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Synthesis of Murine Mammary Tumor Viral Proteins In Vitro

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The coding potential of murine mammary tumor viral genomic RNA was investigated by in vitro translation of various size classes of RNAs isolated from the virions. The major products of translation of full-size 35S polyadenylylated virion RNA were *gag*-related polyproteins of 75,000, 105,000, and 180,000 daltons (P75, P105, and P180, respectively). Studies on the kinetics of translation of these three proteins established that they were synthesized independently and that the smaller proteins were not post-translational cleavage products of the larger proteins. Tryptic peptide mapping showed that almost all of the P75 sequences were contained within P105 and almost all of the P105 sequences were contained within P180. The syntheses of all three proteins were inhibited by m^7GTP, indicating that they were translated from capped mRNA's. Although a 24S polyadenylylated RNA had been identified as the intracellular mRNA for *env* precursor polyprotein, no such protein could be translated from the 24S polyadenylylated RNA isolated from the virions. However, translation of a 14S size class of polyadenylylated virion RNA yielded four prominent proteins of about 36,000, 23,000, 21,000, and 20,000 daltons. These proteins were unrelated to murine mammary tumor viral structural proteins, as suggested from tryptic peptide mapping and immunoprecipitation data. They might be the products of an as-yet-unidentified gene located near the 3' terminus of the murine mammary tumor viral genomic RNA.

Murine mammary tumor virus (MuMTV) is a type B retrovirus and is known to be involved in the induction of mammary carcinoma in several strains of mice. This virus can be transmitted either horizontally through the milk or genetically as a stable provirus integrated in the germ lines of these mice (12, 20). The molecular events leading to the mammary tumorigenesis caused by MuMTV have not been elucidated yet. It is also not known whether the MuMTV genome codes for a transformation-specific protein similar to the proteins of avian sarcoma viruses or murine sarcoma viruses. Investigations along these lines are hampered by the lack of any tissue culture system in which MuMTV causes transformation.

MuMTV virions contain at least seven proteins (19). There are four core proteins (p28, p23, p14, and p10) encoded by the *gag* gene, two envelope proteins (gp52 and gp36) encoded by the *env* gene, and the reverse transcriptase, a product of the *pol* gene. It is presumed that similar to the type C viruses, the *gag*, *pol*, and *env* genes are arranged in the order *gag-pol-env* from the 5' end to the 3' end along the genomic RNA. The intracistronic order of the *gag* proteins has been established as 5'-p10-p23-p28-p14-3' (6, 7, 11, 25). The similar order for the *env* proteins is 5'-gp52-gp36-3' (7, 22, 25). Both *gag* proteins and *env* proteins are synthesized as precursor polyproteins. A 75,000-dalton protein (P75) has been identified as the *gag* precursor, and a 70,000-dalton protein (gp70) has been identified as the *env* precursor (1, 5, 16, 21). The *pol* gene is presumably expressed through the synthesis of a 180,000-dalton *gag-pol* precursor protein (P180).

We and others have shown previously that the P75 *gag* precursor can be translated in vitro from MuMTV genomic RNA or from 35S RNA isolated from MuMTV-infected cells (4, 13, 24). A virion-associated protease can specifically cleave P75 in vitro and may be responsible for the maturation of individual *gag* proteins in vivo (23). The RNA isolated from MuMTV virions is heterogeneous in size (4, 24). We have shown previously that even 24S virion RNA can be translated into P75, but no gp70, the precursor of the *env* proteins, could be translated from any virion RNA (24). However, as we have reported earlier, a 24S polyadenylylated [poly(A)$^+$] mRNA isolated from MuMTV-producing cells can be translated into the *env* precursor gp70 (24).

Translation of 35S virion RNA gives rise to two more polyproteins in addition to P75. These proteins have molecular weights of about 105,000 (P105) and 180,000 (P180) (4, 24). Both

of these proteins contain *gag*-related sequences (4, 24). It has been postulated that P180 is a *gag-pol* fused protein similar to the proteins observed for type C viruses, but there has not been any direct demonstration that this protein contains polymerase sequences. There is no counterpart of P105 in nondefective murine type C viruses. Dickson and his coworkers have thoroughly established that MuMTV P75 and P105 made both in vitro and in vivo are closely related and that all of the tryptic peptides present in P75 are also present in P105 (4, 6). However, the relationship between P105 and P180 has not been established.

In the studies reported here, we extended the above-described observations of Dickson et al. (4, 6) to demonstrate that P105 is an intermediate between P75 and P180. All tryptic peptides present in P105 were also present in P180. We also found that all three polyproteins were translated from capped mRNA's and that they were the products of independent translational events.

We and others have reported previously that MuMTV-infected cells contain an MuMTV-related 14S size class of poly(A)$^+$ RNA (18, 24). It is not known whether this size class of MuMTV-related RNA codes for any protein. We report here that a 14S poly(A)$^+$ RNA isolated from MuMTV virions could be translated into four prominent proteins which were unrelated to any known MuMTV structural proteins. It appears that these four proteins might be the products of an unidentified MuMTV gene.

(Some of these results were presented in a preliminary communication [G. C. Sen, J. Racevskis, and N. H. Sarkar, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, T62, p. 245].)

MATERIALS AND METHODS

Materials. [^{35}S]methionine and [^{3}H]uridine were purchased from New England Nuclear Corp. m^{7}GTP was from P-L Biochemicals. Calf liver tRNA was purchased from Boehringer, and protein A-Sepharose was from Pharmacia Fine Chemicals, Inc. Oligodeoxythymidylic acid-cellulose was from Collaborative Research, Inc. Molecular weight markers were purchased from Bio-Rad Laboratories and were radiolabeled with [^{14}C]iodoacetamide as described previously (24).

Virus. MuMTV produced by dexamethasone-stimulated GR cells was harvested and purified by using procedures described previously (24).

Viral RNA. Total RNA labeled with [^{3}H]uridine was extracted from the virions by using the phenol-chloroform extraction procedure (24). For poly(A) selection, viral RNA was dissolved in a buffer containing 10 mM Tris-chloride (pH 7.5), 10 mM EDTA, and 0.2% sodium dodecyl sulfate and heated at 85°C for 1 min. The RNA solution was then made 0.3 M in NaCl and applied to an oligodeoxythymidylic acid-cellulose column pre-equilibrated with a buffer containing 10 mM Tris-chloride (pH 7.5), 0.5% sodium dodecyl sulfate, and 0.3 M NaCl. The RNA not bound to the column was washed off with the same buffer and pooled with the flow-through fraction. This pooled RNA fraction was reapplied to the column in the same way to ensure the retention of all poly(A)-containing RNAs. The poly(A)-containing RNAs were eluted out with the same buffer without NaCl. The poly(A)-containing RNAs were recycled once more through the oligodeoxythymidylic acid-cellulose column to free them completely from poly(A)$^-$ RNAs.

