JOURNAL OF VIROLOGY, May 1977, p. 247-255

Vol. 22, No. 2
Printed in U.S.A.

Cell-Free Coupling of Influenza Virus RNA Transcription and Translation

JEAN CONTENT,* LUCAS DE WIT, AND MICHEL HORISBERGER

Department of Virology, Institut Pasteur du Brabant, B-1040 Brussels, Belgium, and Research Department, Pharmaceutical Division, CIBA-GEIGY Ltd., 4002 Basel, Switzerland*

Received for publication 13 December 1976

A cell-free coupled system for the transcription and translation of fowl plague virus RNA is described. The system utilizes a new nuclease-preincubated rabbit reticulocyte lysate that has a high sensitivity to exogenous mRNA and a very low level of nuclease activity. Translation of the viral proteins in the coupled system is strictly dependent upon the viral transcriptase activity. In the coupled system the optimal concentration of magnesium is intermediate between the optimum for transcription and that for translation. Translation of the viral proteins seems faithful. The products represent the major viral peptides M and NP and two peptides with the same electrophoretic mobility as HA and P_2. Virion NA is not resolved in the kind of polyacrylamide gels described. Proteins M and NP were immunoprecipitable with monospecific antisera. It is concluded that the virion-associated RNA polymerase transcribes the negative-stranded segments of the viral genome coding for these major structural proteins into fully functional mRNA's.

Influenza viruses are enveloped animal viruses whose genome is composed of a segmented single-stranded RNA, probably entirely "negative stranded" (15, 19). This finding is based on several lines of evidence, as follows: (i) the virion RNA is not a template for viral protein synthesis in the wheat germ cell-free system (20), whereas in similar conditions the cytoplasmic polyadenylic acid [poly(A)]-rich RNA from influenza virus-infected cells promotes the synthesis of the major virus structural proteins (4, 6, 16); (ii) poly(A)-containing mRNA isolated from infected-cell polysomes is entirely complementary to the virion RNA (5, 14); and (iii) the virion contains an RNA-dependent RNA polymerase (19).

Recently one of us described the possibility of stimulating the influenza virion-associated polymerase activity with mammalian cell ribosomes in the absence of manganese ions, but in the presence of magnesium ions, and at low ionic strength (7). These new conditions were compatible with those required for protein synthesis; therefore, it was of interest to determine in a coupled system for transcription and translation whether the products of this activated RNA polymerase could represent fully functional viral mRNA's. In this paper we show that, despite the low activity of the influenza virion-associated polymerase, it is possible to establish a coupled system yielding most of the virus structural proteins, provided that a suitable cell-free system is chosen.

MATERIALS AND METHODS

Cell and virus. Fowl plague virus (FPV), Rostock strain, was grown in the allantoic cavity of embryonated eggs and purified as previously described (7) by polyethylene glycol-NaCl precipitation followed by isopycnic centrifugation on a 5 to 40% (wt/vol) potassium tartrate gradient. The viral band was collected, diluted with 5 volumes of 10 mM Tris-hydrochloride (pH 7.4)-100 mM NaCl-1 mM EDTA (TNE), pelleted for 80 min at 109,000 × *g*, resuspended in TNE, and sedimented in the same conditions. The pellet was resuspended in a small volume of TNE and stored in small portions in liquid nitrogen. The virus protein concentration was 10 to 40 mg/ml in TNE.

[^{35}S]methionine virus, cytoplasmic extracts, and cytoplasmic RNA from control or infected cultures of chicken embryo fibroblasts were prepared by the methodology described recently (4).

Cell-free translation in the reticulocyte lysate translation system. Lysates were prepared from rabbits (2 to 3 kg) that had been made anemic by phenylhydrazine injection as previously described (8). The blood, containing 50 to 80% reticulocytes, was collected and processed as described by Pelham and Jackson (13). Preincubation of the thawed lysate with the calcium-dependent micrococcal nuclease was as described previously (13), with the following modifications. Hemin (1 mM) was dissolved before use in concentrated KOH (21). The concentration of the 19 unlabeled amino acids (L-

methionine was omitted) added to the "master mix" was calculated to be 110 μM in the final reaction mixture. The concentration of L-methionine in the final reaction was optimal at 10 μM. We routinely used 6×10^8 cpm of [^{35}S]methionine per ml. Nevertheless, to keep the specific radioactivity of the methionine as high as possible, we diluted it to 160,000 cpm/pmol, with a final concentration of L-methionine at 4 μM.

To assay protein synthesis, 3- or 5-μl samples from the reaction mixture were spotted onto Whatman 3MM paper, and the hot trichloroacetic acid-insoluble radioactivity was determined (17).

Disruption of the virion and RNA polymerase assay. The purified virions were disrupted in 8 mM HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid; pH 8.0)–0.3% Triton N-101–1% sodium deoxycholate–200 mM urea–10 mM 2-mercaptoethanol–10 mM potassium acetate for 2 min at 20°C (7). The reaction mixture concentrations in the transcriptase assay were: HEPES (pH 8.0), 50 mM; Mg^{2+}, 5 mM (or as indicated); potassium acetate, 110 mM; dithiothreitol, 1 mM; creatine kinase, 80 μg/ml; creatine phosphate, 10 mM; ATP, 1 mM; UTP, GTP, and CTP, 0.5 mM; [^{3}H]UTP (42 Ci/mmol), 100 μCi/ml; and disrupted FPV, 0.4 to 1.6 mg/ml.

