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TRANSLATION OF GENOMIC AVIAN MYELOBLASTOSIS VIRUS RNA IN A CELL-FREE PROTEIN SYNTHESIS SYSTEM FROM RABBIT RETICULOCYTES

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Summary. — Reaction conditions suitable for translation of genomic avian myeloblastosis virus RNA in micrococcal nuclease-pretreated reticulocyte lysates are described. The products of translation were characterised by immunoprecipitation and gel electrophoresis and compared with virus-specific products formed in host cells. Genomic viral RNA directed in a cell-free system the synthesis of precursors to viral structural proteins, namely $Pr76^{gag}$ and $Pr180^{gag,pol}$.

Key words: *avian myeloblastosis virus RNA*; *cell-free protein synthesis*; *rabbit reticulocyte lysates*; *polyprotein precursors*

Introduction

Recent studies on the genetics and biochemistry of avian oncoviruses indicate that the order of the four known viral genes on virion genomic RNA is 5′-gag-pol-env-src-3′ (Wang, 1978). The src gene which is absent from leukosis viruses is believed to be involved in sarcomagenic transformation; env codes for the virion envelope glycoproteins; pol for the RNA-dependent DNA polymerase and gag for the complex of virion internal structural proteins (Eisenman and Vogt, 1978).

The proteins of oncoviruses are synthesized in vivo as large polyprotein precursors which are cleaved to the proteins characteristic of the mature virions (Vogt *et al.*, 1975). There are three types of virus-specific mRNAs in the cells: 30–40 S mRNA for translation of gag and gag-pol genes; 28 S mRNA for translation of env gene; and 20 S mRNA for translation of src gene (Weiss *et al.*, 1977). The genomic RNA isolated from those viruses (30–40 S RNA) was shown to act as mRNA in heterologous cell-free systems and to direct the synthesis of polyproteins related to virion proteins. The primary translation product of gag gene is a 76,000 dalton polyprotein designated $Pr76^{gag}$ (von der Helm and Duesberg, 1975; Pawson *et al.*, 1976) and of gag and pol genes the $Pr180^{gag,pol}$ (Paterson *et al.*, 1977, Purchio *et al.*, 1977; Philipson *et al.*, 1978).

In the present paper we describe the reaction conditions of avian myeloblastosis virus RNA translation in vitro in rabbit reticulocyte lysates rendered dependent on added mRNA by treatment by the micrococcal nuclease technique (Pelham and Jackson, 1976). Pr76^{gag} and Pr180$^{gag, pol}$ were immunoprecipitated from reaction mixtures by antibodies to structural protein of virus (p27) and they coincided in the positions on slab gels with relevant precursors synthesized in vivo in host cells.

Materials and Methods

Substances. Micrococcal nuclease (15,000 units/mg) was obtained from Boehringer; ethylene glycol-bis-(2-amino ethylether) -N, N'-tetraacetic acid from Sigma; ^{35}S-methionine (600—1000 Ci/mmol) from Radiochemical Centre, Amersham; rabbit globin mRNA was a kind gift of Dr. O. Fuchs; and phenylhydrazine-HCl (Lachema), a 2,5 % aqueous filtered solution (pH 7), was a kind gift of Dr. J. Hradec.

Cell-free protein synthesis. New Zealand white rabbits (1.5—2.5 kg) were made anaemic by daily subcutaneous injections of phenylhydrazine solution (0.5 ml/kg body weight) for five days. After a further two days, when reticulocytes comprised more than 90 % of the total cell population, the blood was taken by intracardiac puncture into a solution 0.13 M NaCl — 0.0074 M $MgCl_2$—0.005 M KCl—0.001 % heparin. After washing, the cells were lysed by adding an equal volume of cold water. Cell debris was discarded by centrifugation (17.000 × g, 15 min) and the supernatant (lysate) was stored in liquid nitrogen.