RNAs were size fractionated by centrifugation through a 15 to 30% linear sucrose gradient in a buffer containing 10 mM Tris-chloride (pH 7.5), 50 mM NaCl, 10 mM EDTA, and 0.2% sodium dodecyl sulfate. Centrifugation was for 6.5 h at 40,000 rpm in an SW41 rotor. The viral RNA from each fraction was recovered by precipitation with ethanol after 50 μg of carrier tRNA was added. It was dissolved in a buffer containing 0.3 M NaCl and 10 mM Tris-chloride (pH 7.5) and again precipitated with ethanol to remove traces of residual sodium dodecyl sulfate. Finally, the RNA pellets were dissolved in water and stored at −80°C.

In vitro translation. Viral RNA was translated in vitro in a nuclease-treated rabbit reticulocyte lysate system, as described previously (24). Usually, 1 mCi of [^{35}S]methionine per ml of lysate was used, but for some experiments the amount of radiolabeled methionine was increased by up to 10-fold. If needed, the radiolabeled methionine solution (10 mCi/ml) was lyophilized before use.

Gel electrophoresis. In vitro-synthesized proteins were separated by polyacrylamide gel electrophoresis on a 15% gel (24). The radiolabeled proteins were detected by fluorography of the gels.

In vivo labeling and immunoprecipitation. Cultures of GR cells were labeled with 200 μCi of [^{35}S]methionine per ml for 2 h, and cellular proteins were then extracted as previously described (16). The cell extracts were incubated with anti-MuMTV p28 antiserum, and this was followed by adsorption with staphylococcal protein A antibody adsorbent; then the immunoprecipitates were separated by polyacrylamide gel electrophoresis, as previously described (16).

Tryptic peptide mapping. After electrophoresis, the gels were soaked in water for 10 min and then dried. The dried gels were marked with radioactive ink and were exposed to Kodak BB-5 X-ray film for 1 day. Protein bands were excised from the dried gels and then swollen in 0.05 M NH_4HCO_3–0.1% sodium dodecyl sulfate and homogenized on a flat glass surface. Extraction, precipitation, oxidation, and trypsinization of protein were performed by the methods of Beemon and Hunter (2). The electrophoretic separation of the peptides and subsequent chromatography were performed by the methods of Elder et al. (8), using cellulose-coated thin-layer plates (10 by 10 cm and 20 by 20 cm; EM Laboratories). The thin-layer plates were sprayed with Enhance spray (New England Nuclear Corp.) and then exposed to Kodak XR2 X-Omat film at −70°C.

RESULTS

Translation of poly(A)$^+$ and poly(A)$^-$ virion RNAs of different sizes. We and others have reported previously that RNA isolated

from MuMTV virions is heterogeneous in size (4, 24). It is not clear whether this size heterogeneity is an artifact of the process used for the isolation of the virion RNAs or whether the virions physiologically package RNAs of different sizes. We have also reported that virion RNAs of different sizes can be translated in vitro into *gag*-related polyproteins (24). In an attempt to extend these studies and to further understand the coding potential of MuMTV virion RNAs of different sizes, the following experiments were performed. Total virion RNA was fractionated into poly(A)$^+$ and poly(A)$^-$ RNA populations by oligodeoxythymidylic acid-cellulose chromatography. Both poly(A)$^+$ and poly(A)$^-$ RNAs were then fractionated according to size by centrifugation through a sucrose gradient. The profiles of the size-fractionated RNAs are shown in Fig. 1. The poly(A)$^+$ RNA population yielded several discrete peaks, ranging from about 35S to 14S, whereas the poly(A)$^-$ RNA population peaked around 20S and 5S.

Poly(A)$^+$ and poly(A)$^-$ RNAs were recovered from each fraction and translated individually in a reticulocyte lysate system. The in vitro-translated proteins were analyzed by gel electrophoresis (Fig. 2). A poly(A)$^+$ RNA of about 35S was translated into three major proteins, which had

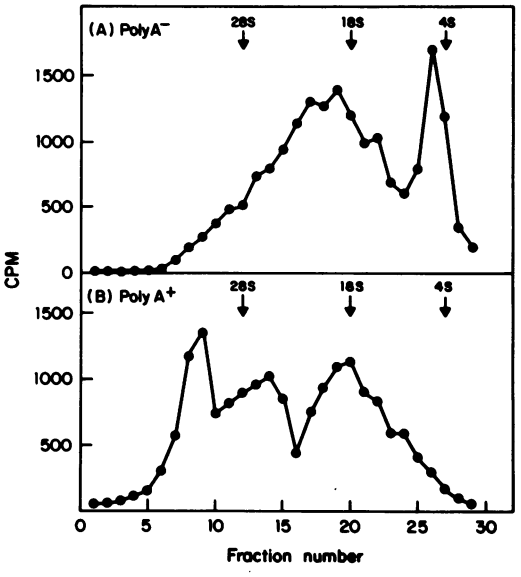

FIG. 1. *Size fractionation of poly(A)$^+$ and poly(A)$^-$ virion RNAs. poly(A)$^-$(A) and poly(A)$^+$(B) ^{3}H-labeled virion RNAs purified by chromatography on oligodeoxythymidylic acid-cellulose were fractionated according to size by centrifugation through a sucrose gradient as described in the text. Equal samples from all fractions were counted for radioactivity. A mixture of ^{3}H-labeled tRNA's and rRNA's was centrifuged in a parallel gradient. The positions of these standard RNAs are shown on the figure.*

molecular weights of 75,000, 105,000, and 180,000 (Fig. 2A, lanes 3 through 6). 35S RNA which has not been selected on oligodeoxythymidylic acid-cellulose also translates into these proteins, as has been reported previously (24). Some smaller proteins were also translated from the full-size virion RNA (24), most probably due to premature termination of the elongating peptide chains. P75, P105, and P180 were all immunoprecipitable with anti-p28 and anti-p10 sera (data not shown). As the size of the poly(A)$^+$ RNA decreased to about 28S, both P105 and P180 disappeared (Fig. 2A, lane 9). Instead, a new protein of about 85,000 daltons appeared in the translation products. As the RNA size decreased further to about 24S, the most prominent translation product was a protein of about 55,000 daltons. No appreciable amounts of P75 and P105 were translated from these RNAs. However, unselected 24S virion RNA can be translated into P75 and P105, as reported previously (24). Translation of much shorter poly(A)$^+$ RNAs (14S to 18S) produced another group of prominent proteins. Four proteins of about 36,000 daltons (P1), 23,000 daltons (P2), 21,000 daltons (P3), and 20,000 daltons (P4) were the most predominant in this group (Fig. 2B). These proteins were also translated efficiently from unfractionated virion RNA either before or after poly(A) selection (data not shown). Translation of poly(A)$^-$ RNA from about 35S to about 22S yielded P75 and P105 as the only major translation products (Fig. 2C). poly(A)$^-$ RNA smaller than 20S could not be translated into any prominent proteins.