The reaction mixture (usually 25 μl) was incubated at 31°C for 60 min and terminated by the addition of 2 volumes of saturated sodium inorganic orthophosphate and sodium inorganic pyrophosphate (1:1) followed by 20 volumes of 10% trichloroacetic acid. After 15 min at 0°C, the precipitates were collected by filtration on Whatman GF/C glass fiber disks or cellulose nitrate filters (Sartorius; 0.45 μm). The filters were washed at least eight times with 5% cold trichloroacetic acid, dried, and counted in a toluene-based scintillating solution, with an efficiency of 55% for ^{3}H.

Experimental conditions for the coupled system. The reaction mixture concentrations were: HEPES (pH 8.0), 25 mM; L-methionine, 3 μM; Krebs ascites cell tRNA, 60 μg/ml; ATP, 1 mM; UTP, CTP, and GTP, 0.5 mM; and disrupted FPV, 1 mg/ml. A total of 80 to 90% of the final reaction volume was comprised of the nuclease-preincubated reticulocyte lysate. When RNA synthesis was monitored, [^{3}H]UTP (42 Ci/mmol) was added at 100 μCi/ml. When protein synthesis was assayed, 6×10^8 cpm of [^{35}S]methionine per ml was added. Incubation, usually in 25 μl, was at 31°C.

Product identification. Samples (2 μl) from the in vitro translation reaction, [^{35}S]methionine-labeled virion, or cytoplasmic extracts were analyzed by electrophoresis on 7 to 20% polyacrylamide slab gel gradients (80 by 125 mm) (4). After fluorography (10), the X-ray films were scanned at 540 nm in a Gilford spectrophotometer equipped for microdensitometry with a model 2520 linear transport system.

Immunoprecipitation. Samples (50 to 100 μl) from the translation reaction were treated with 0.1 mg of pancreatic RNase per ml and 0.1 M EDTA for 15 min at 37°C. After clarification for 5 min at 9,000 $\times$ *g*, the supernatant was incubated for 1 h at 20°C with 0.1 volume of normal rabbit serum and for 30 min with 0.05 volume of goat anti-rabbit immunoglobulin G (IgG) (IgG fraction). After centrifugation for 10 min at 3,000 $\times$ *g*, the supernatants were divided into two fractions and treated with 0.1 volume of either anti-M or anti-NP rabbit antibodies for 1 h at 20°C. After the addition of 0.05 volume of goat anti-rabbit IgG (IgG fraction) for 30 min at 20°C, the samples were kept overnight at 0°C, washed three times by centrifugation for 2 min at 9,000 $\times$ *g* in phosphate-buffered saline containing 1% Triton X-100 and 1% sodium deoxycholate. The pellet was dissolved in 0.05 M Tris-hydrochloride (pH 6.8)–1% sodium dodecyl suflate–1% 2-mercaptoethanol–10% glycerol, heated for 3 min at 100°C, and analyzed by electrophoresis on a polyacrylamide slab gel as described above.

Materials. Micrococcal nuclease (EC 3.1.4.7) (29,000 U/mg) was from P.L. Biochemicals, Milwaukee, Wis., and EGTA [ethyleneglycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid], hemin, and Triton N-101 were from Sigma Chemical Co. (St. Louis, Mo.). The goat anti-rabbit IgG fraction was obtained from Nordic Immunological Laboratories (Tilburg, The Netherlands). Rabbit antibodies anti-X 47 (M) and anti-X 42 (NP) were kindly provided by G. C. Schild (World Health Organization International Laboratory for Biological Standards).

[^{3}H]UTP and [^{35}S]methionine were obtained from the Radiochemical Centre (Amersham, United Kingdom).

RESULTS

Virion-associated polymerase. The virion-associated RNA polymerase of FPV has a relatively low activity in vitro. Figure 1 shows that, with a typical preparation of purified FPV, we could obtain an incorporation of 40 pmol of UMP per mg of viral protein per h.

McGeoch and Kitron (11) and Plotch and Krug (S. J. Plotch and R. M. Krug, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, S114, p. 223) have used some oligonucleotides to stimulate this activity in vitro. We confirmed this observation by using ApG at a concentration of 0.5 mM and found that this substance stimulated 5 to 20 times the transcriptase activity associated with FPV. The optimal magnesium concentration for this reaction has been reproducibly found to be 3 to 5 mM, and this requirement is not influenced by the addition of the dinucleotide (Fig. 1). Under these conditions the amount of [^{3}H]UMP incorporated amounts to 240 pmol of UMP per mg of viral protein per h. This corresponds to 800 pmol of RNA per mg of viral protein per h and 0.2 to 0.4 μg of RNA per ml per h. In some early experiments (data not shown) we also observed a stimulation of the virion-associated polymerase activity by wheat germ ribosomes and to a much lower extent by the wheat germ extract. With the wheat germ lysate, several attempts were made to couple the viral transcription and

