JOURNAL OF VIROLOGY, Oct. 1981, p. 107-117
0022-538X/81/100107-11$02.00/0

Vol. 40, No. 1

In Vitro Translation of Avian Myeloblastosis Virus RNA

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Received 30 March 1981/Accepted 10 June 1981

Avian myeloblastosis virus (AMV)-infected cells contain two viral mRNA's, a genome-sized 34S (7.5-kilobase) mRNA and a 21S (2.5-kilobase) subgenomic mRNA, which contains the AMV-specific sequences (*myb* sequences). We found that AMV virions packaged both the 7.5-kilobase full-length genomic RNA and the 2.5-kilobase subgenomic RNA. In vitro translation of AMV virion RNA sized by sucrose density gradient centrifugation yielded 76,000-, 56,000-, 48,500-, 47,000-, and 32,000-dalton products. The 76,000-dalton protein was coded for by RNA throughout the gradient, but the peak of activity was at 34S to 35S. The 56,000-, 48,500-, and 32,000-dalton proteins were encoded in a 21S RNA, and the 47,000-dalton protein was encoded in an RNA of approximately 24S. The 76,000-dalton protein was identified as Pr76^{gag}, based upon immunoprecipitation with specific antiserum and the presence of the 19* dipeptide. 7-Methylguanosine triphosphate inhibited the syntheses of Pr76^{gag} and the 56,000-, 48,500-, and 32,000-dalton proteins, but not the synthesis of the 47,000-dalton protein. The 56,000-, 48,500-, 47,000-, and 32,000-dalton proteins were not immunoprecipitated by anti-*gag*, anti-reverse transcriptase, or anti-gp85 antiserum. Two-dimensional peptide maps of the 56,000- and 48,500-dalton proteins indicated that they were unique. In vitro translational products of myeloblastosis-associated virus 1 were also analyzed to aid in the identification of the AMV *myb* gene product(s); the translational products analyzed included Pr76^{gag}, p60^{env}, and a 56,000-dalton polypeptide which apparently was not identical to the 56,000-dalton AMV translational product, as determined by two-dimensional peptide mapping. Our data indicated that one of these proteins (56,000, 48,500, or 32,000 daltons) may represent the product of the AMV *myb* gene and, therefore, the putative transforming protein(s) of AMV.

Avian myeloblastosis virus (AMV) is one of the avian acute leukemia viruses. These viruses have been divided into three groups based upon the pathological pictures that they produce (18), the markers that are present on the surfaces of transformed hematopoietic cells (5), hybridization with specific cDNA probes (43), and RNase T_1 oligonucleotide mapping (6, 11, 12). The AMV group includes two virus isolates, AMV and E26, which share sequence homology based upon cDNA hybridization (43). Unlike other avian acute leukemia viruses, which can transform both fibroblasts and hematopoietic cells (5, 16-21), AMV is only able to transform hematopoietic cells (16, 32-35).

AMV is defective (32), requiring the presence of a helper virus to produce infectious virus. Clones of AMV-transformed myeloblasts which do not release infectious virus can be isolated (32). However, such nonproducer clones do release noninfectious virus particles that contain AMV viral RNA (9, 11). Infectious progeny virus can be rescued from these cells after superinfection with a helper virus. The envelope specificity of the rescued virus is determined by the specificity of the helper virus (32). Two helper viruses, one belonging to subgroup A (myeloblastosis-associated virus 1 [MAV-1]) and one belonging to subgroup B (MAV-2), have been isolated from a stock of the standard strain of AMV (46).

A recent analysis of the RNA of AMV by sucrose density gradient sedimentation and glyoxal gel electrophoresis indicated that the size of AMV genomic RNA is 33S to 34S or 7.5 kilobases (kb) (9, 15). Analyses of unintegrated proviral DNA (3, 49) and of a molecularly cloned AMV proviral DNA sequence (48) supported these size measurements.

AMV contains a unique sequence that presumably represents the transforming gene(s) or *onc* gene of AMV (10, 11, 43), which is not related to the *src* gene of Rous sarcoma virus (10, 11, 43, 52, 53) or to the unique sequences of other acute leukemia viruses (11, 43). This sequence (designated *myb*) has been defined both

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by the preparation of AMV-specific cDNA (10, 43) and by AMV-specific RNase T_1-resistant oligonucleotides (11). The *myb* gene is highly conserved among higher vertebrates (43). It is expressed in normal tissue (8, 15), but its level of expression varies with both the tissue examined and the age of the chicken (8). The *myb* sequences are located at the 3′ end of AMV RNA, as determined by ordering of the RNase T_1-resistant oligonucleotides (11) and by heteroduplex mapping (50).

Based upon hybridization with cDNA probes specific for the viral structural protein gene *gag*, the reverse transcriptase gene *pol*, and the envelope glycoprotein gene *env*, AMV has been found to contain most if not all of the *gag* and *pol* genes but none of the *env* gene (9, 15).

AMV-infected myeloblasts contain a 7.5-kb genome length mRNA and a 2.5-kb subgenomic mRNA (9, 15). The subgenomic mRNA contains sequences homologous to the strong stop region, the c region, and all of the *myb*-specific sequences (9, 15).

In this study we sought to identify the translational products of AMV present in AMV-infected myeloblasts and to compare these products with those produced by in vitro translation of viral RNA. The appearance of novel peptides encoded by AMV RNA should be helpful in identifying the *myb* protein(s) of AMV. Specifically, translation of the AMV subgenomic mRNA should yield the *myb* gene product(s).