Effect of m^{7}GTP on the translation of virion RNAs. Translation of capped mRNA's, but not of uncapped mRNA's, is known to be inhibited by structural analogs of the cap, including m^{7}GTP (9). We tested the effects of m^{7}GTP on the efficacy of translation of 35S, 24S, and 14S poly(A)$^+$ virion RNAs for the purpose of determining, in an indirect way, whether these RNAs are capped. We chose only these three size classes of poly(A)$^+$ virion RNA for detailed study because they represented the sizes of the three classes of MuMTV-related poly(A)$^+$ RNA present in MuMTV-producing cells (18, 24). The results of this experiment are shown in Fig. 3. Translation of 35S RNA was strongly inhibited by the inclusion of 0.5 mM m^{7}GTP in the reaction mixture, indicating that 35S RNA is a capped mRNA. On the other hand, translation of both 24S and 14S poly(A)$^+$ RNAs was, if anything, stimulated by inclusion of m^{7}GTP in the translation mixture. Therefore, it seems that 24S and 14S poly(A)$^+$ RNAs isolated from MuMTV virions are most probably uncapped. However, m^{7}GTP-mediated inhibition of translation should not be used as an

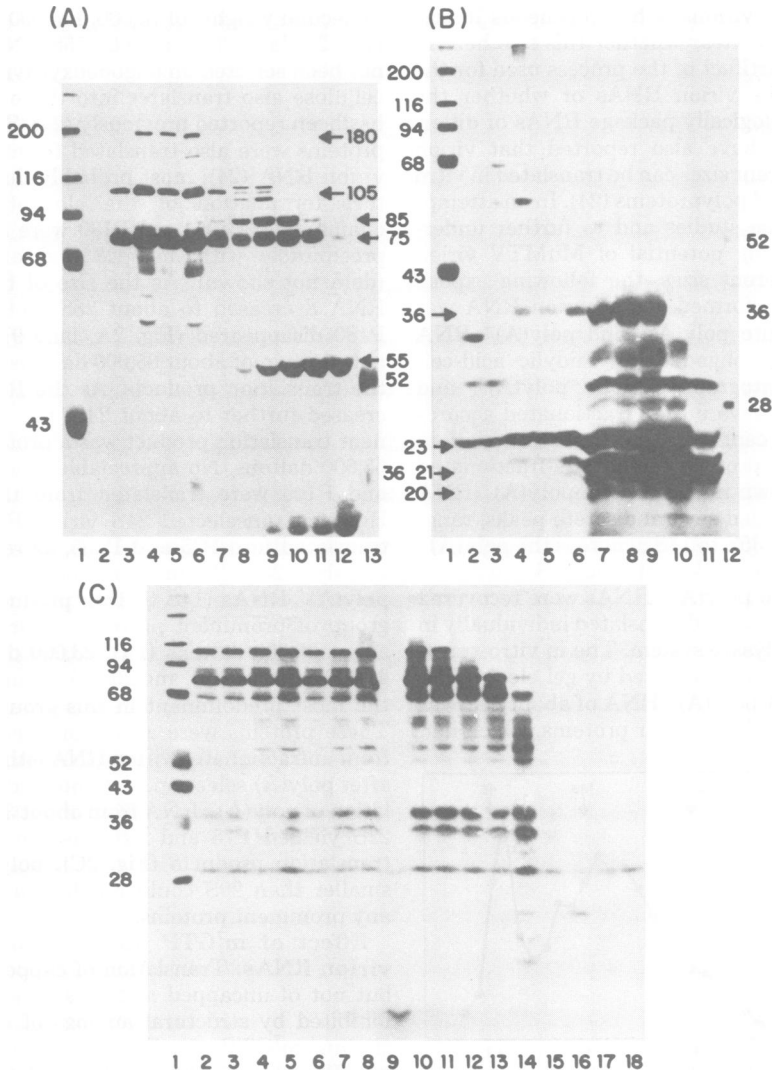

FIG. 2. *Analysis of translation products of virion RNAs of different sizes. poly(A)$^+$ and poly(A)$^-$ virion RNAs of different sizes, obtained from the fractionation shown in Fig. 1, were translated individually. Equal samples of all fractions were used for translation. The translation products were analyzed by polyacrylamide gel electrophoresis. The numbers on the sides indicate the molecular weights (×10^3) of standard proteins. The numbers with arrows indicate the molecular weights (×10^3) of the different proteins translated from the virion RNAs. (A) Lane 1, ^{14}C-labeled standard molecular weight marker proteins; lane 2, translation products without added mRNA; lanes 3 through 12, translation products of poly(A)$^+$ RNAs recovered from fractions 6 through 15, respectively; lane 13, ^{14}C-labeled MuMTV. (B) Lane 1, Standard molecular weight markers; lanes 2 through 11, translation products of poly(A)$^+$ RNAs from fractions 16 through 25, respectively; lane 12, ^{14}C-labeled MuMTV. (C) Lane 1, Standard molecular weight markers; lanes 2 through 8, translation products of poly(A)$^-$ RNAs from fractions 8 through 14, respectively; lane 9, translation products without any added mRNA; lanes 10 through 18, translation products of poly(A)$^-$ RNAs from fractions 15 through 23, respectively.*

absolute index of capped mRNA's (27). The same concentration of m^{7}GTP inhibited the translation of tobacco mosaic virus RNA (an RNA with a known capped structure) by about 80% and stimulated the translation of mengovirus RNA (an RNA without a cap structure) by about 100%, confirming the expected effects under our conditions (data not shown). The strong inhibition of translation of 35S RNA by m^{7}GTP resulted in reduced synthesis of all of the proteins ranging in molecular weight from 180,000 to 28,000. These results indicated that the initi-