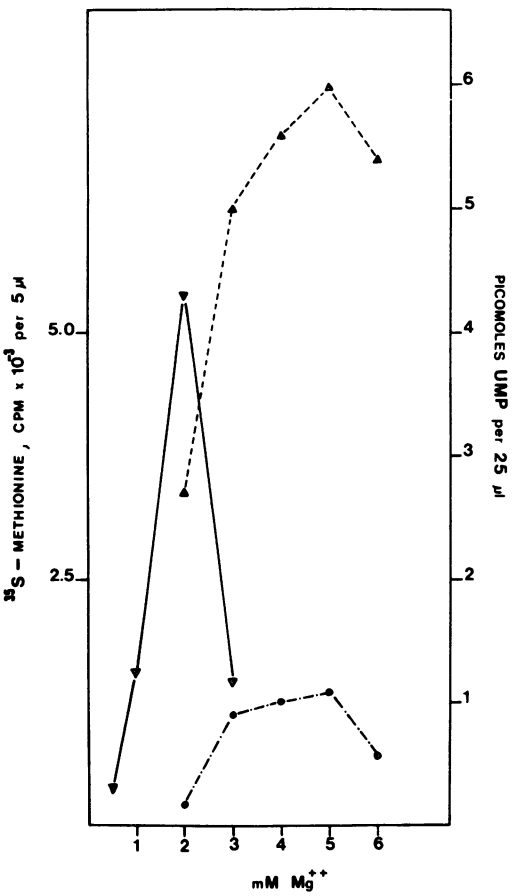

FIG. 1. *Mg^{2+} requirement for the FPV-associated RNA polymerase activity and protein synthesis in the coupled cell-free system. The two dashed lines represent the incorporation of [^{3}H]UMP in a standard transcriptase reaction mixture as a function of the magnesium concentration. Symbols: ⊙-·-·⊙, in the absence of ApG; △----△, with 0.5 mM ApG. The solid line represents the incorporation of [^{35}S]methionine in the coupled cell-free system. Disrupted FPV is added to the nuclease-preincubated reticulocyte lysate, as indicated in the text. In this case, the concentration of Mg^{2+} represents what is added to the preincubated cell-free system; it is not the final concentration since the preexisting Mg^{2+} in the reticulocyte lysate is not known.*

translation by adding a suitable concentration of disrupted FPV to the complete translation mixture in the presence of the four ribonucleotide triphosphates. No significant stimulation of amino acid incorporation was detected. When the products of these reactions were examined by polyacrylamide gel electrophoresis, multiple bands were observed, with molecular weights ranging from 15,000 to 100,000, and at least 30% of the products were of a low molecular weight (below 15,000). None of the products seemed to correspond to the virus structural proteins.

Use of rabbit reticulocyte lysate. Since this failure could be the result of a high RNase content in the wheat germ extract, we decided to use a cell-free system that is known for its very low RNase activity. Recently, Pelham and Jackson (13) described a procedure that eliminates the endogenous mRNA activity of the rabbit reticulocyte lysate by means of a calcium-dependent nuclease, thereby rendering it extremely sensitive to a low level of exogenous mRNA. The efficiency of translation of this system becomes up to five times higher than that of the wheat germ lysate.

A cell-free system was prepared by this procedure and found to be very efficient for the translation of several mRNA's. With purified rabbit globin mRNA or tobacco mosaic virus RNA, the incorporation of labeled amino acids increased linearly between concentrations of 0.5 and 12 µg of RNA per ml. The saturation in mRNA was reached at a concentration of 20 µg/ml.

Such a high sensitivity to exogenous mRNA should allow the translation of the putative mRNA synthetized in vitro by the polymerase reaction, which under the best conditions produces 0.5 µg of RNA per ml per h. The coupling of the two reactions would be possible if the ionic conditions are compatible and if the products of the polymerase reaction are functional mRNA's that are not too firmly bound to the RNA template (19).

We have checked several parameters of the preincubated reticulocyte cell-free system. In this system 80 to 90% of the reaction volume is occupied by the undiluted reticulocyte lysate itself, whose final ionic concentration is unknown. Thus, the ionic parameters of the translation system cannot be measured, except for the 0.4 mM magnesium chloride and 95 mM potassium chloride added during the preparation of the lysate (13). We have observed that any further addition of magnesium is inhibitory for the translating activity. With most mRNA's, except globin mRNA, the translation is stimulated by the addition of 60 µg of mouse (Krebs ascites cells) tRNA per ml. Spermidine (0.05 to 0.4 mM) had no effect on the activity of this cell-free system.

It was of interest to know whether this cell-free system translates accurately and efficiently the influenza virus mRNA obtained from FPV-infected cells. For comparison, the left panel of Fig. 2 shows the electrophoretic distribution of the labeled protein components

