fraction in the presence or absence of S100 or pH 5 enzymes from infected cells resulted in the same set of polypeptides (Fig. 1c, d, and e). Minor amounts of polypeptides with molecular weights between 30,000 and 60,000 are visible (Fig. 1a, c, d, and e), which could not be precipitated with antiserum to bovine serum albumin. These minor products presumably arise by premature termination of translation or proteolytic cleavage of the high-molecular-weight polypeptides. A different result was obtained when the membrane-free 20,000 $\times$ *g* supernatant was incubated *in vitro*. Three to five times less virus-specific polypeptides were present in the immunoprecipitate of this fraction as compared with the 20,000 $\times$ *g* pellet. Gel analysis of the immunoprecipitate of the incubation mixture with added 20,000 $\times$ *g* supernatant showed that a polypeptide with an apparent molecular weight of 65,000 is the only virus-specific product synthesized (Fig.

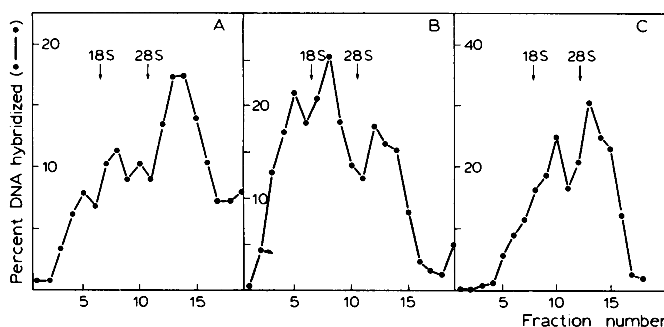

FIG. 3. Gradient analysis of virus-specific RNA from total polyribosomes before and after oligo(dT)-cellulose chromatography. RNA from each fraction was precipitated with ethanol, after addition of 20 μg of 28S RNA from reticulocyte ribosomes as carrier. The relative amount of virus-specific RNA in each fraction was determined by hybridization with R-MuLV complementary DNA (4). (A) Phenol-extracted RNA from total polyribosomes (4) was applied to a 15–30.2% (wt/vol) isokinetic glycerol gradient in TNE (10 mM Tris·HCl pH 7.4, 100 mM NaCl, and 1 mM EDTA) and centrifuged at 35,000 rpm for 13 hr in a Spinco SW41 rotor at 5°; (B) same as (A), except that the RNA was denatured in 10 mM Tris·HCl pH 7.4 and 0.5% $NaDodSO_4$ at 50° for 5 min; (C) analysis of RNA purified on an oligo(dT)-cellulose column. Poly(A)-containing RNA (80 μg) was treated with 85% formamide in 10 mM Tris·HCl pH 7.4 at 37° for 5 min and loaded onto a 5–22.7% (wt/vol) isokinetic sucrose gradient in TNE containing 50% (vol/vol) formamide. Tubes were spun in a Spinco SW41 rotor at 41,000 rpm for 15 hr at 5°.

1b). Among the products synthesized *in vitro* the virion proteins p30 and p15 are not detected, while they are present in minor amounts in the extracts of cells pulse-labeled *in vivo* (Fig. 1f) (3). In the experiments presented, translation *in vitro* was performed at its optimal temperature of 30°. It was, however, not investigated whether this temperature is optimal for processing of the precursor polypeptide.

Virus-Specific Polypeptides Synthesized by Free and Membrane-Bound Polyribosomes. The experiments described above indicate that the synthesis of R-MuLV-specific polypeptides was possible in crude cell extracts. We decided to examine whether translation mediated by purified free and membrane-bound polyribosomes resulted in the biosynthesis of the same set of specific polypeptides. Polyribosomes isolated from different cell fractions were incubated in the subcellular system in the presence of S100 from rabbit reticulocytes. Comparison of the gel analysis of the immunoprecipitated products synthesized by these polyribosomes (Fig. 2d, e, and f) with those synthesized in the crude cytoplasmic extracts (Fig. 1a) shows that the 82,000 and 76,000 M_r polypeptides presumably were not synthesized by the isolated polyribosomes. The major products synthesized by the purified polyribosomes originally present in the 125 × *g* pellet (Fig. 2d) and the membrane-bound polyribosomes (Fig. 2f) were polypeptides with molecular weights of 65,000 and 45,000. This latter polypeptide was present in much smaller amounts in the *in vitro* products of the 20,000 × *g* pellet fraction (Fig. 1c). Minor products were several polypeptides in the region of 30,000–60,000 M_r and a polypeptide of 74,000 M_r (Fig. 2d and f). The latter molecular weight value has been assigned since the mobility of this polypeptide is consistently somewhat faster than that of the 76,000 M_r band. Free polyribosomes synthesize mainly one polypeptide which comigrates with pp65 (Fig. 2b and e). After longer exposure times also a faint band of M_r 74,000 was visible on the autoradiogram (not shown). For comparison, the protein pattern of [14C]amino-acid-labeled R-MuLV is also shown (Fig. 2c). The most striking feature is the occurrence in the virus preparation of a polypeptide coinciding with the putative precursor polypeptide of 65,000 M_r. The ratio of the percentages of virus-specific polypeptides of free and membrane-bound polyribosomes was approximately the same as that between the membrane-rich cytoplasmic pellet fraction and membrane-free supernatant fluid. However, qualitative differences in individual polypeptides between the various fractions do exist. Addition of amino-acid analogues (*p*-fluorophenylalanine, canavanine, and azetidine-2-carboxylic acid) to the cell-free system did not result in the synthesis of higher-molecular-weight polypeptides by these polyribosomes (Fig. 2g, h, and i).