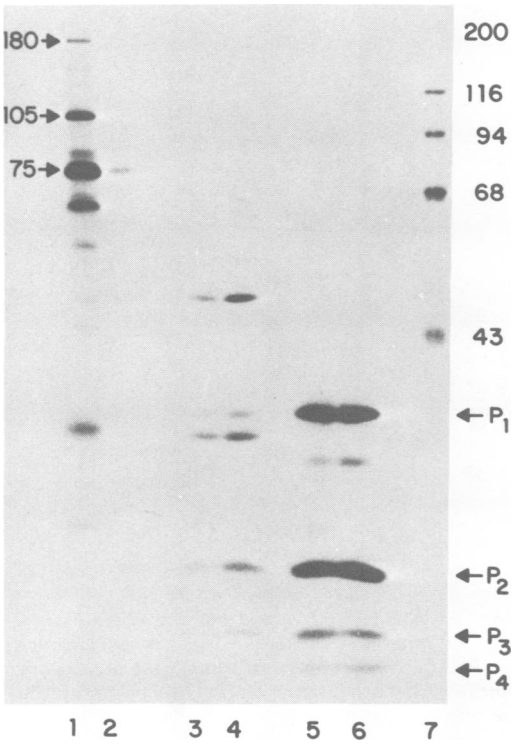

FIG. 3. *Effects of m⁷GTP on the translation of poly(A)⁺ virion RNAs of different sizes. Lanes 1 and 2, Products of translating 35S RNA in the absence and presence of 0.5 mM m⁷GTP, respectively; lanes 3 and 4, products of translating 24S RNA in the absence and presence of 0.5 mM m⁷GTP, respectively; lanes 5 and 6, products of translating 14S RNA in the absence and presence of 0.5 mM m⁷GTP, respectively; lane 7, molecular weight markers. Numbers on sides indicate molecular weights (×10³).*

ation of translation of all of these proteins involved the cap structure on the mRNA, thus ruling out internal initiation.

Kinetics of synthesis of P75, P105, and P180. To determine the relationship among the syntheses of P75, P105, and P180, we studied the kinetics of their syntheses in vitro. If the smaller proteins were being synthesized by post-translational proteolytic cleavage of the larger proteins, then one would expect the appearance of the larger proteins first, followed by the appearance of the smaller proteins. On the other hand, if the translations of the three proteins were independent events, one would expect that the smaller proteins would be synthesized sooner than the larger proteins. Figures 4A and B show the results of experiments in which we studied the kinetics of the appearance of these proteins. In the experiments shown in Fig. 4A, the regular protocol for translation of 35S virion RNA was followed. Equal samples of translation mixtures were taken out at several times, and the products were analyzed. In the experiments shown in Fig. 4B, 5 min after the initiation of translation 0.6 μM edeine was added to the translation mixture to inhibit completely further initiation of new peptide chains. Again, equal samples of the translation mixture were withdrawn at different times, and the products were analyzed. As is evident from the results both in the presence and absence of edeine, P75 was synthesized first, followed by P105 and then P180. These results strongly argue against the hypothesis that P75 is a cleavage product of P105 and P105 is a cleavage product of P180. The data are consistent with the notion that all three proteins are primary translation products and result from three independent translational events.

Tryptic peptide maps of P75, P105, and P180 translated in vitro. As previously reported, P75, P105, and P180 are all *gag*-related proteins since they can be immunoprecipitated with anti-p28 and anti-p10 sera (24). In an attempt to understand further the relationships among these three polyproteins, we compared their tryptic peptide maps. For this purpose [^{35}S] methionine-labeled proteins were synthesized in vitro by translating full-size poly(A)⁺ virion RNA. The newly synthesized proteins were separated by polyacrylamide gel electrophoresis. The three large proteins were recovered individually from the gel and then digested with trypsin. The peptides in the trypsin hydrolysates were separated on cellulose thin-layer plates by two-dimensional analysis, using electrophoresis and chromatography (Fig. 5). The major peptide spots are numbered on the figures and are listed in Table 1 for comparing the different peptides obtained from the three different polyproteins. The labeled spots present right above spot 1, including the very large spot at the top of some plates, were of a diffuse nature and were different from all other spots when viewed on the original X-ray films; they were probably not peptides. Peptides 1 to 11 were present in P75, although peptide 11 was not a very prominent peptide. P105 contained all of the peptides present in P75 except peptide 4. In addition, P105 contained four new tryptic peptides, peptides 12 to 15. Among these, peptides 13, 14, and 15 were very prominent. These results indicate that P105 contains all of P75. P180 contained all of the peptides present in P105 except peptide 5. Among the additional peptides present in P180, the most prominent ones were peptides 20, 22, and 23. It is clear from these data that all of the methionine-containing peptides in P105 were also present in P180.

Tryptic peptide maps of P75 and P105 made in vivo. The tryptic peptide maps of

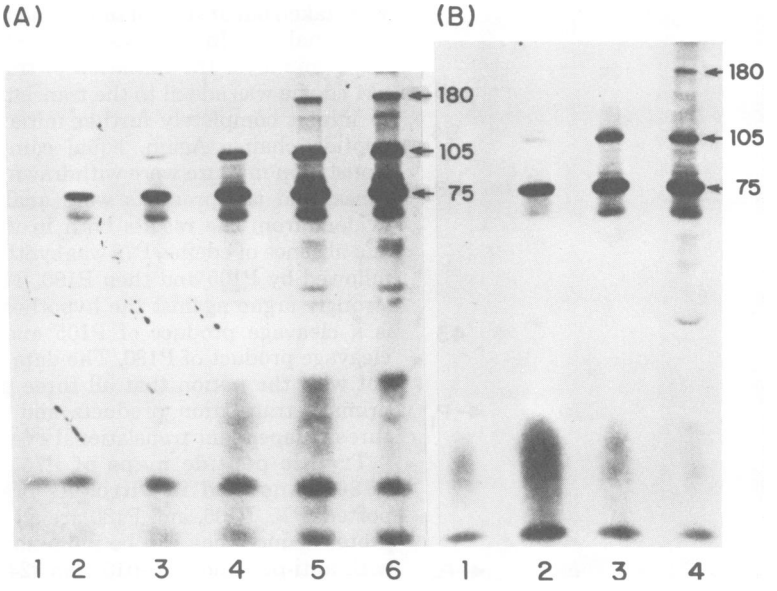

FIG. 4. *Kinetics of the syntheses of P75, P105, and P180. 35S poly(A)$^+$ RNA was translated, and equal portions of the translation mixture were analyzed after different lengths of incubation. (A) Lanes 1 through 6, incubation for 10, 15, 20, 30, 60, and 120 min, respectively. (B) After 5 min of normal translation incubation, 0.6 μM edeine was added. This amount of edeine blocked initiation of new peptide chains by more than 95%. Lanes 1 through 4, Incubations for 5 min (time of edeine addition) and 15, 30, and 60 min, respectively. Numbers on the sides indicate molecular weights (×10^3).*