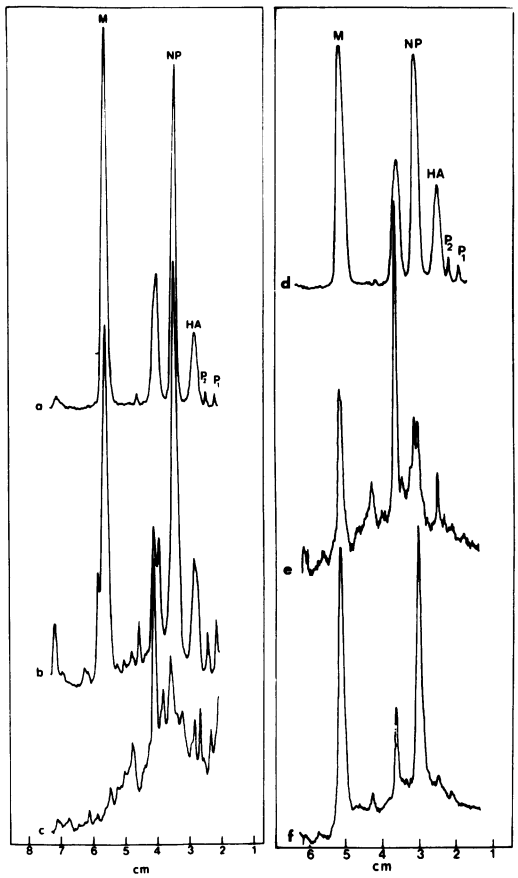

FIG. 2. *Analysis of products synthesized in the mRNA-dependent reticulocyte lysate programmed with total RNA from infected cells. Products were analyzed by slab gel electrophoresis on a 7 to 20% polyacrylamide gradient and scanned at 540 nm after fluorography. (a) [^{35}S]methionine-purified virion. The molecular weights of the virus structural proteins were as follows: P_1, 89,000; P_2, 80,000; HA, 74,000; NP, 59,000; and M, 28,000 (4). (b) Cytoplasm from chicken embryo fibroblasts that had been infected with 1 PFU of FPV per cell and labeled with [^{35}S]methionine from 6 to 9 h after infection. Slightly ahead of HA_1, one can distinguish a residual amount of a major component from the uninfected cell (molecular weight, 47,000) which is probably actin. The small shoulder moving ahead of M probably represents NS_1. (c) [^{35}S]methionine-labeled cytoplasm from uninfected chicken embryo fibroblasts. (d) [^{35}S]methionine-labeled purified FPV. (e) In vitro products in the reticulocyte cell-free system programmed with 200 μg of total RNA per ml from FPV-infected cells. RNA was extracted 2 h after infection. (f) Same as (e), except that RNA was extracted 4 h after infection. The full scale for the microdensitometric scanning was set at an optical density of 1.2 for (e) and 3.0 for all other tracings. Endogenous products of the reticulocyte cell-free system are shown in Fig. 4 and 5.*

from a purified FPV preparation (Fig. 2a), a cytoplasmic extract obtained from FPV-infected cells (Fig. 2b), and uninfected chicken embryo fibroblasts (Fig. 2c). The right panel of Fig. 2 shows the labeled protein components from a purified FPV preparation (Fig. 2d) as compared with the products of the in vitro translation in the reticulocyte cell-free system of total cytoplasmic RNA extracted from chicken embryo fibroblasts at 2 (Fig. 2e) and 4 h (Fig. 2f) after injection with FPV. These cytoplasmic RNAs have not been enriched for poly(A)-containing material by oligodeoxythymidylic acid [oligo(dT)]-cellulose chromatography.

RNA obtained as soon as 2 h after infection promotes the synthesis of proteins M, NP, and HA (Fig. 2e). After 4 h, M and NP predominate at the expense of putative actin, a major host cell component (Fig. 2f), and a minor peak, perhaps corresponding to P_2, becomes detectable (Fig. 2f). Except for their immunoprecipitation with an anti-FPV rabbit immune serum, these in vitro products have not been further characterized. We do not know whether the in vitro peptide at 2.5 cm (Fig. 2d, e, and f) is genuine HA (the precursor of HA_1 and HA_2) and whether it is partially glycosylated. A similar observation was reported very recently (13). It is interesting to note that in the wheat germ cell-free system the synthesis of such a component was never observed with poly(A)-containing mRNA preparations obtained from infected cells (4, 6, 16). The difference between the two systems could reflect a peculiar deficiency or a higher nuclease content of the wheat germ extract. It could also originate from losses occurring during the oligo(dT)-cellulose chromatography utilized in the purification of mRNA for the wheat germ system. This procedure could cause the loss of either some mRNA species with shorter or no poly(A) stretches or some essential tRNA species present in the infected chicken embryo fibroblasts.

Coupled system. (i) Kinetics and Mg^{2+} requirement. FPV was disrupted with urea, sodium deoxycholate, and Triton N-101 and added to the nuclease-preincubated reticulocyte lysate supplemented with the four ribonucleotide triphosphates and either [^{3}H]UTP or [^{35}S]methionine, as described above.

Figure 3 shows that in this reaction the incorporation of UMP continued for 1 to 2 h (Fig. 3A [a and c]), whereas the incorporation of [^{35}S]methionine reached its maximum between 2 and 5 h (Fig. 3B [c]). This incorporation is weak but reproducibly higher than that found in the endogenous reaction (Fig. 3B [a]).

Except for a twofold stimulation after 1 h of

incubation, the stimulatory effect of ApG (0.5 mM) of the RNA polymerase reaction was no longer observed in the presence of reticulocyte lysate (Fig. 3A [b and c]); indeed, ApG exerted a pronounced inhibitory effect on the incorporation of [^{35}S]methionine (at least 50% inhibition of the net counts per minute) (Fig. 3B [b and c]). This effect is also shown in Fig. 5.

As mentioned above, the actual Mg^{2+} concentration in the lysate is not known, but any addition of Mg^{2+} to the reticulocyte lysate inhibits protein synthesis programmed with exogenous mRNA, whereas the optimal Mg^{2+} concentration for the polymerase reaction is 3 to 5 mM (Fig. 1). For this reason we measured the effect of Mg^{2+} on the rate of protein synthesis in the reticulocyte lysate supplemented with disrupted FPV. For this reaction, Fig. 1 shows a very sharp Mg^{2+} optimum at 2 mM. This requirement is thus intermediate between that for protein synthesis and that for RNA synthesis. This may explain why both transcription and translation are suboptimal in the coupled reaction. This is in contrast to an analogous system described recently with vesicular stomatitis virus (VSV) (1), in which both viral RNA translation and transcription are significantly stimulated and prolonged when coupled. Figure 3 shows that the situation is entirely different here. The viral transcriptase alone in the absence of the lysate, but with 0.5 mM ApG and 5 mM Mg^{2+}, was three to four times more active (data not shown).