MATERIALS AND METHODS

Cells. Chicken embryo fibroblasts and yolk sac cells were prepared as previously described (22, 23) from gs^-chf^- embryos obtained from either SPAFAS, Norwich, Conn., or Life Sciences, Inc., St. Petersburg, Fla. Chicken embryo fibroblasts were grown in either F10 medium containing 5% calf serum, 10% tryptose phosphate broth, 10 U of penicillin per ml, and 0.5 mg of streptomycin per ml or Scherer medium containing the same additions. AMV-transformed myeloblasts were grown in either BT-88 medium (33) or RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) containing 5% fetal calf serum, 5% heat-inactivated chick serum, 10% tryptose phosphate broth, 1% 100× BME vitamins (Flow Laboratories, Inc., Rockville, Md.), 0.008 mg of folic acid per ml, 0.5% dimethyl sulfoxide, 10 U of penicillin per ml, and 0.5 mg of streptomycin per ml. AMV nonproducer clones were isolated from soft agar as described recently by Moscovici and Moscovici (35).

Viruses. Plasma from leukemic chickens infected with AMV subgroup A (AMV-A) or AMV-B was obtained from Joseph Beard, Life Sciences Inc. Cloned stocks of AMV were a gift from Carlo Moscovici, Veterans Administration Medical Center, University of Florida, Gainesville. MAV-1 and MAV-2 are the subgroup A and B helper viruses of AMV, respectively. These helper viruses do not transform chicken embryo fibroblasts or chicken yolk sac cells and were isolated by endpoint dilution.

To obtain AMV preparations with a high ratio of transforming virus to helper virus, virus was prepared in chickens (8); forty to sixty 1-day-old chicks were injected intravenously with AMV-A or AMV-B. Plasmas from chickens containing 3×10^8 to 5×10^8 myeloblasts per ml of blood were pooled and used for virus purification and subsequent viral RNA extraction.

Preparation and sizing of viral RNA. Viral RNA was extracted from AMV-containing plasma or culture fluids of MAV-1-infected chicken embryo fibroblasts as described previously (8). Polyadenylic acid [poly(A)]-containing RNA was selected by chromatography on oligodeoxythymidylic acid cellulose (Collaborative Research, Inc., Waltham, Mass.) (10). The poly(A)-containing RNA was dissolved in 0.2 to 0.4 ml of a solution containing 0.01 M Tris (pH 7.4), 0.001 M EDTA, 0.02 M LiCl, and 0.2% sodium dodecyl sulfate (SDS), heated at 85°C for 1.5 min, and layered onto a 15 to 30% linear sucrose gradient prepared in the same buffer. The gradient was centrifuged at 40,000 rpm for 7.5 h at 24°C in a Beckman SW41 rotor. Fractions (0.3 ml) were collected from the bottom of the gradient with a peristaltic pump. The NaCl concentration was adjusted to 0.2 M, and the RNA in each fraction was precipitated with 2 volumes of ethanol.

Preparation of cDNA probes. [^{32}P]DNA complementary to MAV-1 and to the unique sequences of AMV was synthesized and selected by methods described previously for [^{3}H]cDNA (10), with the exception that actinomycin D was omitted from the reaction mixture.

Gel electrophoresis of viral RNA and transfer to diazobenzyloxymethyl paper. Poly(A)-containing viral RNA was precipitated twice with ethanol in a siliconized microfuge tube, dissolved in 5 μl of glyoxal buffer (30), and heated at 50°C for 1 h. A 3-μl amount of tracking dye was added, and the RNA was subjected to electrophoresis in a 1% agarose gel at 6 V/cm until the dye front migrated 20 cm. Diazobenzyloxymethyl paper was prepared by the methods of Alwine et al. (1) and Wahl et al. (55).

The filter was incubated in a prehybrid mixture containing 50% formamide, 5× SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt solution (5× Denhardt solution contains 0.1% bovine serum albumin, 0.1% polyvinylpyrolidone, and 0.1% Ficoll [55]), 500 μg of sonicated, denatured calf thymus DNA per ml, and 0.1% SDS for 12 to 24 h at 41°C. Hybridization was carried out in a solution containing 50% formamide, 5× SSC, 1× Denhardt solution, 100 μg of calf thymus DNA per ml, and 6×10^5 to 10×10^5 cpm of [^{32}P]cDNA probe at 41°C for 48 h in a sealed plastic bag. Approximately 10,000 cpm of the desired probe per cm^2 was used.

After hybridization, the filter was washed once with 2× SSC containing 50% formamide and 0.1% SDS, twice with 2× SSC containing 0.1% SDS, and then twice with 2× SSC. All washes were carried out at 37°C for 30 min per wash.The filter was then blotted dry, and autoradiography was performed by using Du Pont Cronex 2DC film and a Du Pont Lightning Plus intensifying screen.

In vitro translation of viral RNAs. RNA to be translated was precipitated three times to remove any

SDS remaining from the sucrose gradient. In vitro translation was carried out by using the mRNA-dependent reticulocyte lysate system of Pelham and Jackson (39), as described previously (2). 7-Methylguanosine triphosphate (^{7m}GTP; PL Laboratories) was added to some translation reaction to a final concentration of 1 μM to inhibit translation of capped mRNA's.

Immunoprecipitation. The labeled products of in vitro translation were characterized by a sequential immunoprecipitation procedure; 85 μl of RIPA buffer (0.05 M Tris [pH 7.4], 0.15 M NaCl, 0.001 M EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing 1% Trasylol was added to a 13-μl reaction mixture, 5 μl of anti-p19 serum or anti-*gag* serum was added, and the mixture was incubated on ice for 1 h. A 15-μl amount of a 50% solution of protein A-Sepharose was added, and the preparation was mixed on a rotating platform for 1 h. The anti-*gag* immune complexes were pelleted by centrifugation with a Beckman microfuge. The supernatant fluid was removed and immunoprecipitated with rat anti-gp85 serum. This procedure was repeated once more with rabbit anti-reverse transcriptase. The immunoprecipitates were analyzed on 8.5% SDS–polyacrylamide gels (28).