P75 and P105 made in vitro were compared with the tryptic maps of these proteins labeled with [^{35}S]methionine in vivo. These proteins were labeled in vivo, immune precipitated with MuMTV anti-p28 serum, and separated by polyacrylamide gel electrophoresis. Each protein was then digested with trypsin, and the resulting peptides were analyzed (Fig. 6). P75 labeled in vivo contained peptides 1, 2, 6, 7, 8, 9, and 10 present in P75 made in vitro (Table 1). However, peptides 3, 4, 5, and 11 were missing from P75 made in vivo. It is possible that the amino acid sequences present in peptides 3, 4, and 5 are the same as those present in peptides 24, 25, and 26, respectively, and that their differential mobilities, which were highly reproducible from run to run, were due to differences in their post-translational modifications. It is known that P75 made in vivo is highly phosphorylated (13, 17), whereas P75 translated from virion RNA in vitro is probably not phosphorylated. The addition of phosphate groups to a peptide changes its mobility. Therefore, it is possible that peptides 24, 25, and 26 represent the phosphorylated peptides within P75 made in vivo. It is also curious to note that the relative amounts of peptides 1 and 2 were rather low in P75 made in vivo compared with P75 made in vitro. This suggests that P75 made in vivo may be heterogeneous in terms of either two distinct proteins of the same size being present or two forms of the same protein (resulting from different degrees of post-translational modifications) being present. P105 made in vivo contained all of the peptides present in P75 made in vivo. In addition, it contained peptides 13, 14, and 15. These were the three most prominent additional peptides contained in P105 made in vitro compared with P75 made in vitro. P105 made in vivo was missing peptides 3, 4, and 5 and contained peptides 24, 25, and 26 instead. As in P75 made in vivo, spots 1 and 2 were fainter than the corresponding spots in P105 made in vitro. The difference between the peptide maps of P105 made in vitro and P105 made in vivo is puzzling since P105 made in vivo presumably does not get phosphorylated (17). The molecular basis for this difference remains to be determined. There were also some differences in the tryptic peptide maps of P180 made in vitro and P180 made in vivo (data not shown).

Tryptic peptide maps of mature MuMTV *gag* proteins. MuMTV *gag* precursor polyprotein P75 is processed to the four mature *gag* proteins p10, p14, p23, and p28 in vivo. These proteins are the structural proteins of the MuMTV virions. We examined the tryptic peptide maps of the individual mature *gag* proteins to be able to assign the different peptides ob-

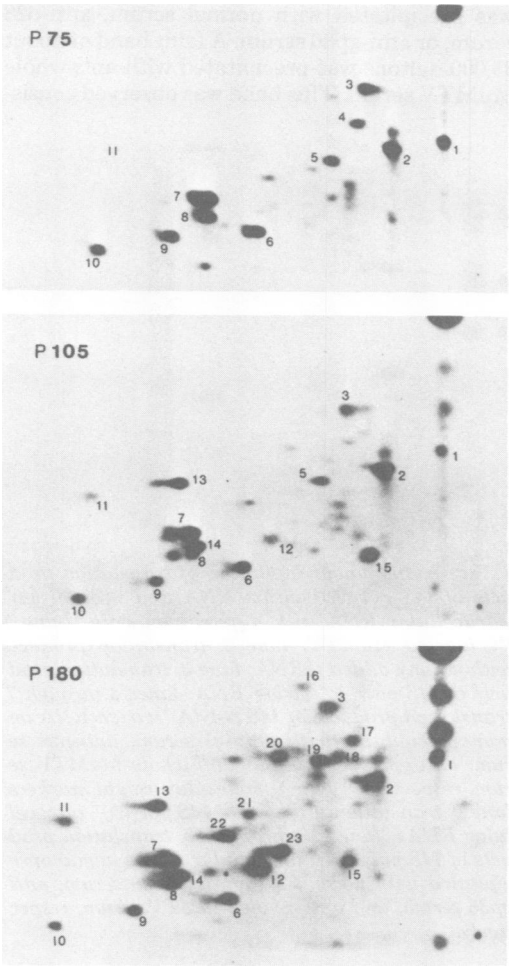

FIG. 5. *Tryptic peptide analyses of P75, P105, and P180 synthesized in vitro. The peptide analyses of ^{35}S-labeled proteins were performed as described in the text. The origins were at the lower right corners. Electrophoresis was from right to left, and chromatography was from bottom to top.*

tained from P75 to the different mature *gag* proteins. [^{35}S]methionine-labeled virus was purified, the constituent proteins were separated by gel electrophoresis, and peptide analysis was performed. Peptide maps of p10, p14, p28, and a mixture of these proteins are shown in Fig. 7. (P23 is methionine deficient.) p10 contained peptides 9 and 10 (Table 1) plus an additional peptide. p14 contained peptide 8. p28 contained peptides 1, 7, 24, and 25 plus an additional peptide. Thus, a mixture of p10, p14, and p28 contained peptides 1, 7, 8, 9, 10, 24, and 25, which were all present in P75 made in vivo, plus two or three additional peptides which were not present in P75 made in vivo. On the other hand, peptides

TABLE 1. *Tryptic peptide compositions of gag polyproteins and mature proteins*[a]

Peptide	Made in vitro			Made in vivo				
	P75	P105	P180	P75	P105	P10	P14	P28
1	+	+	+	+	+			+
2	+	+	+	+	+			
3	+	+	+					
4	+							
5	+	+						
6	+	+	+	+	+			
7	+	+	+	+	+			+
8	+	+	+	+	+		+	
9	+	+	+	+	+	+		
10	+	+	+	+	+	+		
11	+	+	+					
12		+	+					
13		+	+		+			
14		+	+		+			
15		+	+		+			
16			+					
17			+					
18			+					
19			+					
20			+					
21			+					
22			+					
23			+					
24				+	+			+
25				+	+			+
26				+	+			

[a] Data from the results shown in Fig. 5 through 7.

2, 6, and 26, which were present in P75 made in vivo, were missing from this mixture.

Characterization of the translation products of 14S poly(A)$^+$ virion RNA. As mentioned above, poly(A)$^+$ virion RNA of about 14S translated into four major proteins (P1, P2, P3, and P4). We were interested in these proteins because a 14S MuMTV-related poly(A)$^+$ RNA had been detected in various MuMTV-producing cells (18, 24) and the translation products of this intracellular RNA had not been identified. We characterized the four major proteins translated from 14S poly(A)$^+$ virion RNA both immunologically and by tryptic peptide mapping. For the immunological characterization both 14S virion poly(A)$^+$ RNA and 14S intracellular poly(A)$^+$ RNA were translated in vitro, and the translation products were tested for immunoprecipitability with normal serum, anti-p28 serum, anti-gp36 serum, and anti-MuMTV serum (Fig. 8). P1, P2, P3, and P4 were the major translation products of 14S virion poly(A)$^+$ RNA (Fig. 8, lane 3). None of these proteins was precipitated with the four antisera tested. These proteins were also not recognized by anti-gp52 and anti-p10 sera (data not shown). As expected, translation of 14S intracellular poly(A)$^+$ RNA gave

rise to many proteins (Fig. 8, lane 9), including proteins which migrated at the positions of P1, P2, P3, and P4. However, none of these proteins was precipitated with normal serum, anti-p28 serum, or anti-gp36 serum. A faint band of about 35,000 daltons was precipitated with anti-whole MuMTV serum. This band was observed consis-

