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Direct Evidence for Messenger Activity of Influenza Virion RNA

(protein synthesis *in vitro*/gel electrophoresis/radioimmune assay)

W. SIEGERT, G. BAUER, AND P. H. HOFSCHNEIDER

Max-Planck-Institut für Biochemie, 8033 Martinsried bei München, Germany

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ABSTRACT In a cell-free system of *Escherichia coli*, RNA from influenza virus particles is translated into a polypeptide antigenically identical with the ribonucleoprotein and several more proteins, some of which correspond in size to viral structural components.

Replication of influenza virus RNA very likely proceeds in steps similar to those in replication of RNA phages and picornaviruses (1, 2). The RNA in virions codes for the formation of complementary RNA strands, which serve as template for progeny RNA synthesis. These reactions are catalyzed by the virus-specified RNA polymerase that is part of the virion (3–6). However, it is unknown whether the RNA from virus particles or their complementary strands serve as messenger for synthesis of virus-specific proteins. Studies on the nature of polysomal RNA from infected cells led to controversial results. Plus strands as well as minus strands have been detected in preparations of polysomes (7–9).

A direct approach to solving this question is provided by using RNA from influenza virus particles in an *in vitro* protein-synthesizing system and by studying its potential to code for virus-specific proteins.

Recently we demonstrated that RNA from avian myeloblastosis virus is translated with high fidelity in a cell-free system of *Escherichia coli* (10). There is additional evidence that the same is true for RNA of Rauscher murine leukemia virus (ref. 11; W. Siegert, unpublished results), mouse mammary tumor virus (11), and feline leukemia virus (W. Siegert, unpublished results).

We have presented preliminary data suggesting that influenza virus RNA has messenger properties in the *E. coli* system (12). Here we wish to present clear evidence that influenza virus particles contain a messenger-like RNA (plus strand) which can be expressed in the *E. coli* system.

MATERIALS AND METHODS

Influenza virus, fowl plague "Rostock" strain, which was used for pilot experiments, was a gift from Dr. R. Rott, University of Giessen. The experiments reported here were performed with strain A PR8, which was a gift from Dr. R. Siegert, University of Marburg and Dr. H. Bachmayer, Sandoz–Forschung, Vienna. Virus was purified from the allantoic fluid of eggs by a sequence of alternating low- and embryonated high-speed centrifugations followed by two isopycnic sucrose gradients (13). Rauscher leukemia virus and avian myeloblastosis virus were kindly provided by Drs. O'Connor (NIH, Bethesda, Md.) and J. F. Beard (Duke University).

Viral RNA was extracted twice with phenol after digestion with Pronase in the presence of sodium dodecyl sulfate, precipitated twice with ethanol, dissolved in 0.1 M Tris·HCl (pH 7.8) and used for *in vitro* synthesis (10). Preparation of the *in vitro* system, incubation conditions, and analysis of the products on polyacrylamide gels have been described (10, 14, 15).

Radioimmunoassays were performed with *in vitro* product that was prepared by phenol treatment in the presence of sodium dodecyl sulfate and unlabeled methionine and dialyzed against 0.15 M NaCl–15 mM sodium citrate (pH 7.0) for 2 days at 4° (10).

For preparation of antisera, rabbits were injected subcutaneously with influenza strain A PR8 or phage M 12 together with Freund's complete adjuvant. 4 Weeks later the animals received an intravenous booster injection and were bled after 1 week.

Guinea pig antisera against influenza type A and B ribonucleoprotein were obtained from WHO World Influenza Centre, National Institute for Medical Research, Mill Hill, London NW 7, England.

For microimmunodiffusion tests (10, 16) the *in vitro* proteins were mixed with unlabeled influenza virus (10^6 hemagglutinating units per ml) and with phage M 12 particles (10^{15} plaque-forming units per ml) as carriers to effect a visible precipitation. This virus suspension was made 1% with respect to sodium dodecyl sulfate. 15 μl of this mixture was put in each well and immediately allowed to diffuse against antisera.