Rabbit anti-*gag* antiserum that reacted with all of the *gag* proteins and rabbit anti-Rous-associated virus-60 gp85 serum were produced in our laboratory. Rabbit antiserum specific for the individual *gag* proteins was a gift from V. Vogt (Cornell University), and rabbit anti-AMV reverse transcriptase was from Life Sciences, Inc. The anti-*gag* protein reactivity in the anti-reverse transcriptase serum was removed by absorbing the antiserum with 12 μg of purified Rous-associated virus-2 virions disrupted in RIPA buffer per μl of antiserum, as described (13).

Two-dimensional tryptic peptide mapping. Tryptic peptide mapping was performed by a modification of a previously described procedure (41, 42). The desired gel bands were cut out, and the paper backing was removed. Before further processing, gels treated for fluorography with En^{3}Hance required the removal of the precipitated fluorographic agent. En^{3}Hance was removed by first swelling each gel band in 10% methanol for 30 min and then washing the gel band in 50% methanol–50% acetic acid four times for 30 min, after which time the gel band was clear. The gel band was then washed four times (30 min each) in 10% methanol to remove the acetic acid and lyophilized. Then, 0.2 to 0.4 ml of performic acid (a 19:1 mixture of formic acid and hydrogen peroxide kept at room temperature for 1 h) was added to each gel band, and the preparations were incubated on ice for 1 h to oxidize the proteins. The gel bands were lyophilized twice and incubated for 18 to 24 h at 37°C in 1.0 ml of 0.05 M ammonium bicarbonate buffer (pH 8.0) containing 50 μg of L-(tosylamido-2-phenyl)ethyl chloromethyl ketone-treated trypsin (252 U/mg; Worthington Biochemical, Freehold, N.J.). The supernatant fluids were removed and lyophilized twice. Tryptic peptides were analyzed in two dimensions on cellulose-coated thin-layer plates (20 by 20 cm; EM Laboratories, Elmsford, N.J.). Electrophoresis in the first dimension was for 130 min at 600 V in a pH 4.7 buffer containing n-butanol, pyridine, acetic acid, and water (2:1:1:18, vol/vol) at 4°C and was monitored by using 2% orange G and 1% acid fuchsin as marker dyes. The plates were dried at room temperature overnight, and ascending chromotography in the second dimension was carried out in *n*-butanol–pyridine–acetic acid–water (65:50:10:40). The dried plates were sprayed with En^{3}Hance spray (New England Nuclear Corp., Boston, Mass.) and were exposed to Du Pont Cronex 2DC film using Du Pont Lightning Plus intensifying screens for 3 to 14 days.

Assay for the presence of Ac-Met-Glu dipeptide. The presence of acetylmethionyl glutamic acid (Ac-Met-Glu), the characteristic amino-terminal dipeptide of p19 and Pr76^{gag}, was determined as previously described (42).

RESULTS

Presence of AMV-specific subgenomic mRNA within virions. A 21S AMV-specific subgenomic mRNA has been described recently (9, 15). This RNA contains the first 101 nucleotides present at the 5′ end of the viral genome (which is known as the strong stop region), the AMV *myb* sequences, and the c region (9, 15). Previous work with Rous-associated virus-2 21S *env* mRNA (38, 51) and the 21S avian erythroblastosis virus-specific subgenomic RNA (2) demonstrated that these RNAs could be incorporated into virions and could be translated efficiently both in vivo and in vitro. To determine whether the AMV subgenomic RNA was also packaged, poly(A)-containing AMV and MAV-1 virion RNAs were denatured with glyoxal and electrophoresed in a 1.0% agarose gel, and the RNAs were transferred to diazobenzyloxymethyl paper by the method of Alwine et al. (1). The blot was then hybridized with either $cDNA_{AMV}$ or $cDNA_{MAV\text{-}1}$. Figure 1 shows that a small amount of 20S to 21S AMV-specific subgenomic RNA was incorporated into AMV virions (Fig. 1, lane 2). With this preparation of MAV-1 virion RNA, there was very little 21S *env* mRNA incorporated into virions (Fig. 1, lane 3). Therefore, we used the AMV virion-associated 20S to 22S RNA to identify the translational products of this RNA species by in vitro translation.

In vitro translation of AMV virion RNA. AMV virion RNA was purified as described previously (8), and its size was determined with sucrose density gradients. The RNA from each sucrose gradient fraction was translated in vitro by using the rabbit reticulocyte lysate system. Major AMV translational products (Fig. 2A) were observed at 76,000, 56,000, 48,500, 47,000, 43,000, 41,000, 37,000, 35,000, 34,000, 32,000, and 31,000 daltons. Figure 2B shows the translational products of MAV-1 virion RNA that was prepared and measured by sucrose gradient sedimentation in the same manner as AMV RNA. Translational products of 76,000, 60,000, 56,000, 43,000, 41,000, 37,000, 35,000, 34,000, and 31,000

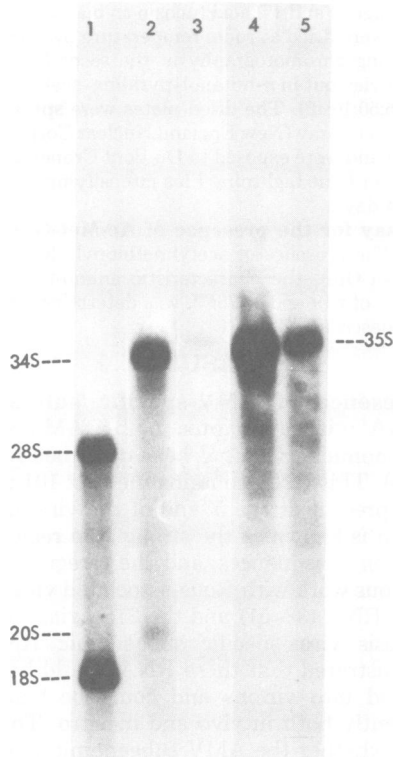

FIG. 1. *Size of AMV-A-specific RNA. A 1.5-μg amount of poly(A)-containing 70S AMV-A RNA or MAV-1 70S RNA was denatured in 1 M glyoxal–10 mM sodium phosphate (pH 7.0) at 50°C for 1 h, electrophoresed in a 1% agarose gel, and transferred to diazobenzyloxymethyl paper. Blots were hybridized with either [^{32}P]cDNA$_{AMV}$ (lanes 2 and 3) or [^{32}P]cDNA$_{MAV\text{-}1}$ (lanes 4 and 5). Approximately 10,000 cpm of probe per cm^2 of filter paper was used in each hybridization. Lane 1, [^{32}P]rRNA; lanes 2 and 4, AMV-A 70S RNA; lanes 3 and 5, MAV-1 70S RNA.*