The reticulocyte lysates were treated with micrococcal nuclease to reduce endogenous mRNA by the method described by Pelham and Jackson (1976) but with doubling the concentration of nuclease. Incubation mixtures for protein synthesis further referred to as basic incubation mixture contained in a final volume of 15—30 μl : 7.5—15 μl of nuclease-treated reticulocyte lysate, each amino acid except methionine at 55 μM, 10—20 μCi ^{35}S-methionine and 0.75 μg-1.5 μg chicken liver tRNA. The presence and concentrations of various fortification components and exogenous mRNA varied as described along with the results. Incubation was carried out at 30 °C. For kinetic studies, trichloroacetic acid precipitable radioactivity was estimated in 2—5 μl portions of incubation mixtures. To characterise the protein products synthesized, the reaction mixture was subjected to immunoprecipitation.

Virus and viral RNA. Avian myeloblastosis virus (AMV) was purified from blood plasma of leukaemic chicks and RNA was extracted from purified virions with phenol-sodium dodecylsulphate (Trávníček and Říman, 1973). The results described below apply to genomic AMV-RNA which sedimented as a single peak with a sedimentation coefficient of 60—70 S in a 10 to 30 % glycerol gradient containing 0.01 M Tris-HCl, pH 7.6, 0.1 M NaCl (Beckman SW 41 rotor, 38.000 rev/min, 4 °C, 3 hr). After repeated ethanol precipitation, the dried RNA was solubilised at a concentration of 1 mg/ml in sterile distilled water, sealed in glass ampoules and kept frozen in liquid nitrogen. The 30—40 S RNA subunits were obtained by heat denaturation of sealed samples of 60—70 S AMV-RNA at 100 °C for 90 sec immediately before adding to the cell-free system. Each sample was melted only once. The RNA samples were simultaneously analysed on cylindrical composite agarose (0.5 %) — acrylamide (1.7 %) gels in a buffer system containing 0.045 M Tris-HCl, pH 8.3, 0.045 M boric acid, 0.014 M ethylene diamine tetraacetic acid (EDTA), 0.2 % sodium dodecylsulphate (SDS) as described by Tiollais et al. (1972).

Radioactive labelling of cells and virus. AMV-producing cultures of leukaemic myeloblasts (Becker *et al.*, 1962) were cultivated in suspension (5 × 10^7 cells/ml) as described by Říman and Beaudreau (1970). In pulse-chase experiments, the cells were incubated for 2 hr at 37 °C in methionine-free growth medium supplemented with 50 % dialysed chicken serum. The medium was removed by centrifugation (600xg, 37 °C) and the cells were incubated once more under the same conditions. After the second prelabelling period, the cells were diluted with the same incomplete growth medium to a concentration of 10^7 cells/ml. The culture was supplemented with 60 μCi ^{35}S-methionine/ml and incubated for 30 min at 37 °C. In pulse labelling, the culture was transferred to ice, the cells were pelleted (2.500xg, 4 °C), rinsed and kept frozen at —60 °C. In chase experiments, "pulsed" cells were washed twice at 37 °C with complete growth medium and then incubated in the same medium at 37 °C for 90 min. The culture was then transferred to ice and processed in the same manner as in pulse experiments. The labelled myeloblasts were melted, homogenized (5 × 10^6cells/2 ml) in lysis buffer (see Immunoprecipitation) and centrifuged at 15,000xg for 15 min at 4 °C. The supernatant was stored at −60 °C.

Labelling of virus (12 hr) was carried out under the same conditions as pulse labelling of cells but the ^{35}S-methionine concentration was 30 μCi/ml. The labelled virus was purified as described (Malý and Říman, 1971) and stored at −60 °C.

Antigens, antisera and immunoprecipitation procedure. For preparation of virion antigens, AMV from chicken leukaemic plasma was extensively purified (Malý and Říman, 1971). Internal structural virion protein p27 was prepared by phosphocellulose ion exchange column chromatography essentially as described for isolation of proteins of murine oncoviruses (Strand and August, 1976). The isolated protein moved in gel electrophoresis as a single band with a mobility identical to that of p27 of the AMV lysate.