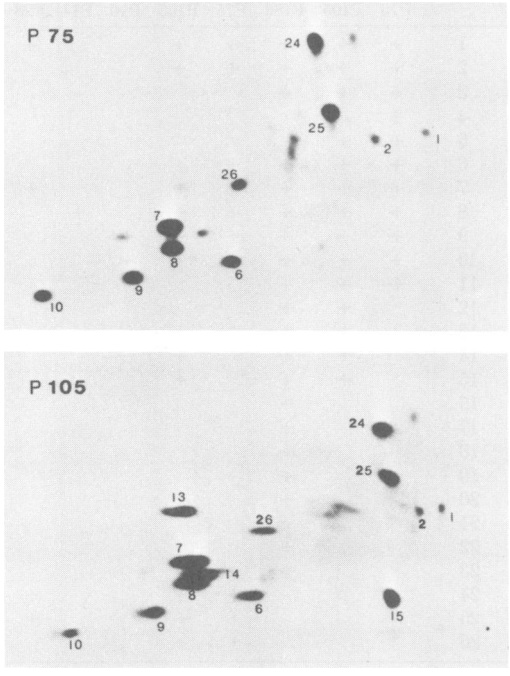

FIG. 6. *Tryptic peptide analyses of P75 and P105 synthesized in vivo. ^{35}S-labeled P75 and P105 were immunoprecipitated from a cell lysate, and their tryptic peptides were analyzed as described in the text. Electrophoresis was from right to left, and chromatography was from bottom to top.*

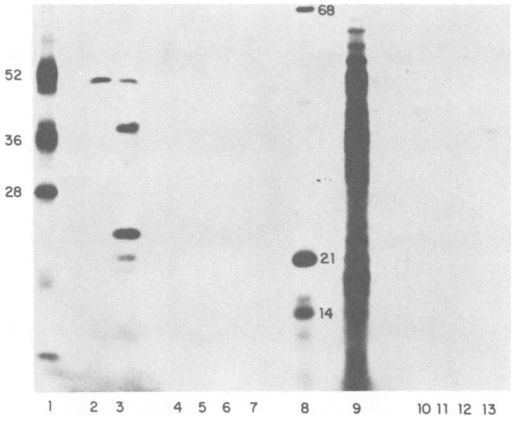

FIG. 8. *Immunoprecipitation of translation products of 14S poly(A)$^+$ virion RNA and 14S poly(A)$^+$ intracellular RNA with different antisera. Lane 1 ^{14}C-labeled MuMTV: lane 2, translation products without any added mRNA; lane 3, translation products of 14S poly(A)$^+$ virion RNA; lanes 4 through 7, translation products of 14S poly(A)$^+$ virion RNA immunoprecipitated with normal serum, anti-p28 serum, anti-gp36 serum, and anti-whole MuMTV serum, respectively; lane 8, molecular weight markers; lane 9, translation products of 14S poly(A)$^+$ intracellular RNAs; lanes 10 through 13, translation products of 14S poly(A)$^+$ intracellular RNAs immunoprecipitated with normal serum, anti-p28 serum, anti-gp36 serum, and anti-whole MuMTV serum, respectively.*

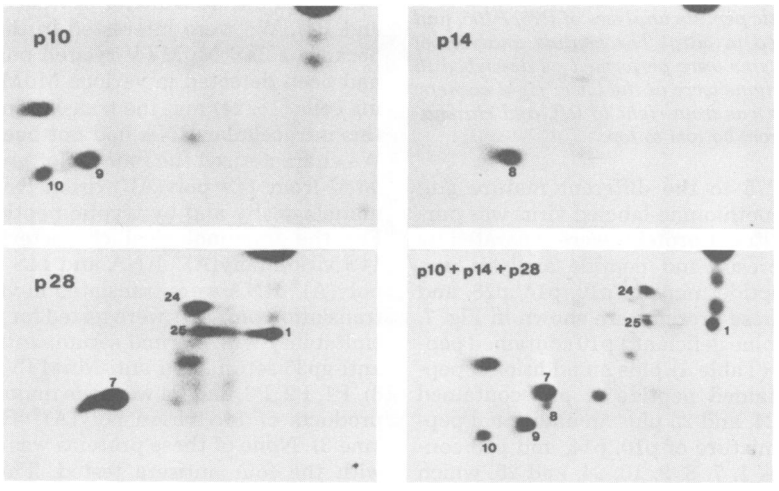

FIG. 7. *Tryptic peptide analyses of virion gag proteins. ^{35}S-labeled p10, p14, and p28 and a mixture of these proteins were analyzed. Electrophoresis was from right to left, and chromatography was from bottom to top.*

tently in many experiments. However, its origin and nature are unclear at this time. The results described above indicate that P1, P2, P3, and P4 are unrelated to any of the structural proteins of MuMTV. To characterize them further, tryptic peptides obtained from in vitro-synthesized [^{35}S]methionine-labeled P1, P2, P3, and P4 were analyzed. The peptide maps of the four individual proteins and the maps of mixtures of P1 and P3 and of P3 and P4 are shown in Fig. 9. The maps of all four proteins had strong similarities, and these maps were quite distinct from the maps of P75, P105, and P180 (Fig. 5). They also did not show any methionine-containing peptides present in the mature MuMTV *gag* proteins, p10, p14, and p28 (Fig. 7). The constituent peptides of the four proteins are listed in Table 2. P1 contained four prominent peptides (marked 1, 2, 3, and 4). Protein P2 also contained all four peptides, but peptide 1 was present in much lower quantities compared with P1. P3 contained peptides 1, 2, and 3 plus two extra peptides, peptides 5 and 6. Moreover, in P3 both spot 1 and spot 5 appeared to be doublets. P4 contained only peptides 2 and 3 common to the other proteins; in addition, it contained three peptides, designated peptides 7, 8, and 9. Peptide 4 in P1 and peptide 7 in P4 had very similar mobilities, and they could be identical. These data indicate that all four proteins share common peptides. However, it is not clear whether the complete primary sequences of the smaller proteins are contained within the larger proteins. Nonetheless, it is evident from these analyses

