Identification of a Large Polypeptide Precursor of Avian Oncornavirus Proteins
(proteolytic cleavage/pulse chase/antibody/tryptic fingerprint)

VOLKER M. VOGT AND ROBERT EISENMAN

Swiss Institute for Experimental Cancer Research, 1011 Lausanne, Switzerland

Communicated by J. D. Watson, April 2, 1973

ABSTRACT Antibody to partially disrupted avian myeloblastosis virus was used to selectively precipitate newly synthesized intracellular viral polypeptides from extracts of infected chicken cells. When analyzed by sodium dodecyl sulfate-gel electrophoresis, immune precipitates from extracts of cells pulse-labeled for 10 min with $^{35}S$-methionine contain none of the major virion polypeptides. Instead they show prominent viral specific polypeptides of molecular weight 76,000 and 12,000, as well as minor quantities of other labeled polypeptides. From pulse-chase kinetics and two-dimensional tryptic fingerprints it appears that the large polypeptide is a precursor of at least the two major virion proteins of molecular weights 24,000 and 11,000, while the smaller is a precursor of the 11,000-dalton virion protein.

The oncornaviruses, or RNA tumor viruses, consist of single-stranded RNA encapsidated by four to eight major proteins (1, 2, 2a) and a lipid-containing coat. In the avian oncorna-viruses, two of the proteins account for 60% of the total amino acid in virions. These two proteins (2), and perhaps two or three lesser ones, are the so-called group-specific antigens that carry determinants common to all avian oncornaviruses (3). Two or three additional proteins with carbohydrate moieties (1, 2, 4) are found on the surface of the virus particles (5) and appear to carry the type specificity (4).

Little is known about the synthesis of oncornavirus proteins. Cells infected with these viruses continue to grow, devoting only a small percentage of cellular protein synthesis to viral proteins. Thus, an investigation of viral-specific translation requires a specific probe. We have prepared a rabbit antiserum to disrupted avian myeloblastosis virus (AMV) and have used this antiserum to selectively precipitate intracellular viral polypeptides from extracts of infected cells labeled with amino acids. From the analysis of these immune precipitates, we conclude that at least the two most numerous AMV proteins are synthesized together as a single large precursor polypeptide.

MATERIALS AND METHODS

Growth and Labeling of Cells and Virus. Primary fibroblasts from 11-day leukemia-free chick embryos (Villejuif, Paris) were infected in suspension by incubation of 50 μl of leukemic chicken serum (approximate titer 10$^{13}$ AMV particles per ml) with 10$^6$ cells in 1 ml of Dulbecco's modified Eagle's medium containing 10% calf serum. After 45 min at 37°C, the cells were diluted with the same medium and serum and seeded onto 10-cm plastic dishes. On the third to fifth day after seeding, cells were washed twice with 5 ml of warm phosphate-buffered saline (pH 7.4) and then incubated for 2 hr with modified Eagle's medium containing 5% dialyzed calf serum and 0.02 mM unlabeled or $^3$H-labeled methionine (50 μCi/ml). Subsequent to this long-term labeling, the cell monolayer was washed as before and then incubated for 10 min at 37°C with 1.5 ml of Earle's saline (6) containing $^{35}S$-methionine (about 100 Ci/mmol) at 30 μCi/ml, or 500 μCi/ml for preparation of precursor for fingerprinting. The cells were either lysed immediately or, for chase experiments, washed with warm saline, incubated one hour at 37°C with normal culture medium, and then lysed. The lysis procedure consisted of transferring each plate onto ice, washing with ice-cold phosphate-buffered saline, and then adding ice-cold 0.02 M Tris-HCl (pH 7.5)-0.05 M NaCl, containing either 0.5% of the nonionic detergent NP-40 (Shell Oil Co.) or 0.5% NP-40 plus 0.5% sodium deoxycholate. The resulting lysate, scraped from each plate into a tube, was vortexed for 30 sec and then centrifuged for 10 min at 10,000 × g. The supernatant, or cell cytoplasm, contained about two-thirds of the incorporated radioactivity.