daltons were observed. Molecular weights were determined by averaging the results observed in approximately 15 to 20 gels. In Fig. 2A, the translation product in lanes 13 through 18 identified as p47 appeared to have a higher molecular weight (lower electrophoretic mobility) than the translation product found in fractions 16 through 20, which was identified as p48. In every other gel examined, the positions of these two bands were reversed. We cannot explain the deviation observed in the gel shown in Fig. 2A.

The presence of Pr76^{gag} across the gradient presumably was due to translation of fragments derived from the 5′ end of the viral RNA. The bands observed in translations of both AMV and MAV-1 at 43,000 and 41,000 daltons appeared when viral RNA of any size class was added to the translation reaction (Fig. 2) (2). The bands at 31,000, 34,000, 35,000, and 37,000 daltons in fractions 16 through 21 were produced by various strains of Rous sarcoma viruses and Rous-associated viruses (R. E. Karess and S. M. Anderson, unpublished data). At this time we do not know what region of the viral genome encodes these proteins or what gene products these proteins might be related to. They were not immunoprecipitible with specific antisera, and their syntheses were not blocked by ^{7m}GTP (see below). The 76,000-dalton protein found in translations of AMV and MAV-1 was Pr76^{gag}; the identity of this protein was confirmed by immunoprecipitation and by the presence of the 19* dipeptide (see below). The 60,000-dalton protein found in MAV-1 translations was the unglycosylated *env* gene product (see below). Proteins of 56,000 daltons were observed in translations of both MAV-1 and AMV RNAs; these proteins were designated p56$_{MAV\text{-}1}$ and p56$_{AMV}$, respectively. The 47,000- and 48,500-dalton proteins observed in translations of AMV virion RNA were not observed in translations of MAV-1 virion RNA. In addition, there was a protein of approximately 32,000 daltons that apparently was translated from AMV RNA but not from MAV-1 virion RNA. Previous studies of labeled cell extracts demonstrated that AMV nonproducer cells contain Pr180$^{gag\text{-}pol}$, Pr76^{gag}, and the individual *gag* proteins p19, p27, p12, and p15 (11; Anderson and Chen, unpublished data). AMV nonproducer cells do not contain any of the known *env* gene products (11; Anderson and Chen, unpublished data). Thus, AMV does not produce a *gag*-related polyprotein encoded by its putative transforming gene(s), as has been described for other acute leukemia viruses. Therefore, we further characterized the unique polypeptides found in the in vitro translation products of AMV virion RNA.

^{7m}GTP block of translation. ^{7m}GTP, a cap analog, is known to block the translation of capped mRNA in both the reticulocyte lysate system (54) and the wheat germ system (24, 25). To determine whether all of the observed AMV translational products were translated from capped mRNA, we performed translations in the presence and absence of 1 μM ^{7m}GTP. As Fig. 3 shows, the translations of Pr76^{gag} and the 56,000- and 48,500-dalton proteins were blocked by ^{7m}GTP, indicating that these proteins were translated from capped mRNA. Translation of the 47,000-dalton protein was not inhibited by ^{7m}GTP. Although the synthesis of the 32,000-dalton AMV-specific translation product did not appear to be blocked to the same extent as the syntheses of the 56,000- and 48,500-dalton proteins in this preparation of AMV virion RNA, the synthesis of this protein was blocked by