RESULTS

Stimulation of Amino-Acid Incorporation by Influenza Virus RNA. To show that RNA from influenza virions can act as a messenger in an *in vitro* system of *E. coli*, we first studied its ability to stimulate incorporation of [^{3}H]histidine and [^{35}S]methionine into trichloroacetic acid-precipitable material. Addition of influenza virus RNA at a concentration of 150 μg/ml gave rise to a 10- to 50-fold stimulation of amino-acid incorporation, whichever labeled amino acid was used (Table 1). As controls, RNAs from M 12 phage, Rauscher leukemia virus, and avian myeloblastosis virus were used in parallel. The incorporation obtained with influenza virus RNA is lower than that obtained with phage M 12 RNA. But influenza virus RNA stimulates amino-acid incorporation better than avian myeloblastosis or Rauscher leukemia virus RNAs. After digestion of influenza RNA by pancreatic RNase no incorporation was obtained.

As a further control, RNA of Newcastle disease virus was used in parallel. Addition of its RNA did not lead to an in-

TABLE 1. *Incorporation of labeled amino acids (cpm) into trichloroacetic acid-precipitable material*

Experiment	No RNA added	Influenza RNA	NDV RNA	Phage M 12 RNA	AMV RNA	RLV RNA
[^{3}H]His						
1	390	22,700	NT	52,000	NT	NT
2	1,000	23,600	NT	36,000	18,000	2,160
3	730	15,000	NT	21,000	9,600	7,500
4	1,650	17,200	1,630	40,000	NT	NT
[^{35}S] Met						
5	2,370	30,600	NT	171,000	NT	NT
6	1,500	18,300	NT	179,000	NT	11,820
7	4,680	24,300	NT	120,000	NT	NT

To 50 μl of reaction mixture (14, 15), 10 μg of viral RNA, dissolved in 10 μl of 0.1 M Tris·HCl (pH 7.8), and 5 μl of labeled amino acid, corresponding to 2 to 5 $\times$ 10^6 cpm, were added. L-[^{3}H]histidine (specific activity 50 Ci/mmol) and L-[^{35}S]methionine (specific activity 109 Ci/mmol) were purchased from the Radiochemical Centre (Amersham, England). Reactions were performed at 37° for 20 min in the presence of 10 mM Mg^{++}. 10 μl of the assay mixture was dried on paper filters, precipitated with cold 10% trichloroacetic acid containing 1% of the amino acid used for labeling, heated to 85° for 15 min in 10% trichloroacetic acid, then washed once with a mixture of ether and ethanol, and once with ether. The washed filters were dried and the radioactivity determined (14, 15).

NDV, Newcastle disease virus; *AMV*, avian myeloblastosis virus; *RLV*, Rauscher leukemia virus. *NT* = not tested.

corporation of amino acids into trichloroacetic acid-precipitable polypeptides. This fact is compatible with earlier findings, which clearly demonstrate that Newcastle disease virus particles contain RNA complementary to polysomal RNA (17, 18).

Amino-acid incorporation depends linearly on the concentration of influenza virus RNA present in the mixture up to 380 μg/ml. At this concentration a plateau is not yet reached (Fig. 1).

Synthesis of trichloroacetic acid-precipitable polypeptides continues linearly for about 10 min and then levels off (Fig. 2).

Characterization of the In Vitro Product by Gel Electrophoresis. To determine if virus-specific proteins were synthesized, the *in vitro* product was analyzed on sodium dodecyl sulfate–polyacrylamide gels. The radioactivity pattern of the *in vitro* proteins was compared with that of proteins of the influenza virion (Fig. 3). The upper curve shows the densitometer profile of a stained gel carrying influenza proteins. The distribution is in accordance with results obtained recently (13). One can detect five peaks, representing the slowly migrating polypeptide P (83,500 daltons), the nucleoprotein NP (60,000 daltons), the hemagglutinin subunits HA_1 and HA_2 (49,000 and 32,000 daltons, respectively), and protein M (26,500 daltons). The neuraminidase NA (45,000 daltons) is not resolved from HA_1 in this gel system. HA_1, HA_2, and NA are glycosylated.