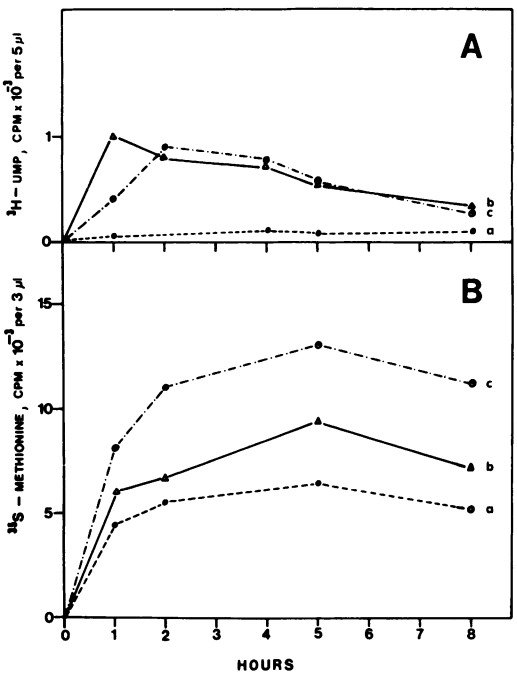

FIG. 3. *Kinetics of incorporation of [^{3}H]UMP (A) and [^{35}S]methionine (B) in the coupled cell-free system. (a) Control reticulocyte lysate without addition of disrupted FPV; (b) reticulocyte lysate with 1 mg of disrupted FPV per ml and 0.5 mM ApG; (c) reticulocyte lysate with 1 mg of disrupted FPV per ml.*

(ii) Product of the coupled reaction. Since the amount of [^{35}S]methionine incorporated in the coupled reaction was rather low, we examined the protein products of this reaction by polyacrylamide slab gel electrophoresis and fluorography. When compared with the distribution of the virion proteins (Fig. 4a), the major products of the coupled reaction coincide with proteins M and NP (Fig. 4b).

An additional protein at 2.6 cm (Fig. 4b) (which seems to correspond to actin) is also detected in the endogenous reaction (Fig. 4c). For unexplained reasons, the synthesis of the endogenous protein was always increased when the disrupted virus was present in the reaction, even when the transcription of viral RNA was inhibited (Fig. 4c and d; see section iv below).

Two distinct peptides run more slowly then NP. When this kind of experiment was repeated, these components, as compared with the products of the infected cytoplasm (Fig. 4f) and the endogenous reaction (Fig. 4h), were better detected in the products of a similar reaction (Fig. 4g). It is clear that they have the same mobility as HA and P_2. P_1 cannot be detected because of the presence of one peptide with the same mobility in the endogenous reaction (Fig. 4h). These components were detected after a long exposure of the gel.

(iii) Inhibition by ApG. It was mentioned earlier that 0.5 mM ApG had an inhibitory effect on the total protein synthesis; this was also demonstrated by a decreased synthesis of M and NP, the major products of in vitro translation. The inhibitory effect of ApG seems to be related to a posttranscriptional event since the transcription of viral RNA was stimulated by ApG (Fig. 1 and 3A). On the other hand, Fig. 5 shows that the inhibition of NP and M syntheses in the coupled system was about 50% under the conditions that do not modify the translation of the endogenous product. Since the same concentration of ApG also had no detectable effect upon the translation of exogenous rabbit globin mRNA in the preincubated reticulocyte lysate or in the wheat germ system (data not shown), the most likely explanation is that ApG prevented some posttranscriptional modification of the mRNA 5′ terminus or introduced an abnormal 5′ terminus. Recent obser-