Rabbit antisera were prepared from New Zealand white rabbits by repeated intracutaneous injections (Vaitukaitis *et al.*, 1971) with either Nonidet P-40-disrupted AMV (polyvalent antiserum against internal AMV proteins) or p27 (monospecific antiserum against p27), both emulsified with Freund' s complete adjuvant. All antisera were clarified (12,000 × g, 4 °C, 5 min) before use.

Samples to be immunoprecipitated were adjusted to 0.25 ml of lysis buffer (0.02 M Tris-HCl, pH 7.4 — 0.05 M NaCl — 0.5 % Triton X-100 — 0.5 % sodium deoxycholate — 0.1 % SDS — 0.1 % methionine — 0.001 M phenylmethylsulfonylfluoride). Antiserum (2 μl) was added, and the mixture was incubated at 4 °C for 20 min. Then 75 μl of a 10 % (w/v) suspension of *Staphylococcus aureus* (Kessler, 1975) were added, and the incubation continued for 20 min. The bacteria were collected by centrifugation and subsequently washed three times by resuspension in 1 ml of lysis buffer and centrifugation. Washed bacterial sediment was resuspended in 50 μl of sample buffer (Laemmli, 1970) and heated at 100 °C for 3 min. After centrifugation, the supernatant was analysed by gel electrophoresis.

SDS-polyacrylamide slab gel electrophoresis was performed in 7.5 % or 15 % gel slabs containing 0.1 % SDS, using the buffer system described by Laemmli (1970). An almost equal amount of trichloroacetic acid-precipitable radioactivity of the samples was added to each slot. After staining and destaining, the gels were treated for fluorography (Bonner and Laskey, 1974). A linear response to radioactivity was obtained by preflashing the X-Omat R film (Kodak) as described by Laskey and Mils (1975). Densitometer tracings of fluorograms were performed at 640 nm in a Pye Unicam SP8-100 UV spectrophotometer.

Results

Preparation of rabbit reticulocyte lysates as described in *Materials and Methods* reliably provided a protein synthesis system with high degree of endogenous incorporation. By treatment of the lysate with micrococcal nuclease following the conditions of Pelham and Jackson (1976), the endogenous incorporation decreased to 1—2 % of the original level and the system became fully dependent on exogenous rabbit globin mRNA. But when a number of preparations of this system effectively responding to globin mRNA were used to translate AMV—RNA, none or only negligible protein synthesis activity was repeatedly observed. Therefore we decided to search for conditions which would be optimal for cell-free protein synthesis directed by genomic RNA of AMV.

Genomic AMV-RNA used as mRNA in our experiments migrated in polyacrylamide-agarose gel electrophoresis as a single peak of 60—70 S in native form and predominantly as 35 S when denatured (Fig. 1). Unless otherwise stated, the denatured 60—70 S AMV-RNA was added to the cell-free system at a concentration of 20 μg/ml.

To determine conditions for efficient translation of genomic AMV-RNA in reticulocyte lysates, the effect of the components used to fortify the cell-free protein synthesis systems was tested in kinetic experiments. As shown in Fig. 2, separate supplementation of the basic incubation mixture with either creatine phosphate and creatine phosphokinase, hemine or dithioerythritol (DTET) and spermidine, respectively, slightly stimulated the non-fortified system, whereas the addition of Mg^{2+} and K^{+} to final concentrations of 1.75 mM and 0.1 M, respectively (Paterson *et al.*, 1977), completely abolished the protein synthesis. When all components with a stimulatory effect were added to the system simultaneously, the incorporation directed by AMV-RNA increased 10—20 times in comparison with that of the non-fortified systems. But when also Mg^{2+} and K^{+} ions were included, the protein synthesis was again completely inhibited (Fig. 2). Therefore, the effect of Mg^{2+} and K^{+} ion-concentration was analysed in detail by changing the concentration of one salt in the fortified system whereas the concentration of the other one was kept constant and identical with that of the non-fortified basic incubation mixture (0.25 mM $MgCl_2$