The lower curve shows the radioactivity pattern of proteins synthesized *in vitro* in the presence of [^{35}S]methionine. It

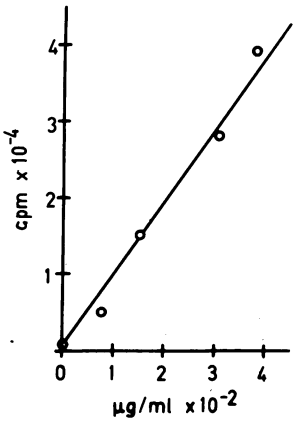

FIG. 1. The effect of various concentrations of influenza virus RNA on incorporation of [^{3}H]histidine into protein. To 50 μl of standard reaction mixture different amounts of RNA were added in 10-μl volumes of 0.1 M Tris·HCl (pH 7.8) resulting in final RNA concentrations as plotted in the diagram. After an incubation of 20 min at 37°, 10-μl aliquots were dried on paper filter disks and processed for detection of radioactivity, as described in the legend to Table 1.

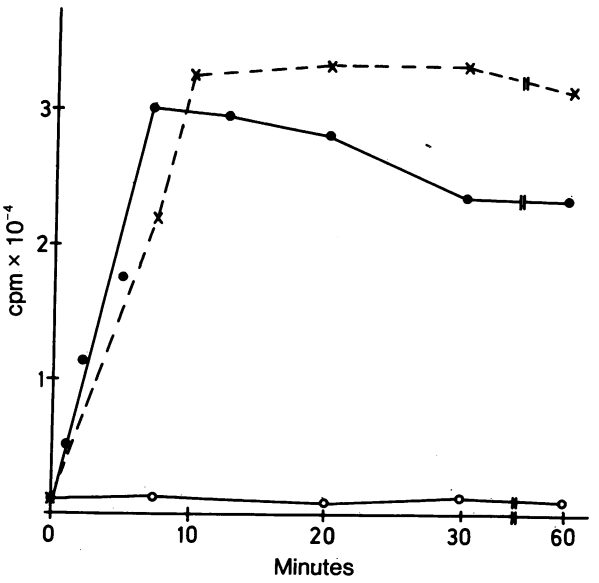

FIG. 2. Time dependence of incorporation of [^{3}H]histidine into protein. At the times indicated 10-μl aliquots were taken out of the standard assay mixture, and the trichloroacetic acid-precipitable radioactivity was determined (see legend to Table 1). Synthesis directed by influenza virus RNA (●) was compared with endogenous (○) and phage M 12 RNA-dependent (×) incorporation.