$^3$H-Methionine- and $^{35}S$-methionine-labeled AMV were prepared by incubation of cells washed with phosphate-buffered saline with 100 μCi/ml of undiluted radioactive amino acid in methionine-free Eagle's medium with 5% dialyzed calf serum. After 2-4 hr, the labeled medium was replaced with normal medium containing unlabeled methionine. This procedure was repeated once a day for several days, with the labeled medium being used again each time, until at least three-quarters of the label had been incorporated. The spent labeled and unlabeled media were then pooled, and the labeled virus was purified by banding to equilibrium in sucrose gradients (2). For preparation of individual virion polypeptides, the purified virus was dialyzed against ammonium carbonate (pH 8.5), lyophilized, redissolved in buffer containing dodecyl sulfate, and electrophoresed as described below.

Immune Precipitation and Gel Electrophoresis. To the pulse-labeled cytoplasm or to purified labeled virus in 1 ml of the lysis buffer were added 5 μg of unlabeled AMV plus 0.1 or 0.2 ml of anti-AMV rabbit serum. The antiserum had been prepared by repeated bimonthly injections of 1 mg of NP-40-disrupted AMV mixed with complete Freund's adjuvant (1:1). The immune precipitate formed in overnight incubation at 4°C was collected by centrifugation for 10 min at 1000 × g. It was washed by resuspending and pelleting in NP-40 lysis buffer twice more and then dissolved in electrophoresis buffer (7) containing 5% sodium dodecyl sulfate. abbreviation: AMV, avian myeloblastosis virus.
and 1% 2-mercaptoethanol. After brief heating at 100°, a 25-μl aliquot of each sample was then layered into one of the wells of a 15% discontinuous polyacrylamide slab gel (8). Parallel samples were separated by at least one empty well. After electrophoresis the slab gel was stained at acid pH with 0.25% Coomassie Blue, destained by diffusion, and soaked for 1 hr in water to reduce acidity. Then for each sample the longitudinal section of the gel marked by the stained protein bands was cut out and sliced into 1-mm strips. These were soaked for 1 day in 0.1 M ammonium carbonate plus 0.1% sodium dodecyl sulfate to elute the labeled proteins. Radioactivity was then counted after addition of water-solubilizing scintillation fluid.

Tryptic Fingerprinting. Slab gels of immune precipitates from cells labeled with 500 μCi/ml of [3H]methionine were prepared and cut as above, but only an aliquot from the eluate from each slice was counted to localize the precursor proteins. Labeled polypeptides from whole virions were prepared similarly but without antibody precipitation. After the addition of 100 μg of bovine-serum albumin as carrier, the eluted proteins were prepared for digestion by precipitation with trichloroacetic acid followed by oxidation in performic acid (9). The oxidized proteins were then digested with 5 μg of TPCK trypsin ( Worthington) in 0.1 ml of 0.05 M ammonium bicarbonate (pH 8.5). After 1 hr at 37°, a second 5 μg of trypsin was added, and an hour later the digestion was terminated by lyophilization. Recovery to this stage was about 50% of starting radioactivity. The dried tryptic digests were taken up in electrophoresis buffer [0.5% pyridine-5% acetic acid (pH 3.5)], centrifuged briefly to remove insoluble material, and then applied to 20-cm × 40-cm thin-

![Fig. 1. Gel electrophoresis of immune-precipitated AMV.](image1)

![Fig. 2. Gel electrophoresis of immune precipitates from pulse-labeled and pulse-chase-labeled cells lysed with NP-40.](image2)

RESULTS

Rabbit antibody to AMV disrupted with NP-40 detergent precipitates at least 80% of [3H]methionine-labeled disrupted virus. In our hands, the two most highly labeled viral polypeptides (molecular weights 24,000 and 11,000, constituting about 65% of total [3H]methionine-labeled AMV) are consistently precipitated in totality, while the polypeptides of molecular weights 12,000 and 19,000 are precipitated in variable amounts. The 10 or more minor polypeptides visible in stained or autoradiographed gels, appear to be precipitated poorly by the antibody. These results are summarized in Fig. 1, which shows that total [3H]methionine-labeled virion proteins and unlabeled marker polypeptides coelectrophoresed with antibody-precipitated [3H]methionine-virion proteins. The positions of the markers as well as the positions of the chief virion glycoproteins (4), labeled with glucosamine and electrophoresed in a parallel gel section, are indicated. A stained gel is also depicted in the upper part of the figure. Viral protein 3, which can be seen to be stained with Coomassie Blue nearly as heavily as protein 4, is barely visible in the radioactive profile as a shoulder to protein 4, presumably because it is poor in methionine.