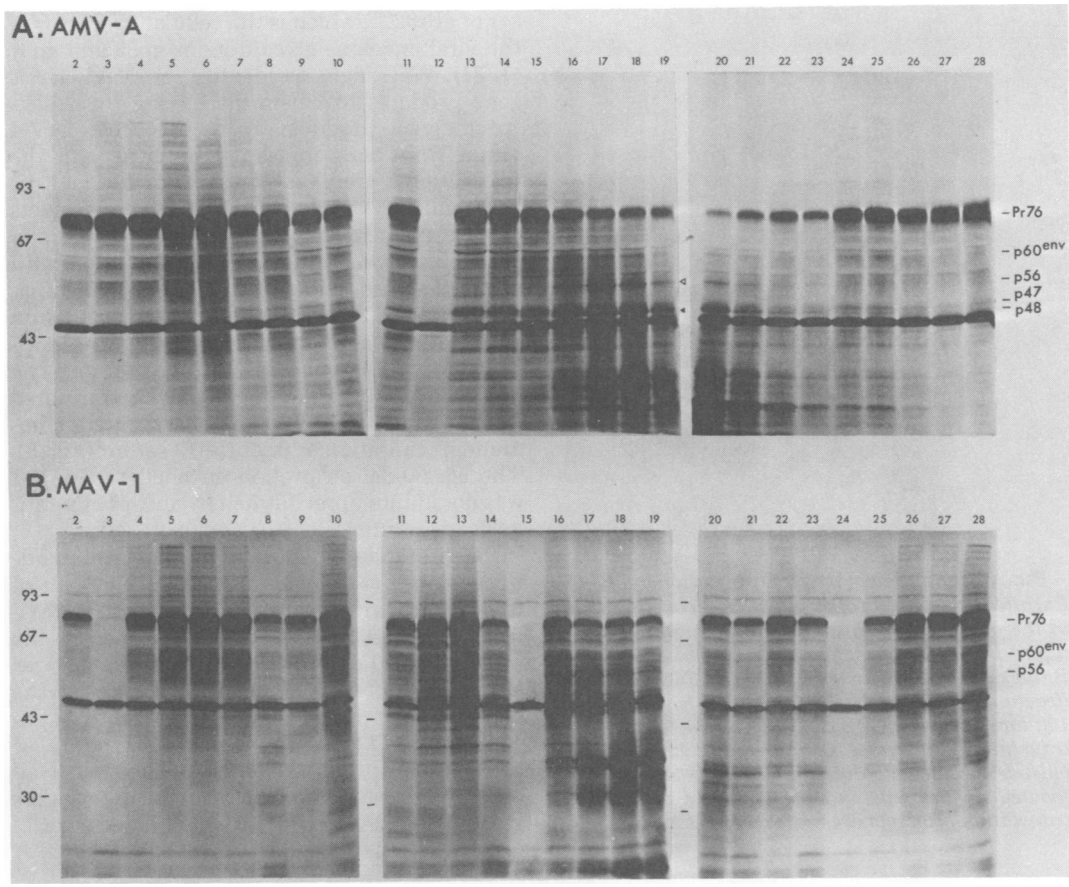

FIG. 2. *In vitro translations of AMV-A RNA (A) and MAV-1 viral RNA (B). Poly(A)-containing virus RNA sizes were determined with a sucrose gradient, and the RNAs in the fractions were concentrated by ethanol precipitation. A 0.25- to 0.5-μg amount of RNA from each of the fractions was translated in vitro by using the rabbit reticulocyte lysate system of Pelham and Jackson (39), and the products were analyzed in an 8.5% SDS-polyacrylamide gel. Molecular weights (×10³) are indicated on the left, and the translation products of interest are indicated on the right. The AMV-specific 48,500- and 56,000-dalton proteins are indicated by the solid and open arrowheads, respectively. Sucrose gradient fraction numbers are indicated across the top. Fraction 6 contained 35S viral RNA, and fraction 17 contained 20S to 22S viral RNA. The RNAs in fraction 12 of AMV-A (A) and fractions 15 and 24 of MAV-1 (B) were lost during experimental manipulation.*

^{7m}GTP in a second preparation of AMV RNA (data not shown).

Immunoprecipitation of AMV in vitro translation products. To identify the translational products that might be related to known viral structural proteins, sequential immunoprecipitations of the translation products with anti-*gag*, anti-gp85, and anti-reverse transcriptase sera were performed. The 76,000-dalton protein was immunoprecipitated by anti-*gag* serum (Fig. 4, lane B), indicating that it was Pr76^{gag}. None of the other AMV translational products were immunoprecipitated by any of the antisera directed against known viral structural proteins (Fig. 4). A protein of approximately 35,000 daltons was immunoprecipitated by anti-gp85 antiserum, but this did not appear to be the 32,000-dalton protein described above. This suggested that the AMV translational products of 56,000, 48,500, 47,000, and 32,000 daltons were not related to any of the viral structural genes. One or more of these proteins may be encoded by the *myb*-specific sequences.

Identification of the MAV-1 *env* gene product. The *env* gene of helper viruses is transcribed and processed to produce 21S *env* mRNA (23, 57). In vitro translation of 21S Rous-associated virus-2 virion RNA yields a 62,000- to 64,000-dalton protein that is immunoprecipitated by anti-gp85 serum (2, 38; Anderson, unpublished data). This translational product presumably represents the unglycosylated precur-

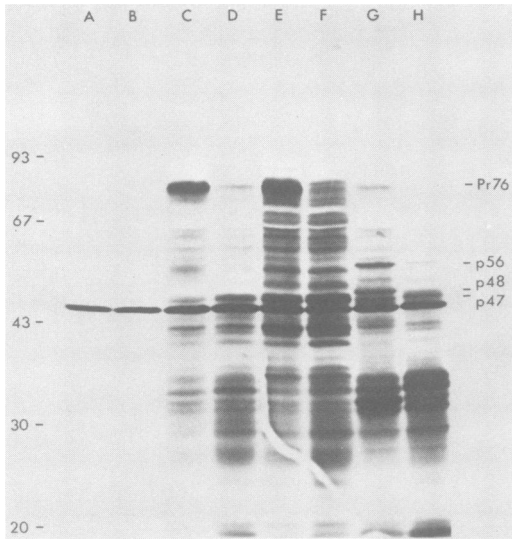

FIG. 3. *Inhibition of in vitro translation by ^{7m}GTP. Parallel translations of virion RNA were carried out in the absence (lanes A, C, E, and G) and presence (lanes B, D, F, and H) of 1 μM ^{7m}GTP. Lanes A and B, no added RNA; lanes C and D, 35S AMV RNA (fraction 5); lanes E and F, 24S AMV RNA (fraction 15); lanes G and H, 21S AMV RNA (fraction 18). The translation products were analyzed on an 8.5% SDS-polyacrylamide gel. Molecular weights (×10^3) are indicated on the left, and the positions of the viral translation products are indicated on the right.*

sor of gPr92^{env}, which is the cellular precursor of the viral envelope glycoproteins gp85 and gp37 (7, 31). We sought to identify the MAV-1 *env* gene products to aid in identifying the AMV-specific gene products. To do this, 21S MAV-1 virion RNA was translated in vitro, and the translational products were analyzed in an 8.5% SDS-polyacrylamide gel (Fig. 5). The products of a parallel translation reaction were immunoprecipitated with anti-gp85 serum and then with anti-p19 serum, and the precipitates were analyzed by gel electrophoresis. A 60,000-dalton polypeptide was immunoprecipitated with anti-gp85 serum but not with anti-*gag* serum. The *gag* gene product Pr76^{gag}, presumably translated from fragmented RNA, was observed after immunoprecipitation with anti-p19 serum (Fig. 5). The 56,000-dalton protein encoded by MAV-1 was not immunoprecipitated by anti-p19 or anti-gp85 antiserum.

