Proc. Nat. Acad. Sci. USA
Vol. 69, No. 4, pp. 888–891, April 1972

Translation of Avian Myeloblastosis Virus RNA in a Cell-Free Lysate of *Escherichia coli*

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

W. SIEGERT, R. N. H. KONINGS*, H. BAUER, AND P. H. HOFSCHNEIDER

Max-Planck-Institut für Biochemie, Munich, and Robert-Koch Institut, Berlin, W. Germany

Communicated by F. Lynen, January 31, 1972

ABSTRACT In a cell-free extract of *E. coli*, RNA from avian myeloblastosis virus directs the synthesis of a protein that is antigenically identical with the group-specific antigen 4, and other proteins, three of which correspond in molecular weight to group-specific antigens 1–3.

According to Temin's provirus hypothesis, the genetic information present in oncogenic RNA viruses is transcribed via an RNA–DNA hybrid into a double-stranded DNA molecule. After being incorporated into the host-cell genome, the DNA functions as a template for the synthesis of progeny RNA molecules (1). Several recent findings, such as the detection of reverse transcriptase (2, 3), host-controlled expression of virus genes (4, 5), rescue of latent viruses (6, 7), and vertical transmission of oncogenic viruses (8), strongly support this hypothesis.

Apart from its function as a template for the synthesis of DNA, no clear role has been ascribed to the viral RNA; it is not known whether the information for the synthesis of virus-specific proteins is conserved in the viral RNA or in its complementary strand, or even in both.

A hint that the viral RNA might have messenger properties is provided by the failure to detect complementary strands in the cytoplasm of infected cells. Complementary strands were detected only in the nucleus (9), and an RNA-dependent RNA polymerase was described (10). Whether or not a given RNA has messenger functions can be demonstrated more directly by a study of its ability to direct the synthesis of specific proteins *in vitro*. That this is the case for avian myeloblastosis virus (AMV)-RNA will be demonstrated in this paper by use of a cell-free lysate of *Escherichia coli*.

MATERIALS AND METHODS

Plasma–AMV, which was a generous gift of Dr. J. W. Beard (Duke University, Durham, N.C., USA) was first purified in a discontinuous sucrose gradient (11) with STE buffer (0.1 M NaCl–0.01 M Tris·HCl (pH 8.0)–1 mM ethylenediamine tetraacetate (EDTA). Virus present in the 35% sucrose layer was collected, diluted by the addition of one volume of NaCl–Tris–EDTA buffer, and pelleted by centrifugation (Spinco SW 50.1; 45,000 rpm; 20 min at 4°).

The pellet was resuspended in NaCl–Tris–EDTA buffer and used for the preparation of viral RNA and viral proteins. The viral proteins were prepared as recently described for RNA-phage M12 proteins (12).

RNA was extracted with a mixture of phenol and *m*-cresol (13) after incubation of the virus with Pronase in the presence of sodium dodecyl sulfate (SDS) at 37°, according to a standard procedure (14). RNA present in the aqueous layer was precipitated twice with cold ethanol; high molecular weight RNA was obtained by linear sucrose density gradient centrifugation (15).

The preparation of the cell-free lysate and of the incubation mixtures, and the extraction of the proteins synthesized *in vitro*, were already described (16).

^{14}C-labeled proteins used in immunodiffusion tests in agar gel were prepared in the same manner (16), except that they were dialyzed against SSC (0.15 M NaCl– 15 mM Na_3-citrate, pH 7.0) for 2 days at 4° and concentrated by evaporation under reduced pressure at room temperature.

Double immunodiffusion in agar gel, in combination with autoradiography, was performed as described (17) with rabbit antiserum against group-specific (gs) antigens 3 and 4 (18). Viral proteins that were extracted by phenol–SDS (19) were used as carriers.

RESULTS

Stimulation of amino-acid incorporation by AMV-RNA

Addition of AMV–RNA to a cell-free lysate of *E. coli* for stimulation of protein synthesis leads to a considerable enhancement of amino-acid incorporation, which is shown in Table 1 by an increase of Cl_3CCOOH-precipitable material. The mixture tested shows only a very low endogenous incorporation of amino acids, since the cpm values in column II, obtained after allowing incorporation for 30 min, are almost identical with those in column I, which were obtained at zero time and, therefore, represent the radioactivity background of the assay.