If AMV-infected chick cells are exposed to radioactive methionine for 16 hr, 1.5% of the incorporated cytoplasmic label can be recovered as viral polypeptides from virus particles released into the growth medium. After a 2-hr labeling period, about 1% of the incorporated methionine is in released virions. Another 0.5% can be recovered as intracellular virion polypeptides by antibody precipitation followed by gel electrophoresis. For labeling times shorter than 1 hr, however, the recovery of viral polypeptides drops sharply. After a
Fig. 3. Gel electrophoresis of immune precipitate from pulse-labeled cells lysed with deoxycholate plus NP-40. Experimental conditions were similar to those described in Fig. 2, except that the $^3$H prior labeling was omitted and 15-times as much $^{35}$S-methionine was used in the pulse. The cells were lysed with NP-40 plus deoxycholate and the cytoplasm was precipitated with antiserum as described. An aliquot of the immune precipitate dissolved in dodecyl sulfate was electrophoresed together with $[^3H]$methionine-labeled total AMV. The numbers and letters are the same as in Fig. 1. ——, $^{35}$S; ———,$^3$H.

Pulse of 10 min, no extracellular labeled virus is detectable, and antibody precipitates of cytoplasmic extracts reveal almost no label at the characteristic mobilities of the major viral poly-peptides. However, several new polypeptides of higher molecular weight appear. This result is depicted in Fig. 2. Cells were labeled for 2 hr with $[^3H]$methionine to mark the stable intracellular proteins precipitated by the antiserum. A subsequent pulse of $^{35}$S-methionine was then followed by rapid lysis in NP-40 buffer, immune precipitation of the cytoplasm, and gel electrophoresis.

Panel A in the figure shows that the antibody has precipitated the $^3$H-labeled intracellular viral poly-peptides 1, 2, and 4, as well as a $[^3H]$poly-peptide (H) of molecular weight 50,000 that is not found in substantial amounts in virions. The $^{35}$S-pulse label from the same cells is resolved into two sharp peaks (P) at molecular weights 76,000 and 12,000, with several smaller peaks, but very little label in viral polypeptides 1 and 4. The difference in the pattern of $^3$H and $^{35}$S indicates that the $^{35}$S peaks are metabolically unstable. This conclusion is supported by panel B, which shows the results of a 1-hr chase after the 10-min $^{35}$S pulse. The sulfur label now follows the tritium profile closely, with only small residual peaks at molecular weights 76,000 and 12,000. Panel C illustrates the result of pulse labeling uninfected cells exactly as in panel A. From a comparison of these two graphs we conclude that the metabolically stable protein H is of host origin. The immune precipitate from uninfected cells also reveals a small $^3$H peak with the same mobility as viral polypeptide 1. We have observed this peak frequently in uninfected-cell extracts and tentatively identified it with the viral group-specific antigen that has been reported in small amounts even in cells not demonstrating other signs of infection (10, 11).

The fact that an antiviral serum precipitates unstable proteins suggests that these proteins are precursors to virion polypeptides. The 12,000-dalton protein could contain the sequence of viral protein 3 with which it comigrates, or protein 4, which is 1000 daltons smaller. The latter possibility seems more likely since nearly as much label is chased out of this unstable protein as that which enters protein 4. After the chase in the experiment described in Fig. 2, 1000 cpm each were recovered in intracellular protein 4 (Fig. 2B) and in protein 4 in extracellular virus particles (not shown). This compares with 1500 cpm in the pulse-labeled precursor.

The 76,000-dalton protein is large enough to contain the sequences of all the viral proteins 1-4. However, in experiments like that described in Fig. 2, generally only 0.05-0.2% of total incorporated cytoplasmic pulse label can be recovered in this protein. This is 8- to 30-times lower than the percentage of viral poly-peptides in virions after long-term labeling periods. Even a summation of all the radioactivity in viral-specific pulse-labeled proteins visible on gels yields a per-

Fig. 4. Tryptic fingerprinting of large precursor and total AMV. The $^{35}$S-labeled 76,000-dalton precursor protein recovered from a dodecyl sulfate gel and total $^{35}$S-labeled AMV were digested with trypsin and then electrophoresed on separate cellulose layers. The sheets were dried, chromatographed together, and then autoradiographed for 4 weeks. Electrophoresis, bottom to top; chromatography, left to right. A, total AMV; B, 76,000-dalton precursor.