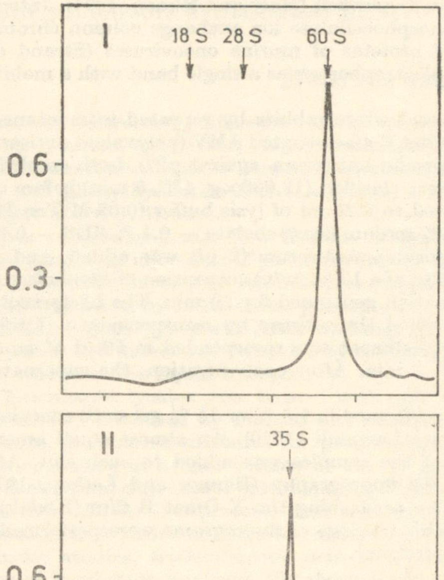

Fig. 1.

Analysis of genomic native (I) and denatured (II) AMV-RNA

Abscissa: migration (mm); ordinate: absorbance at 270 nm. Arrows indicate the location of 18 and 28 S ribosomal RNA markers. Viral RNA samples (30 μg) were electrophoresed on cylindrical composite agarose (0.5 %) — acrylamide (1.7 %) gels.

or 70 mM KCl, respectively). The results (Fig. 3) showed that any further addition of both Mg^{2+} and K^+ ions inhibited the incorporation. Further decrease of $MgCl_2$ and KCl concentrations, which could be obtained only by changing the original procedure of micrococcal nuclease pretreatment (Pelham and Jackson, 1976), had no stimulatory effect.

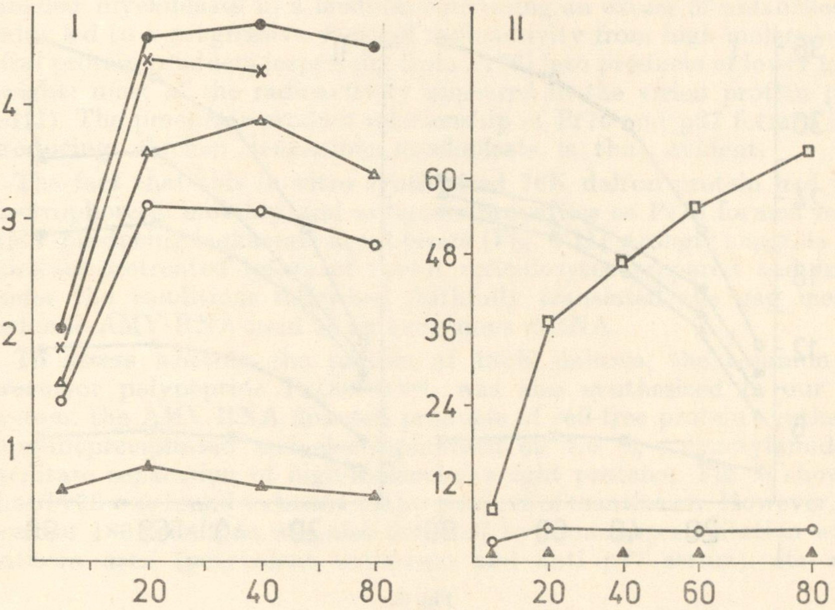

Fig. 2.

Influence of fortification of basic incubation mixture on ^{35}S-methionine incorporation directed by AMV-RNA

I — The basic incubation mixture (30 μl) containing 20 μg/ml 30—40 S AMV-RNA (O) was adjusted to the following final concentrations: 8 mM creatine phosphate and 31 μg/ml creatine phosphokinase (●); 30 μM hemine (×); 25 mM Hepes — 2 mM DTET — 0.5 mM spermidine (△); 1.75 mM $MgCl_2$ — 100 mM KCl (▲).

II — The basic incubation mixture (○) contained all additives (I) (▲) or all additives except Mg^{2+} and K^+ ions (□).

Abscissa: time of incubation (min); ordinate: count/min × 10^{-3} incorporated in 5 μl portions.

Since the concentrations of endogenous nucleoside triphosphates in reticulocyte lysates were unknown, the effect of supplementation with GTP (0.1 mM) and ATP (1 mM) was tested. No (GTP), or only a slightly inhibitory influence (ATP) was observed.