Peptide maps of AMV translational products. Two-dimensional tryptic peptide maps of the major in vitro translation products of AMV were prepared (Fig. 6). A peptide map of the MAV-1 56,000-dalton translational product was also prepared (Fig. 6C). A comparison of the peptide maps of the 56,000-dalton proteins encoded by AMV and MAV-1 indicated that these translational products were not identical. Neither protein appeared to be related to either Pr76^{gag} (Fig. 6A) or the *env* gene products p56^{env}

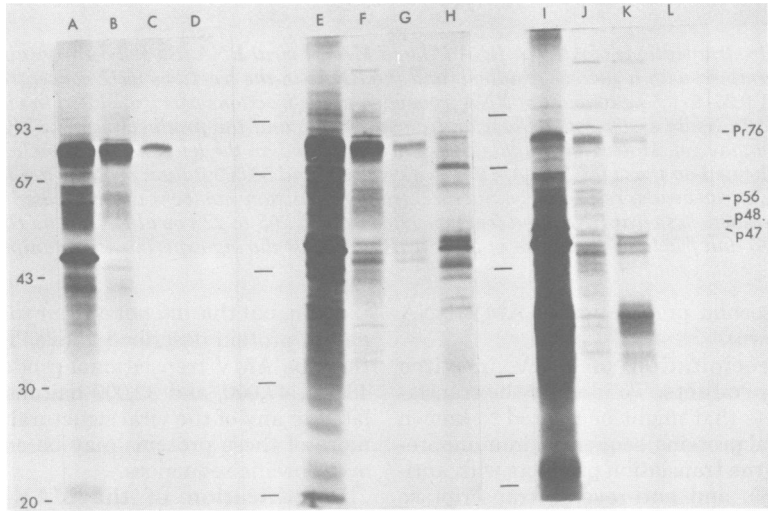

FIG. 4. *Immunoprecipitation of AMV-specific translation products. Parallel translations were performed. The products of one translation were immunoprecipitated sequentially with anti-gag, anti-gp85, and anti-reverse transcriptase antisera. Lanes A through D, 35S AMV RNA (fraction 5); lanes E through H, 24A AMV RNA (fraction 15); lanes I through L, 21S RNA (fraction 18). Lanes A, E, and I show total translation products; lanes B, F, and J were precipitated with anti-gag serum; lanes C, G, and K were precipitated with anti-gp85 serum; and lanes D, H, and L were precipitated with anti-reverse transcriptase serum. Peptides were analyzed on 8.5% SDS-polyacrylamide gels. Molecular weights (×10^3) are indicated on the left, and AMV-specific translation products are indicated on the right.*

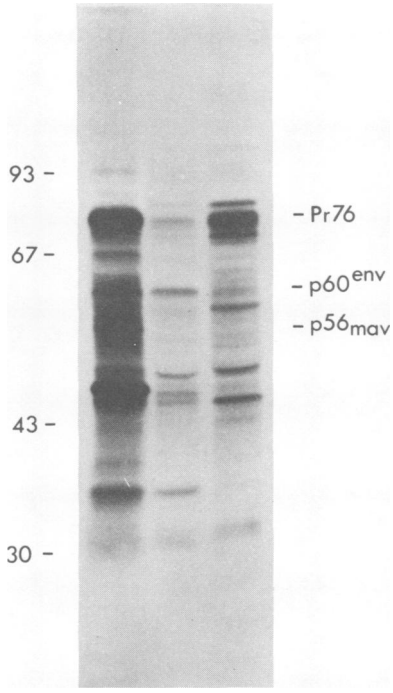

FIG. 5. *Translation products of MAV-1 21S virion RNA. Parallel translations of MAV-1 21S RNA were performed. The products of one translation were analyzed directly (lane A). The translation products of a second translation reaction were immunoprecipitated sequentially with anti-gp85 serum (lane B) and anti-p19 serum (lane C). The translation products were analyzed on an 8.5% SDS-polyacrylamide gel. Molecular weights ($\times 10^3$) are indicated on the left, and selected MAV-1 translation products are indicated on the right.*

and gPr92^{env} (data not shown). Also, the 48,500-dalton translation product was not related to Pr76^{gag} or the *env* gene products (data not shown). In addition, these translation products did not appear to be related to the 46,000- and 48,000-dalton proteins observed in translations of avian erythroblastosis virus 20S to 22S RNA (2; Anderson, unpublished data). The peptide maps of two additional proteins, p35 and p31, were also prepared, and they did not appear to be related to each other or to any of the other AMV translation products.

Presence of the Ac-Met-Glu dipeptide in AMV translational products. Palmiter et al. (37) have determined that the N-terminal amino acid sequence of the Prague strain of Rous sarcoma virus Pr76^{gag} is Met-Glu-Ala-Val-Ilu-Lys . . . ; these workers have also shown that the amino terminus of this protein is blocked, presumably by acetylation. p19 has been mapped to the 5′ end of the *gag* gene (36), and the N-terminal sequences of p19 and Pr76^{gag} are identical (36). These data suggested to Rettenmier et al. (42) that *Staphylococcus aureus* V8 protease, which cleaves at the carboxyl side of glutamic acid residues (26), should release the *N*-acetylmethionyl glutamic acid dipeptide among its digestion products. This was indeed found to be the case, and this dipeptide was found in both Pr76^{gag} and p19, as well as the *gag*-related polyproteins of avian erythroblastosis virus and MC29 virus (42). Thus, the presence of this acetylated dipeptide among the protease V8 digestion products of a protein is diagnostic for the presence of the amino terminus of the *gag* gene.