Fig. 5. Tryptic fingerprinting of smaller precursor and virion protein 4. The $[^3S]$methionine-labeled 12,000-dalton precursor protein and the 11,000-dalton virion protein 4, both recovered from dodecyl sulfate gels, were digested and fingerprinted as described in Fig. 4. A, virion protein 4; B, 12,000-dalton precursor.
percentage of viral-specific synthesis much lower than that given by long-term labeling. Thus, if the 76,000-dalton protein is indeed a viral precursor, much of it must be lost at some stage in the preparation. One possibility for such loss is the preliminary centrifugation that removes nuclei, mitochondria, and particulate debris. Alternatively, the precursor protein could simply be less efficiently precipitated by the antibody, or it could be degraded nonspecifically in the overnight incubation with antiserum.

In an attempt to find conditions for improved recovery of the precursor protein, we treated cells with stronger detergent mixtures before immune precipitation of the cytoplasm. Fig. 3 illustrates the electrophoretic profile of such an experiment. Total [3H]methionine-labeled AMV proteins were co-electrophoresed as a marker with pulse-labeled, antibody-precipitated proteins from cells lysed with 0.5% NP-40 plus 0.5% deoxycholate. This lysis method reproducibly yields 0.25-1.0% of cytoplasmic label as the 76,000-dalton precursor protein, a significantly higher recovery than that given by NP-40 lysis. Other features of the gel profile also differ from those in Fig. 2A. Deoxycholate has reduced the peaks subsidiary to the 76,000-dalton precursor, including the host peak H and the unstable 12,000-dalton peak. In several experiments with NP-40-deoxycholate mixtures, we have frequently also found a pulse-labeled protein slightly smaller than virion protein 1. Fig. 3 shows only a very small peak at this position.

From the results portrayed in Figs. 2 and 3 and from similar experiments, we can identify five unstable proteins specific to AMV-infected cells: a major protein of molecular weight 76,000 with two minor ones of molecular weights about 66,000 and 55,000, a protein with variable yield of molecular weight 23,000, and a protein of molecular weight 12,000 seen in large amounts only in cells lysed with NP-40. The fact that conditions increasing the yield of the 76,000-dalton precursor decrease that of the other labile polypeptides suggests that these latter polypeptides are all secondary products generated by proteolytic cleavage. Antiserum to AMV also precipitates an unstable protein, of molecular weight 70,000-80,000, from extracts of cells infected and transformed by Rous sarcoma virus (data not shown) that contains structural proteins antigenically closely related to those of AMV (3).

To prove that the unstable 76,000-dalton AMV protein is indeed a precursor of viral polypeptides, we recovered this protein from sodium dodecyl sulfate gels and prepared tryptic fingerprints of it. Fig. 4 compares such a fingerprint and one made of total AMV. After elution from the polyacrylamide gel, the [35S]methionine-labeled precursor shown in Fig. 3 was oxidized and digested with trypsin. Oxidized total AMV was digested in parallel. The resulting tryptic peptides were separated on cellulose thin layers in two dimensions, and those peptides containing methionine were then visualized by autoradiography. Comparison of the two patterns reveals that they are nearly identical. 10 of the 12 spots characteristic of total AMV also appear in the precursor fingerprint. Since 65% of the [35S]methionine label in AMV is in virion proteins 1 and 4, the similarity of these fingerprints implies that the amino-acid sequences of at least these two major proteins are included in the large precursor.

Tryptic peptide maps also demonstrate that the 12,000-dalton precursor seen most prominently in Fig. 2 is related to a virion protein. In Fig. 5 the fingerprint of this labile protein is compared with that of virion protein 4. Both polypeptides were recovered from sodium dodecyl sulfate gels, digested, and then electrophoresed and chromatographed. Of the seven main spots visible in the pattern of the precursor, five appear identical in the virion protein. The fingerprint of the virion protein shows one very dark spot, which is absent in the precursor. The similarity of patterns, in addition to the pulse-chase kinetics, indicates that virion protein 4 is thus probably clipped from the 12,000-dalton precursor.