Translation of Oligo(dT)-Cellulose Purified mRNA. Previous reports demonstrated that virus-specific RNA was present in polyribosomes from cells infected with MSV(MLV) and R-MuLV (6, 7). Virus-specific 35S mRNA

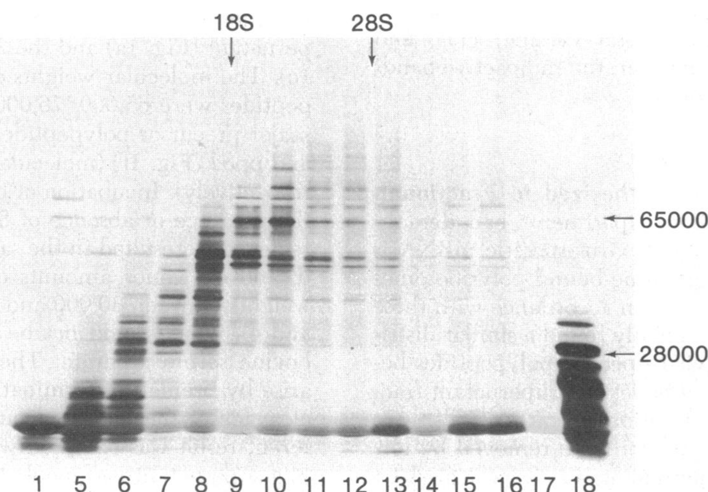

FIG. 4. $NaDodSO_4$-polyacrylamide gel autoradiogram of the polypeptides synthesized *in vitro* by poly(A)-containing RNA, fractionated according to size. RNA from each fraction of the gradient, shown in Fig. 3C, was dissolved in 50 μl of distilled water. Cell-free synthesis was performed in a final volume of 25 μl containing 3 μl of test solution, 12 μl of rabbit reticulocyte lysate, 4 μl of energy and amino-acid mixture as in legend of Fig. 1, and 2 mM magnesium acetate, 65 mM KCl, and 60 mM potassium acetate. Reaction mixtures were incubated at 26° for 150 min. Numbers 5 to 17 refer to the fractions of the gradient. (*1*) Polypeptides synthesized in the lysate, without exogenous RNA; (*5* to *17*) with RNA from the fractions indicated; (*18*) with 10S lens mRNA.

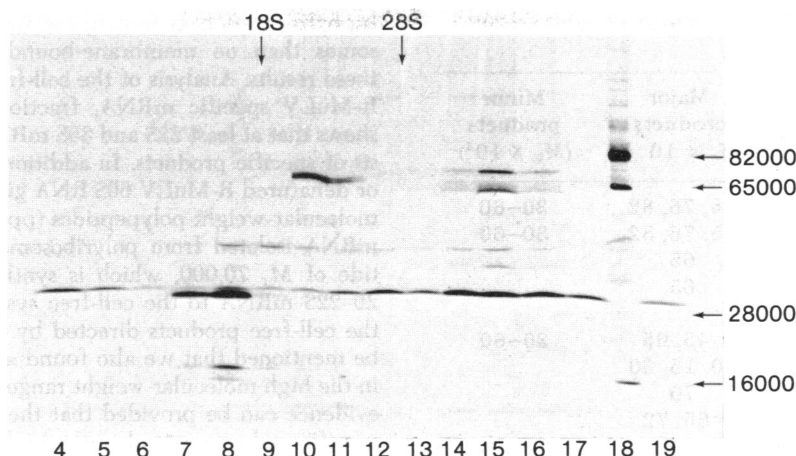

FIG. 5. NaDodSO$_4$-polyacrylamide slab gel analysis of the virus-specific polypeptides synthesized by the fractionated mRNA. Polypeptides synthesized in the cell-free system programmed with RNA from each fraction, as described in legend of Fig. 4, were subjected to indirect immunoprecipitation using an antiserum against R-MuLV. Likewise, an aspecific immunoprecipitation was performed on the polypeptides synthesized in the lysate without exogenous RNA. (*4 to 17*) Virus-specific products synthesized under direction of each fraction of the gradient; (*18*) polypeptides synthesized *in vivo* (as in Fig. 1); (*19*) aspecific control.