When homologous messenger RNA, i.e., phage M12 RNA, is added to the mixture to direct protein synthesis, the Cl_3CCOOH-precipitable radioactivity increases by a factor of up to 80; it is known (12, 16) that this is due mainly to the synthesis of phage-specific proteins. As shown in column IV (Table 1), AMV–RNA leads to a stimulation of amino-acid incorporation that is clearly more than the background of the assay and ranges up to 20% of the incorporation obtained under the direction of M12–RNA. The increase in amino-acid incorporation is linear for at least 20 min and is dependent on the amount of AMV–RNA added and on the concentration of magnesium, the optimum concentration being 10 mM.

Abbreviations: AMV, Avian Myeloblastosis Virus; SDS, sodium dodecyl sulfate; gs, group-specific.

* Present address: Laboratorium voor Moleculaire Biologie, Katholieke Universiteit, Nijmegen, Netherlands.

Characterization of the product synthesized *in vitro* by gel electrophoresis

For detection of whether the observed stimulation of amino-acid incorporation led to the synthesis of AMV-specific proteins, the *in vitro* products were subjected to electrophoresis on SDS-polyacrylamide gels. The pattern obtained was compared with that given by the proteins of AMV (Fig. 1). The *upper curve* (Fig. 1) shows the densitometer profile of the stained gel containing the proteins of AMV. The protein pattern is comparable to that described (17, 19), although peaks 1 and 2 are not clearly separated. Whether this is due to differences in the virus strains or in the techniques used is unknown at present. The molecular weights of the peaks (*from right to left*) of 13,500–16,000; 24,000; 28,000; 38,000; and 60,000–100,000, estimated by comparison with marker proteins (Fig. 1, *upper gel*), are also in good agreement with earlier reports. Peaks 1 through 4 represent the internal antigens of the virus (18), while peaks 5 and 6 correspond to the two viral glycoproteins, which were both shown to contain type-specific (Ve) antigenic activity (17, 19, 20).

The *lower curve* shows the radioactivity pattern of a gel run with proteins, synthesized *in vitro*, as described in Exp. 1, column IV of Table 1. Essentially, the same pattern was obtained when purified high molecular weight RNA was used instead of total AMV-RNA to direct protein synthesis. The radioactivity pattern of the proteins synthesized *in vitro* shows a high degree of similarity to that of the proteins synthesized *in vivo*. Thus, products synthesized *in vitro* are found that correspond, in position and relative amount, to antigens 1–4. This result shows that, *in vitro*, at least four proteins are synthesized that correspond to the proteins 1–4 of the virus particle with respect to molecular weight. This may also be true for the protein synthesized *in vitro* that is found in the position of protein 5 that is synthesized *in vivo*. Since protein 5 is glycosylated (17, 20), it cannot be excluded that these proteins are different. Not enough is known as to how glycosylation influences migration in the gel, and it is also not known whether glycosylation takes place in the mixture used. Apparently, no product that is synthesized *in vitro* is formed corresponding to peak no. 6 that is synthesized *in vivo*. However, peak no. 6 contains another glycosylated protein of the virus (17–19), and it is conceivable that only the nonglycosylated protein component has been synthesized *in vitro* and that it may migrate elsewhere in the gel.

TABLE 1. *Incorporation of [^3H]histidine and [^{14}C]amino-acid mixture (cpm) into Cl_3CCOOH-precipitable material*

		No RNA added		Phage M12-RNA added	AMV-RNA added
		At 0 time	After 30-min incub.	After 30-min incub.	After 30-min incub.
Exp.		I	II	III	IV
1	[^3H]Histidine	1010	1230	52,800	4,990
2	[^3H]Histidine	946	1050	80,400	18,100
3	[^3H]Histidine	not tested	1320	63,200	9,640
4	[^{14}C]Amino-acid mixture	2880	3250	32,200	6,110
5	[^{14}C]Amino-acid mixture	2250	2620	94,900	7,770

To 50 μl of reaction mixture, 10 μg of phage M12 or AMV-RNA, dissolved in 10 μl of 0.1 M Tris·HCl (pH 7.8) and 5 μl of labeled amino acid, corresponding to 2–5 × 10^6 cpm were added. L-[^3H] histidine (specific activity 50 Ci/mmol) and [^{14}C]amino-acid mixture (specific activity 54 Ci/mg-Atom of carbon) were purchased from the Radiochemical Centre (Amersham, England). Reactions were performed at 37° for 30 min in the presence of 10 mM Mg^{++}. At the times indicated, 10-μl aliquots of the assay mixture were dried on paper filters, precipitated with cold 10% Cl_3CCOOH, heated to 85° for 15 min in 10% Cl_3CCOOH, then washed once with a mixture of ether and ethanol, and once with ethanol. The washed filters were dried, and the radioactivity was determined (12, 16).