**DISCUSSION**

The technique of immune precipitation has enabled us to isolate intracellular proteins of avian oncornavirus-infected cells and thus to study viral-specific translation. Shanmugan et al. (12) have also used this method to recover labeled intracellular viral proteins from cells infected with murine leukemia virus. We have demonstrated that antiserum to disrupted AMV precipitates virion polypeptides from extracts of cells labeled for long periods. However, from extracts of pulse-labeled cells, the antibody precipitates chiefly a large polypeptide of molecular weight 76,000 that is not found in virions. Kinetic studies and tryptic peptide mapping establish that this polypeptide contains the sequences of at least the two major methionine-labeled virion proteins 1 and 4, and thus that it is a precursor to them. This precursor may include the sequences of other viral proteins as well, since less than half of its molecular weight is taken up by the two major proteins.

Present evidence suggests that the large precursor is cleaved first into intermediate-sized pieces, which in turn are processed into virion proteins. Whatever the exact nature of the processing scheme, the existence of an unstable polypeptide containing the sequences of more than one viral protein raises the possibility that the primary viral translational product is a still larger polypeptide, perhaps representing an entire 30S oncornavirus RNA subunit. Such a polypeptide would have a molecular weight of 200,000-300,000. The RNA genome of the two best studied picornaviruses, polio and encephalomyocarditis virus, are translated in this manner as single polypeptide chains (13, 14). If there is a larger oncornavirus precursor, however, it may be difficult to detect. Antibody to disrupted virions may not be able to recognize such a molecule, or, like the largest poliovirus polypeptide, it may be cleaved already in the nascent state. The poliovirus polypeptide equivalent to the full genome can be recovered only if proteolytic activity is inhibited in the presence of amino-acid analogues (15). The two analogues most effective in inhibiting polio processing, fluorophenylalanine and canavanine, have no effect on the electrophoretic pattern of antibody precipitates from AMV-infected cells (data not shown).

Both 70S RNA and its constituent 30S subunits from AMV have been reported to be translated by an Escherichia coli protein-synthesizing system in vitro (16, 17). The products of this reaction are heterogeneous, but the largest labeled peaks in dodecyl sulfate-gel electrophoresis coincide with virion polypeptides 1-4. The in vitro-synthesized protein corresponding to polypeptide 1 is also antigenically similar to that protein. These results imply that virion RNA is the messenger for virion proteins. Since we have demonstrated that in vitro the virion polypeptides are not the primary translational products of the RNA, the in vitro synthesis of AMV proteins implies further that bacterial extracts contain a pro-
teolytic activity very similar to that of infected chicken cells. This conclusion is surprising, since bacterial proteins known to be degraded with short half lives in vitro are completely stable in crude extracts in vitro (18, 19).

Since both cells infected by Rous sarcoma virus and AMV, a leukemia virus, exhibit a polypeptide precursor, probably other avian RNA tumor viruses behave similarly. Because of their close structural and biological similarity, we surmise that mammalian and reptilian oncornaviruses will also synthesize some of their proteins as larger precursor molecules.

Indeed, proteolytic cleavage appears to be a general phenomenon for animal RNA viruses. In addition to the picornaviruses and oncornaviruses, arboviruses (20, 20a) have also been reported to synthesize precursor polypeptides. The evolutionary significance of proteolytic cleavage may be, as suggested by Baltimore (21), that mammalian and avian cells are unable to initiate the translation of separate cistrons on a single messenger. Thus, for viruses whose genomes consist of RNA molecules coding for more than one protein, it may have been simpler to evolve a mechanism for cutting a precursor protein at specific sites than to evolve a mechanism for replicating viral RNA in two pools, one with large molecules to be encapsidated, and one with smaller ones to serve as messengers. At least for oncornaviruses, however, it is also possible that, as in certain bacteriophages (7, 22), proteolytic cleavage is a step in virus maturation. Or, perhaps, the existence of polypeptide precursors simply reflects the advantages of coordinate control of the synthesis of virion proteins.

We thank Heidi Diggelmann and Bernhard Hirt for encouragement and criticism, and Tom McPherson, Robert Braschler, and Bernhard Hirt for expert technical assistance. We are also grateful for a gift of leukemic chicken plasma from J. W. Beard and the N.C.I. This work was supported by postdoctoral fellowships from the American Cancer Society (V.M.V.) and from the Damon Runyon Foundation (R.E.), and by Grant 3.412.70 from the Fonds National Suisse.