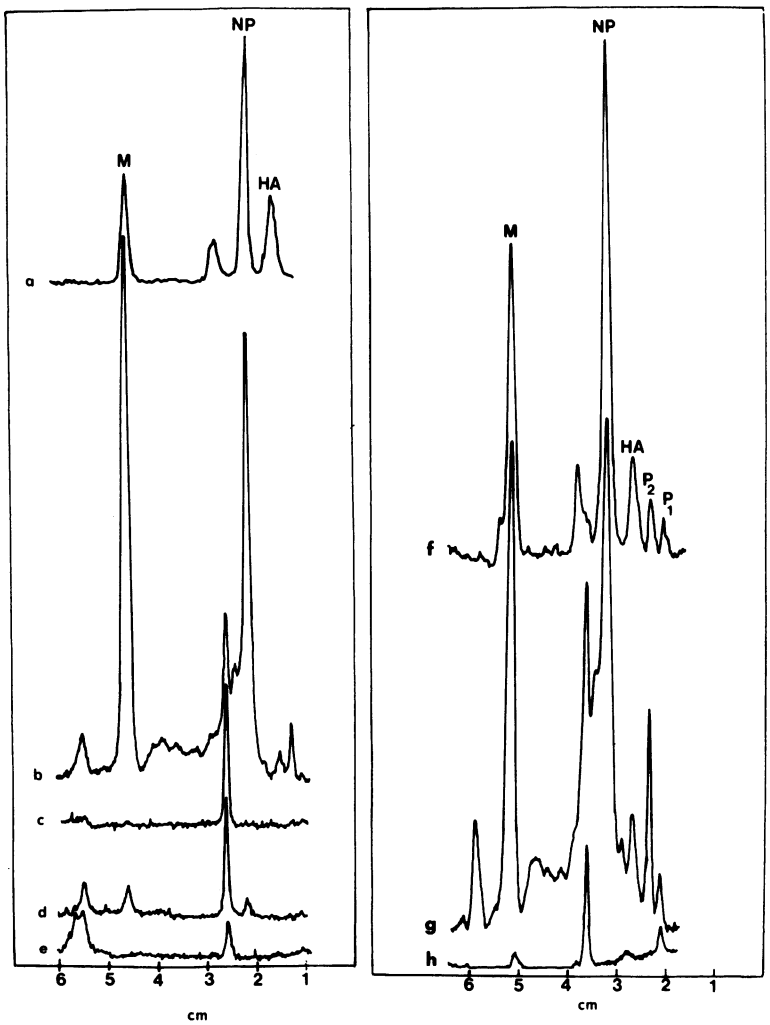

FIG. 4. *Analysis of products synthesized in the coupled reaction under different conditions. (a) [^{35}S]methionine-purified FPV; (b) in vitro products obtained in the coupled cell-free system with 1 mg of disrupted FPV per ml and incubated for 2 h at 31°C; (c) products of the same coupled reaction supplemented with 0.1 mM spermidine hydrochloride; (d) products of the coupled reaction without spermidine and the four ribonucleotide triphosphates; (e) endogenous products of the reticulocyte cell-free system reaction without disrupted FPV; (f) ^{35}S-labeled cytoplasmic proteins from FPV-infected cells; (g) coupled reaction incubated for 3.5 h at 31°C; (h) endogenous reaction (without disrupted FPV). For (b), (c), (d), and (e), the exposure time was 96 h. The full scale for microdensitometric scanning was set at an optical density of 6.0 for (a) and 3.0 for the other tracings. The products were analyzed as described in the legend to Fig. 2. The exposure time was 10 days for (g) and (h) and 24 h for (f). The full scale for the microdensitometric scanning was set at an optical density of 3.0.*

vations showing that ApG or GpG (11; Plotch and Krug, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, S114, p. 223), when used to prime the viral transcriptase, are incorporated at the 5′ end of the newly synthesized RNA would support the second hypothesis.

(iv) Effect of some inhibitors of transcription. If the translation of the viral proteins in the system described here results from its coupling with viral mRNA synthesis and not from the presence of some mRNA contaminants in the virion (4) or from the virion RNA itself (20), it should be possible to prevent it by simply inhibiting the transcriptase reaction. This can be done very easily by omitting the four ribonucleotide triphosphates from the reaction mixture. It must be noted that no addition of GTP or ATP is required for translation in the prein-

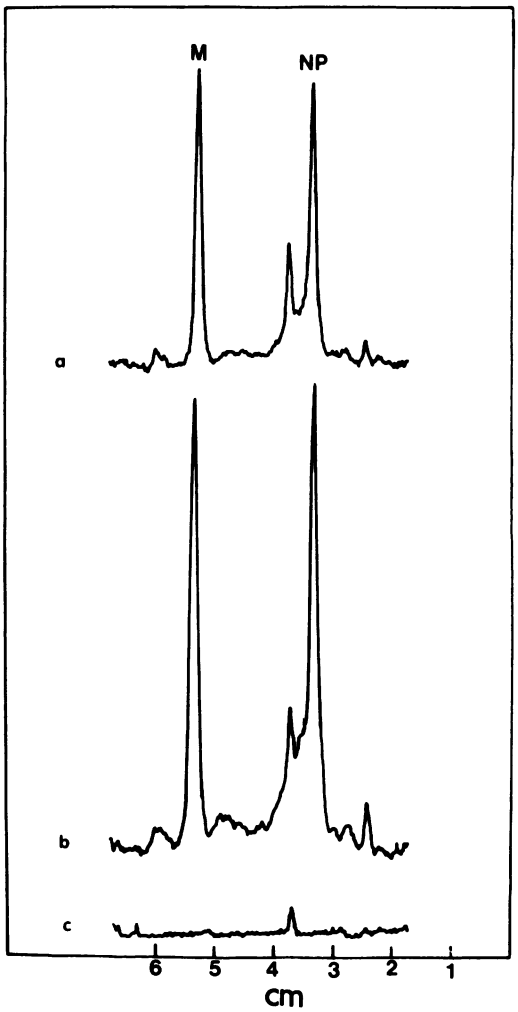

FIG. 5. *Effect of ApG on protein synthesis in the coupled reaction. (a) Coupled reaction supplemented with 0.5 mM ApG; (b) coupled reaction; (c) endogenous reaction. Incubation was for 8 h at 31°C. The products were analyzed as described in the legend to Fig. 2. The exposure time was 24 h for (a), (b), and (c). The full scale was set at an optical density of 3.0.*

cubated reticulocyte system (13). When the ribonucleotide triphosphates were omitted (Fig. 4d), the cell-free system continued to synthesize putative actin (an endogenous peptide), but the synthesis of viral proteins M and P was reduced by at least 95% (compared with Fig. 4b). The observed residual amount of viral peptides may result from the reticulocyte endogenous pool of nucleotide triphosphate.

As mentioned earlier, 0.05 to 0.4 mM spermidine has no effect on the translating efficiency of the reticulocyte lysate programmed with exogenous mRNA. Nevertheless, it completely inhibits the FPV RNA polymerase reaction (18) (M. Horisberger, unpublished data). In the coupled system the addition of 0.1 mM spermidine completely abolished the synthesis of viral proteins (Fig. 4c).