was the predominant RNA species present in free polyribosomes. On the other hand, membrane-bound polyribosomes contained, in addition to 35S RNA, also smaller RNA molecules with sedimentation coefficients of about 14 S and 20–22 S, respectively. First, we were interested in the translation products coded for by the 35S mRNA. Further, we wanted to examine whether or not the 20–22S and 14S RNAs are functional messengers. Evidence that these RNAs can be translated in specific polypeptides provides valuable information about the relation between this mRNA and the 35S mRNA. For this purpose it was necessary to prevent any aggregation of mRNA during fractionation on sucrose gradients. Therefore, the effect of heat-denaturation or formamide treatment (the latter result not shown, but almost identical to that of heat treatment) of polyribosomal RNA in sucrose gradients was studied. It appeared that denaturation of the RNA resulted in a shift of virus-specific RNA from the 35S to the 22S and 14S region (Fig. 3A and B). Fig. 3C illustrates the profile of virus-specific poly(A)-rich mRNA after gradient centrifugation in the presence of 50% formamide. The hybridization profile of this gradient is almost identical to that shown in Fig. 3B, except that the RNA purified on oligo(dT)-cellulose contains less material sedimenting in the 14S region. Fractions from the formamide gradient were translated in a reticulocyte system and the resulting polypeptides were analyzed on a polyacrylamide slab gel. The analysis showed that there was no aggregation of the bulk of the mRNA in the gradient, as there existed a good correlation between the molecular weight of the products synthesized and the S value of the mRNA in each fraction (Fig. 4).

After immunoprecipitation by the indirect method, several distinct polypeptides could be detected (Fig. 5). The 35S mRNA synthesizes two precipitable polypeptides with molecular weights of 65,000 and 72,000 (Fig. 5, *15*), of which the first comigrated with the 65,000 M_r polypeptide (pp65) synthesized *in vivo* (Fig. 6a, b, and c). RNA present in the 20–22S region gives rise to a product of M_r 69,000 (Fig. 5, *10*) which is not synthesized *in vivo* (Fig. 6d and e). Messenger RNA present in the 14S region directs the synthesis of low-molecular-weight polypeptides of about 30,000 and 15,000 M_r (Fig. 5, *8*). In a control experiment immunoprecipitation was carried out on polypeptides synthesized by the endogenous reticulocyte system. One polypeptide with a molecular weight of 32,000 was precipitated by the R-MuLV antiserum, which was present in variable amounts in all immunoprecipitates (Fig. 5). Native or denatured R-MuLV 60S RNA directed the synthesis of the same polypeptides (M_r 65,000 and 72,000) as were coded for by the 35S mRNA (16).

DISCUSSION

After labeling *in vivo* of R-MuLV infected cells with radioactive amino acids, two newly synthesized virus-specific polypeptides were identified (3, 4). A precursor polypeptide of M_r 65,000 was precipitated with a monospecific antiserum against p30, while antiserum against the glycoprotein gp69/71 revealed a second high-molecular-weight polypep-

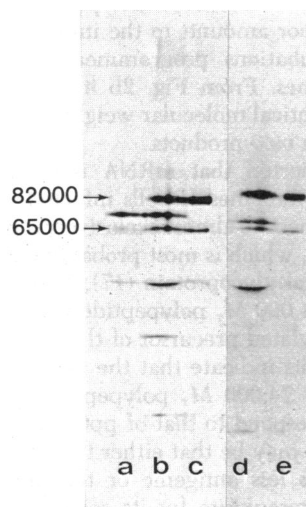

FIG. 6. Comparison of *in vitro* and *in vivo* synthesized virus-specific polypeptides by co-electrophoresis on a NaDodSO$_4$-polyacrylamide slab gel. (a) Immunoprecipitate of polypeptides synthesized *in vitro* by mRNA from fraction 15 (Fig. 4); (b) co-electrophoresis of the immunoprecipitates of polypeptides synthesized *in vivo* and by the mRNA from fraction 15 *in vitro;* (c) and (e) immunoprecipitate of polypeptides synthesized *in vivo;* (d) co-electrophoresis of the immunoprecipitates of polypeptides synthesized *in vivo* and by mRNA from fraction 10 *in vitro.*