The kinetics of protein synthesis directed by increasing concentrations of AMV-RNA was investigated under standard reaction conditions (Fig. 4). Saturation was achieved at concentrations of 20 μg/ml of incubation mixture of added AMV-RNA. The fortified basic incubation mixture with final concentrations as stated in Fig. 4 will be further referred to as standard incubation mixture. The fortifications were shown in Figs 2 and 3.

Immunoprecipitation with defined antisera against AMV structural proteins (monospecific anti p27 serum and polyvalent antiserum directed against p27, p19 and to a lesser extent also to p15/p12) were used to characterize the products of cell-free protein synthesis directed by denatured genomic AMV-RNA under standard reaction conditions. Chick leukaemic myeloblasts pulse-labelled for 30 min and chased for 90 min were used as standards for AMV-specific protein synthesis. Immunoprecipitates from the reticulocyte lysate together with those of chick leukaemic myeloblasts were submitted to electrophoresis on the same SDS-polyacrylamide gel (Fig. 5, Plate I). Standard proteins of known molecular weight were run in parallel.

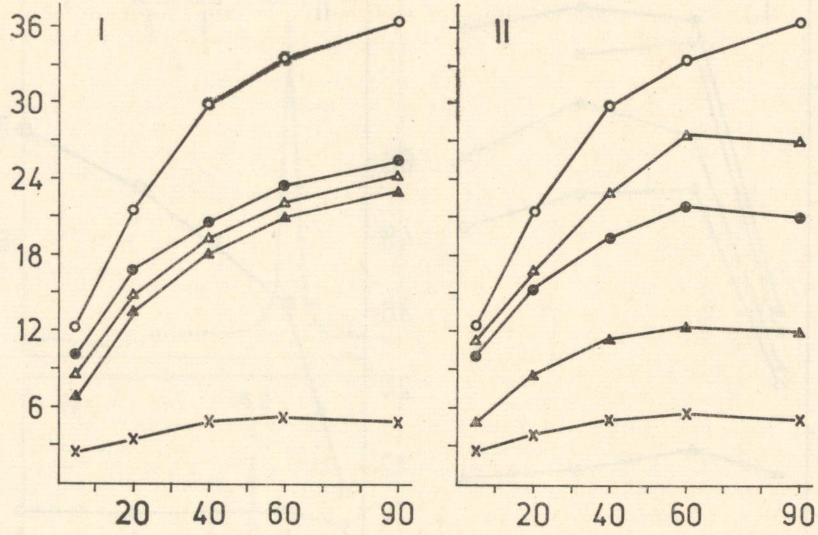

Fig. 3.

Influence of $MgCl_2$ (I) and KCl (II) on ^{35}S-methionine incorporation directed by AMV-RNA Basic incubation mixture (30 μl) containing 20 μg/ml 30—40 S AMV-RNA was fortified in all cases as given in Fig. 2 (II, □). I — $MgCl_2$ (final concentration): 1.75 mM (▲); 1.25 mM (●); 0.75 mM (△); 0.25 mM (○). KCl was kept constant at 70 mM. II — KCl (final concentration): 144 mM (▲); 119 mM (●); 94 mM (△); 70 mM (○). $MgCl_2$ was kept constant at 0.25 mM. The reactions without added RNA contained 0.25 mM $MgCl_2$ and 70 mM KCl (×).
Abscissa: time of incubation (min); ordinate: count/min x 10^{-3} incorporated per 5 μl portions

In the reticulocyte lysate programmed by denatured genomic AMV-RNA, most of the protein products formed a distinct band of 76K daltons after immunoprecipitation both with anti p27 serum (Fig. 5-I) and with antiserum directed against "group-specific antigens" (Fig. 6-I). No radioactivity was immunoprecipitated from the lysate incubated without added RNA.