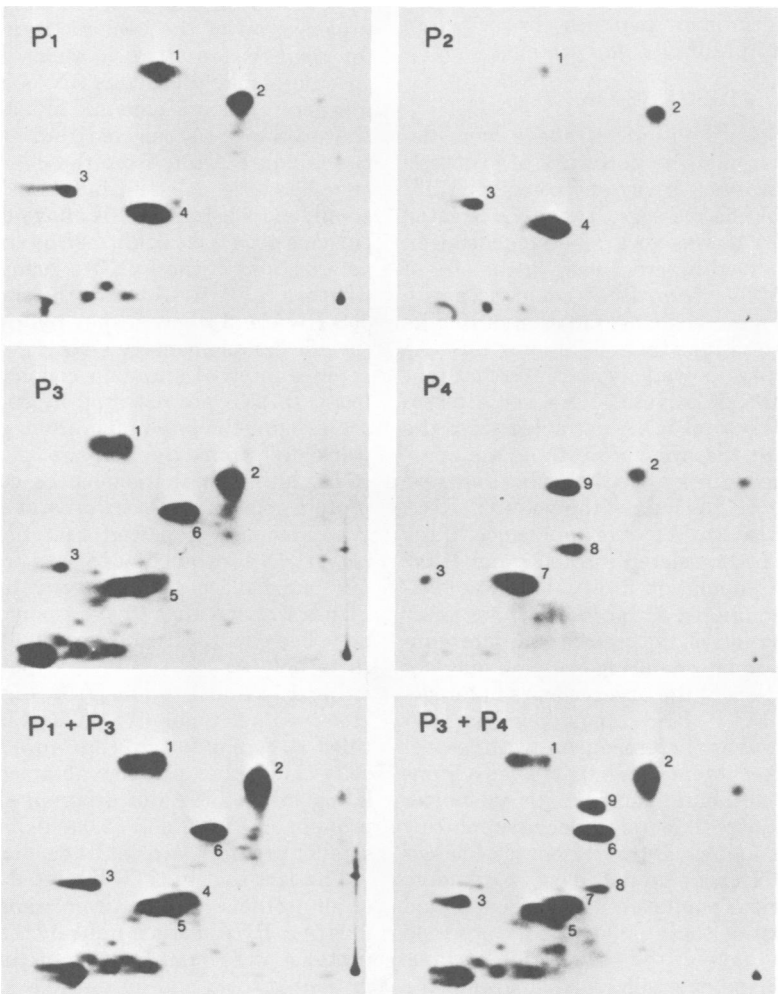

FIG. 9. *Tryptic peptide analyses of the translation products of 14S poly(A)$^+$ virion RNA. P1, P2, P3, and P4 were analyzed individually. Mixtures of P1 plus P3 and of P3 plus P4 were also analyzed. Electrophoresis was from right to left, and chromatography was from bottom to top.*

TABLE 2. *Tryptic peptide compositions of proteins translated from 14S poly(A)⁺ virion RNA*[a]

Peptide	Composition			
	P1	P2	P3	P4
1	+	+	+	
2	+	+	+	+
3	+	+	+	+
4	+	+		
5			+	
6			+	
7				+
8				+
9				+

[a] Data from the results shown in Fig. 9.

that P1, P2, P3, and P4 form a family of proteins which share common peptides distinct from those present in MuMTV *gag* proteins.

DISCUSSION

The experiments described above were designed to determine the pathways of synthesis of MuMTV proteins in vitro by using MuMTV virion RNA as the message. The RNA isolated from these virions was always heterogeneous in size. The observed heterogeneity in the size of isolated MuMTV virion RNA could be partly attributed to nonspecific degradation during its isolation. If we assume that all full-length RNAs in the virions are polyadenylated, the fact that a large quantity of poly(A)⁻ RNA could be selected from the total RNA extracted from the virions suggests that the virion RNAs were partially degraded during virus purification and RNA extraction. The bulk of the poly(A)⁻ RNA population was MuMTV related since these RNAs could be translated into P75 and P105. The 4S RNA population in MuMTV was relatively small compared with other retroviruses.

An examination of the translation data indicates that degradation during isolation may not be the only cause of the observed size heterogeneity of MuMTV RNA. It is possible that MuMTV virions are heterogeneous with respect to their RNA contents and that some virions may contain authentic genomic RNAs shorter than 35S. The experimental evidence supporting this idea is as follows. If the various size classes of poly(A)⁺ RNA were produced only by random nicking at various points on the RNA, one would expect that all of these poly(A)⁺ RNAs would be colinear, having the same 3′ terminus [poly(A) end] and extending farther toward the original 5′ end as their lengths increase. In other words, as the RNA size decreased, more and more sequences from the 5′ end of the full-size virion RNA would be missing. Thus, the *gag* gene would be eliminated before the *pol* gene as the size of poly(A)⁺ RNA decreased. However, if we presume that P180 represents the *gag-pol* fused protein, the translation data are inconsistent with the above-described consequences of random nicking of virion RNA. Full-length 35S virion RNA translated into P75, P105, and P180 (Fig. 2A). From the peptide analysis, it appears that P105 is an intermediate protein between P75 and P180, implying that the extra sequence in P105 as compared with P75 is encoded by sequences at the 3′ end of the *gag* gene. Therefore, it was quite surprising to find that as the poly(A)⁺ RNA size decreased to about 26S to 28S (Fig. 2A, lanes 9 and 10), a large amount of P75 was still translated from it but no P105 or P180 was obtained. Although these polyproteins have not been characterized fully yet and the organization of the genomic sequences coding for them is tentative, it seems reasonable to speculate that these 26S RNAs contained the *gag* gene at their 5′ ends and intact 3′ ends [since they were poly(A) selected], but some portion in the middle starting from the 3′ end of the *gag* gene had been deleted from them. However, we should emphasize that the above-described conclusions need to be confirmed by structural characterizations of these RNAs, namely, by testing whether these RNAs have the same 5′ ends as 35S RNA and whether they hybridize with *pol*-specific complementary DNA.

Since internal initiation codons in a polycistronic mRNA are not used by eucaryotic ribosomes (10), the *env* polyprotein, gp70, was not translated from the full-size poly(A)⁺ virion RNA, although the sequences coding for the protein must have been present in this RNA. We previously reported that non-poly(A)-selected RNA of about 24S was translated into P75 and P105 but no gp70 was produced (24). Figure 2 shows that the *gag* polyproteins must have been synthesized from poly(A)⁻ 24S RNA, since poly(A)⁺ 24S RNA did not give rise to either P75 and P105 or gp70 (Fig. 2B, lane 2). However, a 24S poly(A)⁺ mRNA has been identified as coding for gp70 in MuMTV-producing cells (24). Other proteins obtained from translating virion RNAs of different sizes (namely, proteins of 85,000 and 55,000 daltons and some smaller proteins) are yet to be characterized.