Table 1. R-MuLV-specific polypeptides* synthesized *in vitro*

Fraction used for translation	Major products ($M_r \times 10^{-3}$)	Minor products ($M_r \times 10^3$)
125 × *g* supernatant	65, 76, 82	30–60
20,000 × *g* pellet	65, 76, 82	30–60
20,000 × *g* supernatant	65	
Free polyribosomes	65	
Membrane-bound polyribosomes	45, 65	30–60
14S mRNA	10, 15, 30	
20–22S mRNA	70	
35S mRNA	65, 72	
35S viral RNA	65, 72	

* Polypeptides synthesized *in vivo*: major, M_r 82,000 and 65,000; minor, M_r 74,000, 70,000, 28,000, and 16,000.

tide of M_r 85,000 (4), probably identical with polypeptide pp82 described in this paper. Recently we were able to demonstrate that pp82 is glycosylated, since [^{3}H]glucosamine was incorporated into this polypeptide *in vivo*. A second polypeptide, glycosylated *in vivo*, with a molecular weight of approximately 72,000 was also detected (unpublished observations).

In this study, we present evidence that crude cell-free extracts of R-MuLV infected cells are capable of directing the synthesis of virus-specific products *in vitro* (compare Table 1). All cytoplasmic fractions synthesize pp65, which is also detectable *in vivo*. However, only the membrane-rich fractions synthesize the glycoprotein pp82 and a polypeptide with a molecular weight of about 76,000. The latter polypeptide either may be not completely glycosylated or represents one of the first processing products of pp82 to the viral glycoprotein gp69/71. Addition of purified free and membrane-bound polyribosomes to a cell-free system results in the synthesis of pp65, while no synthesis of the glycoprotein pp82 was detected. Moreover, a polypeptide of M_r 74,000 was present in minor amounts in the immunoprecipitate of the *in vitro* incubations programmed with membrane-bound polyribosomes. From Fig. 2b it can be seen that a polypeptide of identical molecular weight is also present as a faint band in the *in vivo* products.

It has been reported that mRNA isolated from membrane-bound polyribosomes of cells infected with vesicular stomatis virus synthesizes almost exclusively a polypeptide of M_r 63,000 *in vitro*, which is most probably a nonglycosylated form of the viral glycoprotein (17). Therefore, it might well be that the 74,000 M_r polypeptide is a partly glycosylated or nonglycosylated precursor of the viral glycoprotein. However, the results indicate that the ratio of the amounts of the 65,000 and 74,000 M_r polypeptides synthesized *in vitro* does not correspond to that of pp65 and pp82 *in vivo*. The reason for this may be that either the protein moiety of the glycoprotein is less antigenic or that association with membranes is a prerequisite for its correct synthesis. That membranes are involved in the synthesis of virus-specific polypeptides is evident from the more general observation that they are synthesized in relative high amounts in membrane-rich fractions or by polyribosomes isolated from these fractions. The earlier finding of our group (7) and others (12) that the relative content of virus-specific RNA, as measured by hybridization, is four to ten times less on free polyribosomes than on membrane-bound polyribosomes supports these results. Analysis of the cell-free products coded for by R-MuLV specific mRNA, fractionated according to size, shows that at least 22S and 35S mRNA can direct the synthesis of specific products. In addition, it appeared that native or denatured R-MuLV 60S RNA gives rise to the same high-molecular-weight polypeptides (pp65 and M_r 72,000) as 35S mRNA isolated from polyribosomes. However, a polypeptide of M_r 70,000, which is synthesized after addition of 20–22S mRNA to the cell-free system, is not detectable in the cell-free products directed by the viral RNA. It should be mentioned that we also found a number of minor bands in the high molecular weight range. At present, however, no evidence can be provided that these components are virus-specific and correspond to the high molecular weight polypeptides reported by the group of Arlinghaus (18, 19). Since more complete data are still lacking, we can only speculate about the nature of the products synthesized by the different mRNA fractions. In conclusion we may state that a polypeptide of M_r 65,000, which is most probably the precursor of the lower-molecular-weight virion proteins, is synthesized by all fractions tested, except 14S and 20–22S mRNA. Further characterizations with monospecific antisera and the availability of sufficient labeled material for tryptic peptide analysis will be necessary to show the authenticity of the products synthesized and their interrelationship.

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