We examined the in vitro translation products of AMV for the presence of this marker dipeptide. This peptide, which is designated 19*, was present in AMV Pr76^{gag} synthesized in vitro and in MAV-1 Pr76^{gag} labeled in vivo, (Fig. 7). No other translation product of AMV (including the 56,000-, 48,500-, 47,000-, 43,000-, 41,000-, 37,000-, 35,000-, 34,000-, 32,000-, and 31,000-dalton proteins) released the 19* dipeptide after digestion with V8 protease (data not shown). This indicated that the synthesis of these proteins does not begin at the *gag* gene initiation codon with at least the first two amino acid codons of the *gag* gene present.

DISCUSSION

We studied the in vitro translation products of AMV by using the rabbit reticulocyte lysate system. Recently, several investigators have described the viral proteins present in AMV-transformed cells, as detected by immunoprecipitation (11, 44). Duesberg et al. have described the virus-related proteins present in AMV nonproducer cells. These cells contain Pr180$^{gag\text{-}pol}$, Pr76^{gag}, and the individual *gag* proteins. No other *gag* fusion proteins or putative *myb* gene products were described by these authors. The results of our studies on in vivo-labeled lysates from AMV nonproducer cells are in total agreement with these findings. Silva and Baluda (44) described two glycoproteins immunoprecipitated by anti-gp85 antiserum (gp120 and h27) and one protein (p100) immunoprecipitated from myeloblasts by antisera prepared against the individual *gag* proteins. The glycoproteins gp120 and h27 were found in normal yolk sac and spleen cells. The p100 protein was not found in yolk sac cells infected with AMV. We did not detect any of these three proteins in the AMV-transformed myeloblasts which we examined, and thus we assume that these proteins are unique to the cell lines or antisera used by these authors. In SPAFAS yolk sac cells transformed in vivo by AMV we did observe a 110,000-dalton protein, which may be the same protein found

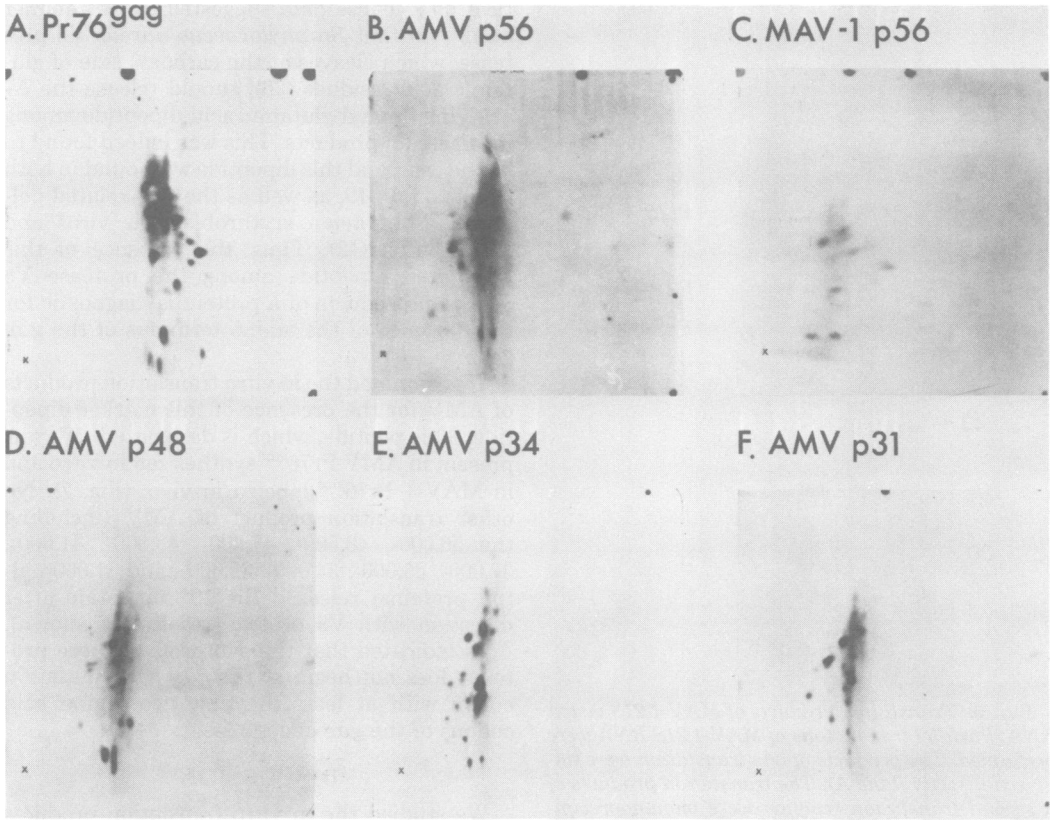

FIG. 6. *Two-dimensional tryptic fingerprints of AMV-A and MAV-1 in vitro translation products. Translation products were separated on 8.5% SDS-polyacrylamide gels, and the bands of interest were located, excised, and processed as described in the text. The samples were spotted in the lower left corners of plates, (×), and electrophoresis was toward the cathode at pH 4.7; this was followed by ascending chromatography. (A) Pr76. (B) AMV 56,000-dalton peptide. (C) MAV-1 56,000-dalton peptide. (D) AMV 48,500-dalton peptide. (E) AMV 34,000-dalton peptide. (F) AMV 31,000-dalton peptide.*

by Silva and Baluda (44). We did not observe this protein in all of the AMV myeloblast clones analyzed.

AMV produces two mRNA's, 34S full-length AMV virion RNA and 21S subgenomic mRNA which contains AMV-specific *myb* sequences. This 21S mRNA is packaged within AMV virions along with the 34S genomic RNA. Therefore, we used virion RNA for in vitro translation experiments. We found four AMV-specific translational products that were not related to any of the known viral structural proteins, either by immunoprecipitation or by peptide mapping. These proteins had molecular weights of 56,000, 48,500, 47,000, and 32,000. Based upon two-dimensional peptide mapping, the 56,000- and 48,000-dalton proteins did not appear to be related to $Pr76^{gag}$, the MAV-1 *env* gene products, or the proteins produced by translation of avian erythroblastosis virus subgenomic 21S mRNA (Anderson, unpublished data). They did not contain the 19* dipeptide diagnostic for the N terminus of *gag* gene products (41) and thus were not translated from the *gag* gene initiation codon.