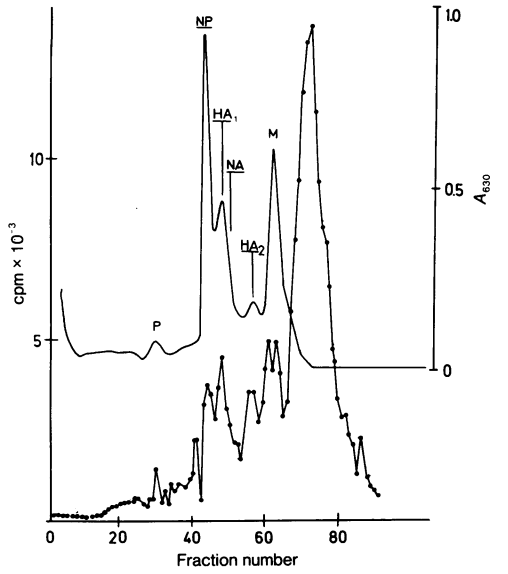

FIG. 3. Gel pattern of the proteins of influenza virus particles (——) and of the product synthesized *in vitro* under the direction of influenza virus RNA (●——●) labeled with [^{35}S]methionine. 10% acrylamide–bisacrylamide gels, 10 cm long, were prepared in the presence of 0.1% sodium dodecyl sulfate and 4 M urea, according to Weber *et al.* (21). They were loaded with 100 μg of influenza virus proteins or labeled proteins synthesized *in vitro* and were run at 2.5 mA per gel at room temperature until the dye marker reached the bottom of the tube. The gels loaded with virus proteins were stained with Coomassie blue and scanned at 630 nm in a Gilford spectrophotometer. For determination of radioactivity, the gels were sliced and disolved in hydrogen peroxide and Protosol Solubilizer (New England Nuclear).

P indicates slowly migrating polypeptide; *NP*, nucleoprotein; HA_1 and HA_2, hemagglutinin subunits; *NA*, neuraminidase; *M*, membrane protein.

demonstrates that influenza virus RNA directs the *in vitro* synthesis of high-molecular-weight proteins, most of which correspond in size to individual viral proteins. The nature of polypeptides that do not correspond in size to virion proteins, especially the material in fractions 40 and 41 and 65–80, will be discussed later.

Analysis of the In Vitro Product by Immunodiffusion. The fact that influenza virus RNA codes for synthesis of proteins that correspond in size to the proteins present in the virion supports the concept that the RNA is translated correctly. To obtain further evidence for the identity of the *in vitro* proteins with virion proteins, we tested whether among the synthesized proteins there is one that has the antigenic properties of ribonucleoprotein A. Influenza *in vitro* proteins labeled with [^{35}S]methionine were mixed with unlabeled influenza virions as carrier and allowed to diffuse toward various antisera to viral proteins (Fig. 4). Among the precipitation lines detected, there is one that contains ribonucleoprotein A antigen (Fig. 4*a*), and it is obvious from the autoradiogram (Fig. 4*b*) that radioactive material is coprecipitated with unlabeled virion antigen. Precipitation lines corresponding to the hemagglutinin and the neuraminidase (16) are not clearly separated, but also contain radioactivity.

A control experiment was performed to exclude the possibility that radioactive material is bound unspecifically to

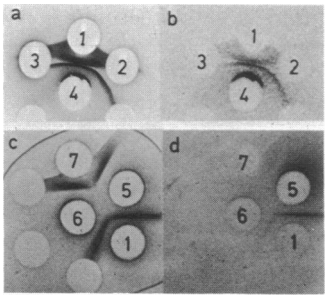

FIG. 4. (*a* and *b*) Immunodiffusion assay with proteins synthesized *in vitro*. A mixture of unlabeled influenza proteins (10^6 hemagglutinating units per ml) with [^{35}S]methionine-labeled influenza proteins, synthesized *in vitro* (*4*) was allowed to diffuse toward rabbit antiserum in influenza A PR8 (*1*), and against guinea pig antisera to ribonucleoprotein A (*2*) and to ribonucleoprotein B (3). (*c* and *d*) Controls were a mixture of unlabeled influenza A PR8 and phage M 12 (*6*) and a mixture of unlabeled influenza A PR8, phage M 12, and [^{35}S]methionine-labeled influenza protein synthesized *in vitro* (*5*) against rabbit antisera to influenza A PR8 (*1*) and to phage M 12 (*7*). (*a* and *c*, photographs of stained immunodiffusion plates; *b* and *d*, autoradiograms.)

the immunoprecipitates. In the experiment shown in Fig. 4*c* a mixture of unlabeled influenza and phage M 12 proteins was put in wells *5* and *6*. Well *5* additionally contained influenza *in vitro* proteins labeled with [^{35}S]methionine. These protein mixtures were allowed to diffuse against antisera to influenza virions (*1*) and to phage M 12 proteins (*7*). The corresponding autoradiogram shows that influenza proteins made *in vitro* are only precipitated by the homologous antiserum.