Analysis of the product by immunodiffusion

The correspondence of the molecular weights of several products synthesized *in vitro* with those of the proteins of the virus particle makes it very likely that the AMV-RNA is translated in the *E. coli* lysate with high fidelity. Further evidence of this may be obtained by comparison of the antigenic properties of the products synthesized *in vitro* with those of the virus proteins. Since the amount of product synthesized *in vitro* was not sufficient for any direct serological evaluation, [^{14}C]amino-acid-labeled product was mixed with un-

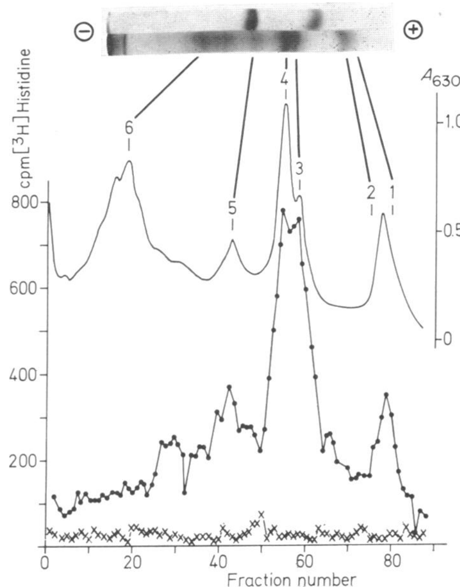

FIG. 1. Pattern of the proteins of AMV (*lower gel* and *upper curve*, ——) and of the product synthesized *in vitro* under direction of AMV-RNA (*middle curve*, ●——●) in polyacrylamide gels, and without addition of RNA (*lower curve*, ×——×). 10% acrylamide–diacrylate gels, 10 cm long, were prepared in the presence of 0.1% SDS and 4 M urea, according to the method of Viñuela *et al.* (32). Gels were loaded with 100–150 μg of AMV-proteins, labeled proteins synthesized *in vitro* (5000–10,000 cpm/gel), or molecular-weight marker proteins (*upper gel*): RNase I from *Escherichia coli* (molecular weight 17,500) and GDH-monomer (glycerol-1-phosphate-dehydrogenase EC 1.1.1.8, molecular weight 35,000), 20 μg per marker protein per gel. Gels were run at 2.5 mA/gel at room temperature until the dye marker reached the bottom of the gel. The gels loaded with virus proteins or reference proteins were stained with Coomassie blue and scanned at 630 nm in a Gilford spectrophotometer. For determination of radioactivity, gels were sliced and dissolved in a mixture of Hyamine hydroxide and piperidine (12, 16).