Immunoprecipitation of chick leukaemic myeloblasts after pulse labelling with anti p27 serum revealed the gag precursor polyprotein of 76K daltons (Pr76) as the major virus-specific protein and a number of smaller products with antigenic determinants of p27 (68K, 64K, 56K, 48K, 45K, 43K, 32K dalton proteins being the most prominent ones) including the p27 as the end product of the posttranslational cleavage (Fig. 5). Incubation of pulse-labelled myeloblasts in a medium containing an excess of unlabelled methionine led to a progressive chase of radioactivity from high molecular weight viral protein products (especially from Pr76) into products of lower molecular weight; most of the radioactivity appeared in the virion protein p27 (Fig. 5-III). The precursor-product relationship of Pr76 and p27 formed in AMV-producing chicken leukaemic myeloblasts is thus evident.

The fact that this in vitro synthesized 76K dalton protein had identical electrophoretic mobility and antigenic properties as Pr76 formed in vivo in AMV-producing leukaemic myeloblasts (Fig. 5-II) strongly suggests that the nuclease-pretreated lysate of rabbit reticulocytes prepared and incubated under the conditions described faithfully translated the gag message of genomic AMV-RNA used as an exogenous mRNA.

To assess whether the protein of 180K daltons, the common gag-pol precursor polypeptide $Pr180^{gag,\,pol}$, was also synthesized in our cell-free system, the AMV-RNA directed products of cell-free protein synthesis were immunoprecipitated and electrophoresed on 7.5 % polyacrylamide gel to facilitate separation of high-molecular-weight proteins. Fig. 6 shows again that Pr76 was found to be the major product of translation. However, a minor peak of 180K daltons was also detected by immunoprecipitation with both antisera used (polyvalent antiserum and anti p27 serum). Its antigenic

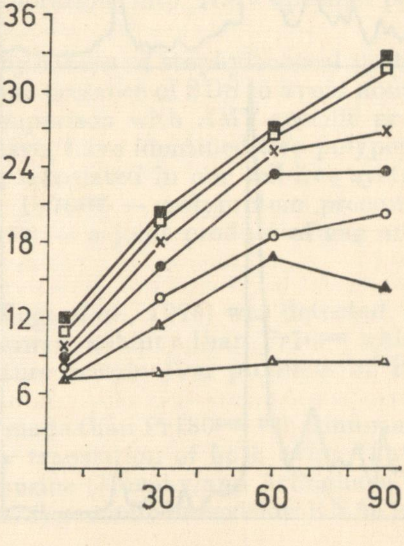

Fig. 4.
Influence of AMV-RNA concentration on ^{35}S-methionine incorporation
Abscissa: time of incubation (min); ordinate: count/min × 10^{-3} incorporated per 3 μl portions.
Incubation was performed in standard incubation mixtures (15 μl) containing: 7.5 μl reticulocyte lysate pretreated by micrococcal nuclease; 25 mM Hepes (pH 7.6); 70 mM KCl; 0.25 mM $MgCl_2$; 0.5 mM spermidine; 2 mM DTET; 30 μM hemine; 55 μM amino acids (minus methionine); 8 mM creatine phosphate; 31 μg creatine phosphokinase/ /ml; 50 μg tRNA/ml and 600 μCi of ^{35}S-methionine/ml. The amount of denatured AMV-RNA (30 – 40 S) per 1 ml was: 100 μg (■), 33 μg (□), 17 μg (×), 10 μg (●), 7 μg (○), 3 μg (▲), none (△).

properties, electrophoretic mobility and the fact that the radioactivity of 180K daltons protein represents approx. 5 – 10 % of that of Pr76 indicate that this protein represents Pr180$^{gag, pol}$ – the common gag-pol precursor polypeptide (Jamjoom *et al.*, 1977; Paterson *et al.*, 1977; Purchio *et al.*, 1977; Philipson *et al.*, 1978).