DISCUSSION

Analysis of the proteins synthesized *in vitro* shows that several distinct proteins are made. The molecular weights of most of these proteins correspond to those of structural components of the virus particle. It has been shown that one of the proteins synthesized *in vitro* has immunological properties of ribonucleoprotein A, an antigen common to all influenza type A strains.

Furthermore, the immunodiffusion tests provide some evidence that the neuraminidase and the hemagglutinin are made *in vitro*. However, this evidence has to be examined in more detail by using monospecific antisera against the individual viral antigens.

These facts indicate that the RNA from influenza virions, in contrast to virion RNA of Newcastle disease virus, has messenger properties that can be expressed in the *E. coli* system. However we do not know if all the virion RNA is present in a translatable form (plus strand). It might be possible that some genes are conserved in a complementary base sequence (minus strand) which first must be transcribed by the virion polymerase to allow translation.

Also we cannot tell with certainty if nonstructural proteins are made. It is possible that the fast-migrating material synthesized *in vitro* corresponds to the protein NS recently described by Klenk *et al.* (19) and Skehel *et al.* (20), but we cannot exclude the possibility that it represents incomplete nascent polypeptide chains. Another protein preferentially detected in the infected cell is the precursor molecule for the two hemagglutinin subunits. According to Klenk *et al.* (19), it exists in a glycosylated (HA) and a nonglycosylated form

(HA_0). One should expect that the nonglycosylated molecule also is synthesized in the *E. coli* lysate. However, since its molecular weight is only slightly larger than the nucleoprotein it cannot be identified with certainty. We need additional data to conclude that the labeled material migrating slower than nucleoprotein (fractions 40 and 41) corresponds to HA_0.

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1. Blair, C. D. & Duesberg, P. H. (1970) *Annu. Rev. Microbiol.* **24**, 539–574.
2. Stavis, R. L. & August, J. T. (1970) *Annu Rev. Biochem.* **39**, 527–560.
3. Scholtissek, C., Becht, H. & Rott, R. (1971) *Virology* **43**, 137–143.
4. Scholtissek, C. & Rott, R. (1969) *J. Gen. Virol.* **4**, 125–137.
5. Skehel, J. J. & Burke, D. C. (1969) *J. Virol.* **3**, 429–438.
6. Chow, N. & Simpson, R. W. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 752–756.
7. Nayak, D. P. (1970) in *The Biology of Large RNA Viruses*, ed. Barry, R. D. & Mahy, B. W. J. (Academic Press Inc., New York), pp. 371–391.
8. Pons, M. W. (1972) *Virology* **47**, 823–832.
9. Pons, M. W. (1973) *Virology* **51**, 120–128.
10. Siegert, W., Konings, R. N. H., Bauer, H. & Hofschneider, P. H. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 888–891.
11. Gielkens, A. L. J., Salden, M. H. L., Bloemendal, H. & Konings, R. N. H. (1973) *FEBS Lett.* **28**, 348–352.
12. Siegert, W. & Hofschneider, P. H. (1973) *FEBS Lett.* **34**, 145–146.
13. Klenk, H.-D., Rott, R. & Becht, H. (1972) *Virology* **47**, 579–591.
14. Konings, R. N. H., Ward, R., Francke, B. & Hofschneider, P. H. (1970) *Nature* **226**, 604–607.
15. Ward, R., Konings, R. N. H. & Hofschneider, P. H. (1970) *Eur. J. Biochem.* **17**, 106–115.
16. Schild, G. C., Winter, W. D. & Brand, C. M. (1971) *Bull. W.H.O.* **45**, 465–471.
17. Bratt, M. A. & Robinson, W. S. (1967) *J. Mol. Biol.* **23**, 1–21.
18. Huang, A. S., Baltimore, D. & Bratt, M. A. (1971) *J. Virol.* **7**, 389–394.
19. Klenk, H.-D., Scholtissek, C. & Rott, R. (1972) *Virology* **49**, 723–734.
20. Skehel, J. J. (1972) *Virology* **49**, 23–36.
21. Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412.