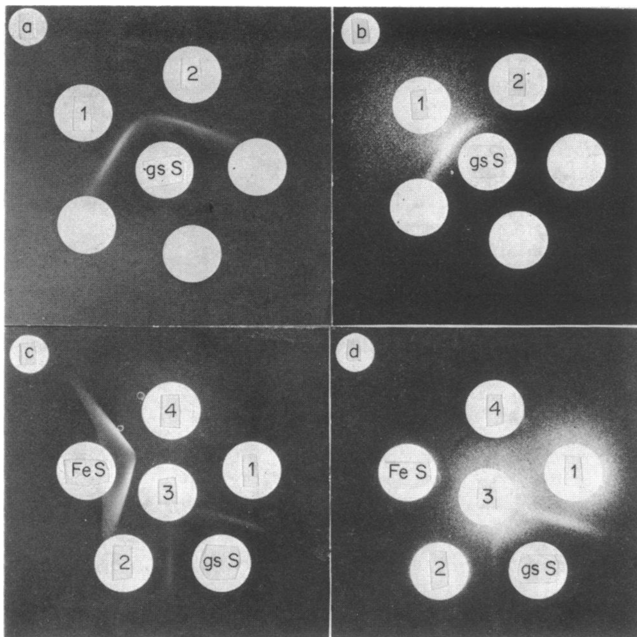

FIG. 2(*a* and *b*). Immunodiffusion of unlabeled viral gs antigen [2] and a mixture of unlabeled and [^{14}C]amino-acid-labeled AMV protein that is synthesized *in vitro* [1] against rabbit antiserum A 912 to gs AMV antigen [gs S]. *a*, photography; *b*, autoradiography. (*c* and *d*) Control experiment with ferritin [4] and ferritin mixed with ^{14}C-labeled virus protein [3] *against* rabbit antiserum to ferritin [FeS]. As positive control, gs antigen was allowed to react with antiserum to gs antigen; unlabeled plus ^{14}C-labeled AMV protein [1]; unlabeled protein [2]. *c*, photography; *d*, autoradiography.

labeled virion proteins and reacted by immunodiffusion. The immunoprecipitation bands were photographed, and then the immunodiffusion plate was treated for autoradiography. Fig. 2*a* and *b* show the photography and the autoradiography of the same set, and it is quite clear that radioactive material is coprecipitated with unlabeled virion antigen. This represents gs antigen 4 and, possibly, antigen 3 (18), which is, however, not separated from it. To exclude the possibility that ^{14}C may be bound unspecifically in antigen–antibody precipitates, several radioactive materials were tested in heterologous antigen–antibody reactions. As an example of these control experiments, it is shown that proteins of AMV that are synthesized *in vitro* were not coprecipitated in a ferritin–antibody complex (Fig. 2*c* and *d*). Proteins of *E. coli* and phage M12 that are synthesized *in vitro* gave the same negative results in immunoprecipitation tests with gs antigen and ferritin.

DISCUSSION

The analysis of the products synthesized by a cell-free lysate of *E. coli* under the direction of AMV–RNA shows that at least four proteins that are synthesized *in vitro* correspond to proteins isolated from the virus particle with respect to molecular weight. By immunological techniques, it could further be demonstrated that one of these proteins synthesized *in vitro* is identical to gs-4 antigen present in the virion. From these facts, it can be concluded that the viral RNA has messenger properties. Yet, the results do not completely exclude the possibility that the complementary strand can serve as a messenger for some virus-specific proteins. However, if we accept Temin's hypothesis of the participation of a DNA-intermediate in AMV–RNA replication, one would have to postulate a symmetrical transcription of AMV–DNA, which is rather unlikely.

We hope, by using other specific antibodies or by studying the several subunits of AMV–RNA (21, 22), to characterize each of the as yet unidentified *in vitro* products or other proteins that are possibly encoded in the viral RNA. In particular, it would be of interest to establish whether cell-surface antigens that are tumor specific (23, 24) are encoded in the virus RNA or are only induced by the virus.

Our results reinforce previous reports that RNA from mammalian and plant viruses can be translated in a cell-free extract from *E. coli* (25–34), a result that strongly favors the universality of the genetic code. Such a close correspondence between the proteins synthesized in animal cells and those synthesized in an *E. coli* lysate must imply that the sense codons of the two systems are largely identical. No clear indication is given whether the different punctuation marks (initiation and termination sites, etc.) are recognized with the same efficiency. The weak stimulation of amino-acid incorporation obtained with AMV–RNA in comparison to that obtained with homologous phage M12-RNA suggests that this is not the case.

For the moment, however, one cannot exclude the possibility that, under our conditions, the secondary and tertiary structure of the RNA interferes with its messenger function. When high molecular weight RNA is denatured before addition to the system, the stimulation of amino-acid incorporation increased. In this connection, it is also of interest to mention again that the relation of the products 1–4 that are synthesized *in vitro* to each other is almost identical with that of the corresponding virion proteins. This points to the conclusion that at least some regulatory signals are recognized with the same degree of specificity.

We thank Drs. J. W. Beard and M. Chirigos for the generous gift of AMV, P. K. Vogt for his interest, and the Deutsche Forschungsgemeinschaft (SFB 51) for financial support. W. S. thanks the Stiftung Volkswagen-Werk for a fellowship.