(v) Further characterization of in vitro products obtained in the coupled reaction. Since the amounts of labeled proteins obtained from the coupled reaction were too low for a determination of their [^{35}S]methionine tryptic peptide maps, one further characterization was performed by immunoprecipitation with two different monospecific rabbit antibody preparations. A comparison with the distribution of the labeled proteins from an FPV-infected cytoplasmic extract (Fig. 6a) shows that, after immunoprecipitation with an anti-NP rabbit antiserum (Fig. 6b) followed by polyacrylamide gel electrophoresis, more than 80% of the precipitated radioactive material comigrates with protein NP, whereas with an anti-M rabbit antiserum more than 95% of the precipitated material runs as a single component, with the same electrophoretic mobility as protein M (Fig. 6c).

DISCUSSION

We have established a cell-free coupled system for the transcription and translation of FPV mRNA. In this system, translation of the viral proteins is strictly dependent upon the functional integrity of the transcriptase reaction. Both transcription and translation are faithful and result in the synthesis of the main viral proteins M and NP and probably P_2 and HA. These proteins have been characterized by their electrophoretic mobility on polyacrylamide gels. Only M and NP have been characterized by their antigenicity. Protein NA, even from purified virion, has not been resolved on the kind of gels used in these experiments. P_1, which is the least abundant of all the virion proteins, cannot be distinguished from one of the endogenous components of the translating cell-free system.

The coupled reaction has to be supplemented with Mg^{2+}; the final concentration of Mg^{2+} is practically halfway between the optimal concentration of the viral polymerase (3 to 5 mM) and that required for the translation of exogenous mRNA in the reticulocyte cell-free system (where no addition of Mg^{2+} is required). Due to this different requirement for the two reactions, which may be taken as one example, it is not surprising that the coupled system used here translated with a relatively low efficiency and that there was no cooperation or reinforcement between the two reactions, as was observed in the case of VSV (1). The virion-associated RNA polymerase of VSV is much more active (20 to 50 times), and the ion require-

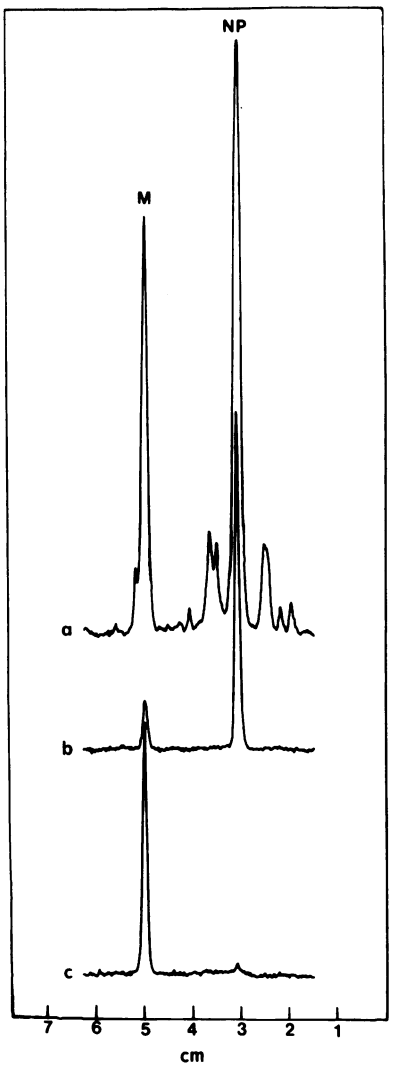

FIG. 6. *Product analysis of the coupled reaction after immunoprecipitation with anti-FPV IgG. (a) ^{35}S-labeled cytoplasmic protein from FPV-infected cells; (b) immunoprecipitate obtained from the standard coupled reaction with anti-NP rabbit antiserum; (c) immunoprecipitate of the same reaction with anti-M rabbit antiserum. The exposure time was 4 h. The full scale was set at an optical density of 3.0.*

ments are very similar for transcription and translation; thus, both reactions are activated and prolonged as a result of the coupling.

In the present work we have taken advantage of a newly described preincubated reticulocyte cell-free system (13). Because of its efficiency, high sensitivity to low amounts of mRNA, and probably a very low RNase content, this system could be used here in conjunction with a virus-associated RNA polymerase of very low activity.

The maximal concentration of viral RNA synthesized by FPV-associated transcriptase was about 0.5 μg per ml per h in the presence of ApG. In the coupled reaction, where both transcription and translation are suboptimal, for reasons stated above, the amount of RNA synthesized (as calculated from Fig. 3A) would not exceed 0.4 μg per ml per h. This is at least 20 times less than the amount of VSV RNA synthesized in the coupled cell-free system described by Ball and White (1). In addition, the amount of virus used is 20 times higher with FPV than with VSV. Nevertheless, it must be kept in mind that the translation efficiency of the mRNA generated in such a coupled cell-free system might be much higher than that of an exogenous mRNA (1).

Inhibition of translation of the viral products by ApG, which was observed exclusively in the coupled system, might be posttranscriptional since the dinucleotide did not inhibit the translation of exogenous mRNA and stimulated the virion-associated transcriptase. This could be explained if ApG serves as a primer for the transcriptase and is incorporated at the 5′ terminus of the native mRNA's as suggested recently (11, 18; Plotch and Krug, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, S114, p. 223). It is conceivable that the presence of ApG at the 5′ end of the mRNA's may introduce a wrong signal for an eventual capping enzyme or for the correct initiation of translation (9). To test this possibility, it will be of interest to measure the extent of mRNA capping and methylation in this cell-free system.