The 47,000-dalton protein is produced by 22S to 24S RNA, whereas the 56,000- and 48,500-dalton proteins are produced by 20S to 22S RNA, which is the size of the AMV subgenomic mRNA species. Translation of the 48,500- and 56,000-dalton proteins, but not of the 47,000-dalton protein, is inhibited by ^{7m}GTP, a cap analog known to block translation of capped mRNA's (24, 25, 54, 56). This may indicate that the 47,000-dalton protein is not translated from a capped mRNA species, but instead is translated from an RNA fragment. Taken together, these facts indicate that either the 56,000-dalton protein or the 48,500-dalton protein or perhaps both represent the translational product(s) of the *myb* gene. In addition to the three proteins discussed above, there is a protein of approxi-

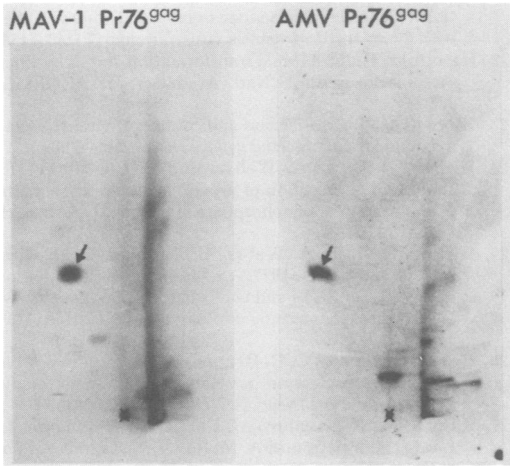

FIG. 7. *Presence of the 19* dipeptide in in vivo-labeled MAV-1 Pr76gag and in vitro-synthesized AMV Pr76gag. [35S]methionine-labeled MAV-1 Pr76gag and AMV Pr76gag were digested with V8 protease, and the digestion products were spotted onto the centers of plates* (×). *Electrophoresis was at pH 6.5 in the horizontal direction, with the anode at the left. Ascending chromatography was from bottom to top. The negatively charged 19* dipeptide is indicated by arrows.*

mately 32,000 daltons that is translated from 21S virion RNA and is not observed in translations of MAV-1 RNA. Like the other AMV-specific translation products, this protein is not immunoprecipitated by antisera prepared against virion structural proteins, nor does it contain the 19* dipeptide characteristic of proteins whose translation begins at the *gag* gene initiation codon. In one preparation of AMV virion RNA, the synthesis of this peptide was blocked by ^{7m}GTP. This translation product is also a candidate for the *myb* gene product.

A question must be raised concerning whether one or both of the 56,000-dalton proteins could be related to the ability of AMV and certain MAV isolates to cause osteopetrosis (45). Competition hybridization experiments have indicated that there may be sequences responsible for the induction of osteopetrosis (50). This possibility can not be resolved by the data presented here, but it poses several interesting questions for future research.

We cannot readily explain why there apparently are multiple products of the *myb* gene of AMV; the 32,000-, 48,500-, and 56,000-dalton proteins are apparently encoded by the 21S *myb* mRNA. The size of the AMV unique mRNA is about 2.5 kb (9, 15) which includes a poly(A) tract of about 0.2 kb, a common region of about 0.7 kb (23), and a leader tract of about 0.3 to 0.4 kb (23; G. P. Gasic and W. S.Hayward, personal communication). Thus, the actual coding region of this RNA is 1.2 to 1.5 kb long, which corresponds to a coding capacity of 47,000 to 55,000 daltons. From heteroduplex mapping of AMV, Souza et al. (50) have estimated that the AMV-specific region is 0.9 ± 0.16 kb long, which corresponds to a smaller coding capacity of 33,000 to 38,000 daltons. This assumes that there are no other 3′ viral sequences, such as part of *env*, which are translated as part of the *myb* gene. This means that all three proteins cannot be read in the same reading frame. However, it is possible that these proteins are coded for by the same region of proviral DNA in different reading frames produced by different splicing patterns. Multiple splicing patterns account for the production of several proteins by the same region of DNA in the simian virus 40 early gene (4, 40), the polyoma virus early gene (14, 27, 47), and gene 8 of influenza virus (29). If this is true, then it is possible that there are multiple *myb* gene products.

Resolution of the question of which protein is the *myb* gene product of AMV will require the preparation of an anti-*myb* serum which can recognize one or more of these proteins. Another possible approach would be to compare the peptide map of the *gag*-related polyprotein of E26, which also contains *myb* sequences (43), and identify E26-specific peptides. The map of the unique peptides of the E26 *gag*-related polyprotein should be related to the unique peptides of the transformation protein of AMV. Studies are in progress to answer this question. Ultimately, this question may also be answered from the DNA sequence of the λ-AMV clone (48) and a determination of whether one or more open reading frames exist in this part of the genome.

ACKNOWLEDGMENTS

We thank V. M. Vogt for antiserum specific for each of the individual *gag* proteins, R. Karess for the rabbit reticulocyte lysate used in this study, and J. B. Beard, D. Beard, and K. Miyagi for the generous supply of AMV plasma. We thank Ben Neel and Hidesaburo Hanafusa for comments on the manuscript and Nancy Britt for assistance in preparing the manuscript. Excellent technical assistance was provided by Nancy Aaronson.

This investigation was supported by Public Health Service grants CA14935 and CA18213 from the National Institutes of Health to H. Hanafusa and by American Cancer Society grant MV-20 to J.H.C. S.M.A. was supported by Public Health Service institutional training grant T32CA09256 from the National Institutes of Health.

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