1. Temin, H. M. (1964) *Virology* **23,** 486–494.
2. Temin, H. M. & Mizutani, S. (1970) *Nature* **226,** 1211–1213.
3. Baltimore, D. (1970) *Nature* **226,** 1209–1211.
4. Payne, L. N. & Chubb, R. C. (1968) *J. Gen. Virol.* **3,** 379–391.
5. Weiss, R. & Payne, L. N. (1971) *Virology* **45,** 508–515.
6. Hanafusa, T., Hanafusa, H. & Miyamoto, T. *Proc. Nat. Acad. Sci. USA* **67,** 1797–1803.
7. Weiss, R., Friss, R. R., Katz, E. & Vogt, P. K. (1971) *Virology* **46,** 920–938.
8. Bentvelzen, P. (1968) Genetical Control of the Vertical Transmission of the Mühlbock Mammary Tumour Virus in the GR Mouse Strain (Hollandia Publ., Amsterdam).
9. Biswal, N. & Benyesh-Melnick, M. (1969) *Proc. Nat. Acad. Sci. USA* **64,** 1372–1379.
10. Watson, K. F. & Beaudreau, G. S. (1969) *Biochem. Biophys. Res. Commun.* **37,** 925–932.
11. Bauer, H. (1970) *Zentralbl. Veterinaermed., Reihe B* **17,** 582–630.
12. Konings, R. N. H., Ward, R., Francke, B. & Hofschneider, P. H. (1970) *Nature* **226,** 604–607.
13. Kirby, K. S. (1965) *Biochem. J.* **96,** 266–269.
14. Bauer, H. (1966) *Z. Naturforsch.* **21b,** 453–460.
15. Erikson, R. L. (1969) *Virology* **37,** 124–131.

16. Ward, R., Konings, R. N. H., & Hofschneider, P. H. (1970) *Eur. J. Biochem.* **17**, 106–115.
17. Bolognesi, D. P., Bauer, H., Gelderblom, H. & Hüper, G., *Virology*, in press.
18. Bauer, H. & Bolognesi, D. P. (1970), *Virology* **42**, 1113–1126.
19. Bolognesi, D. P. & Bauer, H. (1970) *Virology* **42**, 1097–1112.
20. Duesberg, P. H., Martin, G. S. & Vogt, P. K. (1970) *Virology* **41**, 631–646.
21. Duesberg, P. H. (1968) *Proc. Nat. Acad. Sci. USA* **60**, 1511–1518.
22. Duesberg, P. H. & Vogt, P. K., *Proc. Nat. Acad. Sci. USA* **67**, 1673-1680.
23. Gelderblom, H., Bauer, H. & Graf, Th., *Virology*, in press.
24. Kurth, R & Bauer, H., *Virology*, in press.
25. Olson, K. C., Deeney, A. O. C., & Beaudreau, G. S. (1968) *Biochim. Biophys. Acta* **161**, 532–547.
26. Clark, I. M., Chang, A. Y., Spiegelman, S. & Reichmann, M. E. (1965) *Biochemistry* **54**, 1193–1197.
27. Warner, I., Madden, M. I. & Danell, I. E. (1963) *Virology* **19**, 393–399.
28. Tsugita, A., Fraenkel-Conrat, H., Nirenberg, M. W. & Matthaei, I. H. (1962) *Biochemistry* **48**, 846–853.
29. Rekosh, D. M., Lodish, H. F. & Baltimore, D. (1970) *J. Mol. Biol.* **54**, 327–340.
30. Rekosh, D. M., Lodish, H. F. & Baltimore, D. (1969) *Cold Spring Harbor Symp. Quant. Biol.* **34**, 747–751.
31. Říman, J., Trávníček, M. & Vepřek, L. (1967) *Biochim. Biophys. Acta* **138**, 204–207.
32. Viñuela, E., Algranati, I. D. & Ochoa, S. (1967) *Eur. J. Biochem.* **1**, 3–11.
33. Laycock, D. G. & Hunt, I. A. (1969) *Nature* **221**, 1118–1122.
34. Van Ravenswaay-Claasen, I. C., Van Leeuwen, A. B. I., Duijts, G. A. H. & Bosch, L. (1967) *J. Mol. Biol.* **23**, 535–544.