Discussion

Lysates of rabbit reticulocytes (Pelham and Jackson, 1976) as well as extracts of other mammalian cells (Murphy and Arlinghaus, 1978) pretreated with micrococcal nuclease (Pelham and Jackson, 1976) to destroy endogenous mRNA have been widely used as cell-free systems for translation of various

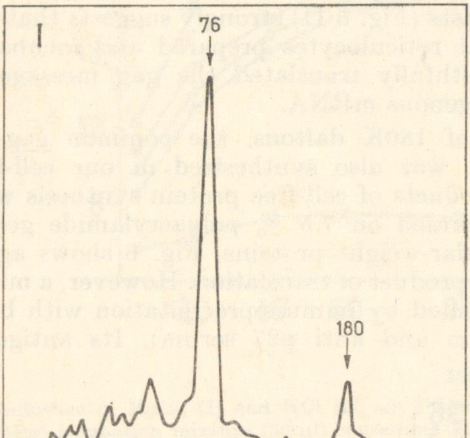

Fig. 6.

A MV-specific polypeptides generated in the cell-free system and resolved on a SDS-polyacrylamide (7.5 %) slab gel. 30 – 40 S AMV-RNA was translated in standard reaction mixture and products were immunoprecipitated:
I – with polyvalent antiserum against AMV
II – with anti p27 serum
Densitometric tracing at 640 nm of the X-ray film after fluorography of the dried gel.

exogenous mRNA. But since reports describing both the preparation of cell-free systems and especially the composition of incubation mixture suitable for an efficient translation of oncovirus RNA differ considerably, the main aim of our study was to define conditions which would be optimal for the faithful translation of genomic RNA of AMV as the necessary background for a detailed study of AMV-specific protein synthesis.

From the present results the following points should be briefly emphasized:

a) The described mode of preparation of the reticulocyte lysate reliably provides an mRNA-dependent protein synthesizing system with very low (1 – 2 %) background incorporation, which allows quite minor protein products to be recognized. Moreover, the majority of the residual incorporation is very probably due to the incomplete washing out of the free ^{35}S-methionine (Kamine and Buchanan, 1977; Murphy *et al.*, 1978).

b) In view of the fact that published data describing optimal conditions for translation of oncovirus genomic RNAs in reticulocyte lysates differ considerably both in the number and concentrations of various fortification components and, surprisingly, also in concentrations of Mg^{2+} (0.75 – 2mM) and K^+ (70 – 140 mM) ions, we analysed this question in detail. We found, in contradistinction to others (Salden and Bloemendal, 1976; Beemon and Hunter, 1977; Kamine and Buchanan, 1977; Paterson *et al.*, 1977; Philipson *et al.*, 1978), that any increase in Mg^{2+} and K^+ concentration above the level of 0.25 mM $MgCl_2$ and 70 mM KCl (Pelham and Jackson, 1976), respectively, was followed by a substantial inhibition of the protein synthesis directed by genomic RNA of AMV.

With regard to this finding and together with the results of our analysis of the qualitative and quantitative representation of fortification components, a "standard incubation mixture" was developed, which reliably provides a high level of ^{35}S-methionine incorporation into AMV-specific protein products.

c) Immunoprecipitation performed by means of staphylococcal protein A antibody adsorbent (Kessler, 1975), in the presence of SDS to avoid nonspecific binding, and gel-electrophoretic comparison with AMV-specific proteins formed in pulse-chase experiments in vivo, have identified two polypeptides with gag protein antigenicity as being translated in our cell-free system in response to 35 S genomic AMV-RNA: $Pr76^{gag}$ – polyprotein precursor of internal virion proteins; and $Pr180^{gag, pol}$ – a joint product of gag and pol genes (Oppermann *et al.*, 1977).

No posttranslational modification (Vogt *et al.*, 1975) was detected. Faint immunoprecipitable protein bands of lower mobility than $Pr76^{gag}$ which we had observed might represent premature termination products of $Pr76^{gag}$ (Purchio *et al.*, 1977).

There is about 20 times more $Pr76^{gag}$ made than $Pr180^{gag, pol}$, conforming to the estimates of this ratio obtained by translation of both avian (Paterson *et al.*, 1977; Purchio *et al.*, 1977) and murine (Murphy and Arlinghaus, 1978; Murphy *et al.*, 1978; Philipson *et al.*, 1978) genomic oncovirion RNAs in cell-free systems. In this regard, the reticulocyte lysate programmed by genomic AMV-RNA mimics an in vivo situation both qualitatively and quantitatively.

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