The fact that m^7GTP inhibited the synthesis of all proteins resulting from translation of 35S poly(A)⁺ RNA made it unlikely that the smaller proteins were translated from internal genes present at the 5′ ends of shortened RNAs, which were produced by degradation of 35S RNA during the incubation for translation because these presumptive shorter RNAs would not have caps at their 5′ ends and their translation would not

be inhibited by m^7GTP. The most plausible explanation for the synthesis of all of these proteins is that they all initiate at a common site on the mRNA but then chain elongation is terminated at several points along the mRNA's, giving rise to this family of proteins. These multiple terminations could be either due to premature release of ribosomes at certain "weak" points on the mRNA or due to occasional read-through of legitimate termination signals on the mRNA. It is also possible that we are dealing with a family of mRNA's of very similar sizes but containing deletions of different termination signals. In addition, for the proteins smaller than P75, the possibility that they are the products of post-translational proteolytic cleavage has not been ruled out. However, for P180, P105, and P75 kinetic experiments definitely have established that they are synthesized independently.

The tryptic peptide mapping of the polyproteins made in vivo and in vitro established that they shared most major peptides. P75 made in vivo and in vitro contained seven common peptides, but the mobilities of three other peptides were different. It is probable that these peptides contain sites of post-translational modification in vivo. P28 is known to be phosphorylated in vivo, and two of these three different peptides were contributed by p28 (Table 1). However, Dahl and Dickson observed that only one peptide migrated differently between P75 made in vitro and P75 made in vivo (4). P75 made in vivo contained seven peptides contributed by the mature proteins P10, P14, and P28. In principle, the extra methionine-labeled peptides present in P10 and P28 (Fig. 7) could be converted to the extra peptides (peptides 2, 6, and 26) in P75 if p23 was introduced inbetween P10 and P28 according to the intracistronic *gag* gene order (6, 11). It is not clear whether P75 contains any extra methionine-containing tryptic peptide(s) in addition to those contained in the four mature viral *gag* proteins.

P105 seems to contain all of the methionine-containing tryptic peptides present in P75. This was true for both in vivo- and in vitro-synthesized proteins. A similar observation was made by Dahl and Dickson (4). However, P105 made in vitro and P105 made in vivo differ in some peptide contents. It remains to be seen whether this was due to different post-translational modifications. Although it seems that P75 is contained within P105, it is not known whether the extra sequences present in P105 are toward the C-terminal or N-terminal side of P75. It is possible that the virion RNA was extracted from a mixture of defective and nondefective MuMTV virions. However, if translations of P75 and P105 from separate mRNA's of very similar sizes are ruled out, one has to postulate that P105 was synthesized by extension of P75 at the C terminus. Otherwise, about 1,000 nucleotides, coding for the extra 30,000-dalton protein, has to be on the 5′ side of the *gag* gene coding for P75. Moreover, internal initiation has to be invoked for the synthesis of P75. The strongest argument for the hypothesis that the extra peptides in P105 are on the C-terminal side of P75 came from the tryptic peptide analysis. Four methionine-containing tryptic peptides present in P105 were absent from P75 but were present in P180 (Table 1). Since it is presumed that P180 is a fused *gag-pol* product, it seems that P105 is an intermediate in this read-through process. One can speculate from the molecular weights of the polyproteins and the molecular weight of the reverse transcriptase that P105 covers the coding potential of the nucleotides right up to the beginning of the *pol* gene. As mentioned above, P180 contained all of P105, along with a number of extra methionine-containing tryptic peptides. The molecular mechanism involved in regulating the relative amounts of P75, P105, and P180 synthesized from 35S RNA remains to be understood.

We have reported previously that GR mammary tumor cells contain a 14S to 18S size class of MuMTV-related poly(A)$^+$ RNA (24). Robertson and Varmus detected this species in many other cells infected with MuMTV (18). According to the data of these authors, this species originates from the 3′ end of the virion RNA and contains no spliced sequence at its 5′ end. The latter finding is surprising if this is a bona fide MuMTV-coded mRNA since in Rous sarcoma viruses both the *env* mRNA and the *src* mRNA have a spliced sequence at their 5′ ends (3). MuMTV *env* mRNA also has a 5′ spliced sequence (18). It is not known whether the MuMTV genome contains any transforming gene. However, if any such gene exists, it seems from the present state of knowledge about other transforming retroviruses that the gene would be located at the 3′ side of the *env* gene. Therefore, this 14S intracellular MuMTV-related poly(A)$^+$ RNA is an attractive candidate for the putative mRNA for the transforming protein. Whether it codes for any protein could not be directly tested, however, since many other cellular poly(A)$^+$ RNAs of the same size were present in the cells. Rous sarcoma virus *src* mRNA codes for a 60,000-dalton protein. It has been shown that Rous sarcoma virions package poly(A)$^+$ RNAs which can be translated into *src* proteins (15). Similarly, murine sarcoma viruses also package RNAs which can be translated into the transforming proteins in vitro (14, 26). Therefore, we decided to test for the coding

potential of 14S poly(A)$^+$ RNAs isolated from MuMTV virions. As Fig. 2 shows, these RNAs were very efficient messages in vitro and were translated into four prominent proteins. Similar observations have been made by Dickson (personal communication). Among the four, P1 and P2 (the larger ones) were more prominent than P3 and P4. We established that all four share some common peptides and, specifically, that P1 and P2 seem to be very closely related. These proteins are probably not related to any structural proteins of MuMTV, as suggested by the immunoprecipitation data and the tryptic peptide mapping data. However, the possibility that these proteins are derived from the 3′ end of the *env* gene and contain sequences common to gp36 has not been rigorously ruled out. What is the nature of the RNAs which code for these proteins? It appears that there are three possibilities. First, these could be bona fide MuMTV mRNA's which code for some nonstructural proteins and fortuitously are packaged in the virions; second, they could be cellular mRNA's randomly packaged in the virions and may have no role in MuMTV gene expression; and third, they may originate from the 3′ end of the full-size virion RNA by random nicking during isolation of the RNA. The third possibility seems to be the most viable one for the following reasons. If these were authentic mRNA's, one would expect them to be capped, and therefore their translation would be inhibited by m^{7}GTP. This was not the case, as shown in Fig. 3. Furthermore, the preponderance of these RNAs in the virions, as inferred from the efficient translation of the small proteins from unfractionated total virion RNA, argued strongly against the hypothesis that these RNAs are randomly packaged cellular mRNA's. Another observation strongly favored the idea that these RNAs are produced by nicking of the full-size virion RNA; namely, our preliminary experiments showed that poly(A)$^+$ 35S RNA did not translate into the small proteins as such, but when the same RNA was randomly degraded by heating at a slightly alkaline pH, poly(A)$^+$ RNAs could then be selected from the resulting RNA population and were translated into P1, P2, P3, and P4 (data not shown). The above-described observations suggest that the RNAs coding for these small proteins are artificially produced from the MuMTV genomic RNA during its isolation, but nonetheless they also provide evidence for the first time that the MuMTV genome contains a potentially translatable gene in an open reading frame situated between the *env* gene and the 3′ terminus. Whether this gene is expressed in cells under physiological conditions and, if it is expressed, what the function of its products is remain to be examined in the future.

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