One striking observation that can be derived from the coupled cell-free system is that the proportion of the different proteins synthesized is not too different from that existing in vivo. It would therefore be possible to determine whether this proportion is regulated at the translation or transcription level, or even whether it depends on some posttranscriptional modification of the mRNA's (3, 9).

Finally, the fact that one can synthesize in vitro most of the authentic viral proteins in a cell-free translating system that is only fed with disrupted FPV particles and the four ribonucleotide triphosphates proves that the virion-associated RNA polymerase transcribes faithfully and completely the corresponding negative-stranded segments of the virion RNA. Until now the products of the virion-associated RNA polymerase had been examined by electrophoresis and annealing to purified virion RNA (2, 19). From the present in vitro observations it can be safely concluded that this RNA polymerase really functions as a viral transcriptase.

ACKNOWLEDGMENTS

We thank G. C. Schild for providing monospecific antisera, A. Ball for advice, B. Mechler for suggesting the use of the nuclease-preincubated reticulocyte cell-free system, H. R. B. Pelham for communicating a preprint on the subject, and L. Thiry for critically reading the manuscript. H. Van Haelen is gratefully acknowledged for expert technical assistance.

Two of us (J.C. and L.D.) were supported by grants from the Belgium Fonds de la Recherche Scientifique Médicale, Fonds National de la Recherche Scientifique, and the Fondation Rose et Jean Hoguet.

LITERATURE CITED

1. **Ball, L. A., and C. N. White.** 1976. Order of transcription of genes of vesicular stomatitis virus. Proc. Natl. Acad. Sci. U.S.A. 73:442–446.
2. **Bishop, D. H. L., J. F. Obijeski, and R. W. Simpson.** 1971. Transcription of the influenza ribonucleic acid genome by a virion polymerase. II. Nature of the in vitro polymerase product. J. Virol. 8:74–80.
3. **Breindl, M., and J. J. Holland.** 1976. Studies on the in vitro transcription and translation of vesicular stomatitis virus mRNA. Virology 73:106–118.
4. **Content, J.** 1976. Cell-free translation of influenza virus mRNA. J. Virol. 18:604–618.
5. **Etkind, P. R., and R. M. Krug.** 1974. Influenza viral messenger RNA. Virology 62:38–45.
6. **Etkind, P. R., and R. M. Krug.** 1975. Purification of influenza viral complementary RNA: its genetic content and activity in wheat germ cell-free extracts. J. Virol. 16:1464–1475.
7. **Horisberger, M.** 1976. The in vitro stimulation of the influenza transcriptase by ribosomes. FEBS Lett. 63:134–136.
8. **Housman, D., M. Jacobs-Lorena, U. L. Rajbhandary, and H. F. Lodish.** 1970. Initiation of haemoglobin synthesis by methionyl tRNA. Nature (London) 227:913–918.
9. **Krug, R. M., M. A. Morgan, and A. J. Shatkin.** 1976. Influenza viral mRNA contains internal N^6-methyladenosine and 5'-terminal 7-methylguanosine in cap structures. J. Virol. 20:45–53.
10. **Laskey, R. A., and A. D. Mills.** 1975. Quantitative film detection of ^3H and ^{14}C in polyacrylamide gel by fluorography. Eur. J. Biochem. 56:335–341.
11. **McGeoch, D., and N. Kitron.** 1975. Influenza virion RNA-dependent RNA polymerase: stimulation by guanosine and related compounds. J. Virol. 15:686–695.
12. **Morrison, T. G., and H. F. Lodish.** 1975. Site of synthesis of membrane and nonmembrane proteins of vesicular stomatitis virus. J. Biol. Chem. 250:6955–6962.
13. **Pelham, H. R. B., and R. J. Jackson.** 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. Eur. J. Biochem. 67:247–256.
14. **Pons, M. W.** 1973. The inhibition of influenza virus RNA synthesis by actinomycin D and cycloheximide. Virology 51:120–128.
15. **Pons, M. W.** 1975. Influenza virus RNA(s), p. 145–170. *In* E. D. Kilbourne (ed.), The influenza viruses and influenza. Academic Press Inc., New York.
16. **Ritchey, M. B., and P. Palese.** 1976. In vitro translation of influenza virus messenger RNA's. Virology 72:410–420.
17. **Roberts, B. E., and B. M. Paterson.** 1973. Efficient translation of tobacco mosaic virus RNA and rabbit globin 9S RNA in a cell-free system from commercial wheat germ. Proc. Natl. Acad. Sci. U.S.A. 70:2330–2334.
18. **Rochovansky, O. M.** 1976. RNA synthesis by ribonucleoprotein-polymerase complexes isolated from influenza virus. Virology 73:327–338.
19. **Simpson, R. W., and W. J. Bean.** 1975. Influenza transcriptase activity of cells and virions, p. 125–143. *In* E. D. Kilbourne (ed.), The influenza viruses and influenza. Academic Press Inc., New York.
20. **Tekamp, P. A., and E. E. Penhoet.** 1976. Message activity of influenza viral RNA. J. Virol. 18:812–816.
21. **Weber, L. A., E. R. Feman, and C. Baglioni.** 1975. A cell free system from HeLa cells active in initiation of protein synthesis. Biochemistry 14:5315–5321.