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Suppression of Murine Retrovirus Polypeptide Termination: Effect of Amber Suppressor tRNA on the Cell-Free Translation of Rauscher Murine Leukemia Virus, Moloney Murine Leukemia Virus, and Moloney Murine Sarcoma Virus 124 RNA

EDWIN C. MURPHY, JR.,[1]* NORMA WILLS,[2] AND RALPH B. ARLINGHAUS[1]

Department of Biology, University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77030,[1] and Howard Hughes Medical Institute, Department of Biology, University of Utah, Salt Lake City, Utah 84132[2]

The effect of suppressor tRNA's on the cell-free translation of several leukemia and sarcoma virus RNAs was examined. Yeast amber suppressor tRNA (amber tRNA) enhanced the synthesis of the Rauscher murine leukemia virus and clone 1 Moloney murine leukemia virus $Pr200^{gag\text{-}pol}$ polypeptides by 10- to 45-fold, but at the same time depressed the synthesis of Rauscher murine leukemia virus $Pr65^{gag}$ and Moloney murine leukemia virus $Pr63^{gag}$. Under suppressor-minus conditions, Moloney murine leukemia virus $Pr70^{gag}$ was present as a closely spaced doublet. Amber tRNA stimulated the synthesis of the "upper" Moloney murine leukemia virus $Pr70^{gag}$ polypeptide. Yeast ochre suppressor tRNA appeared to be ineffective. Quantitative analyses of the kinetics of viral precursor polypeptide accumulation in the presence of amber tRNA showed that during linear protein synthesis, the increase in accumulated Moloney murine leukemia virus $Pr200^{gag\text{-}pol}$ coincided closely with the molar loss of $Pr63^{gag}$. Enhancement of $Pr200^{gag\text{-}pol}$ and $Pr70^{gag}$ by amber tRNA persisted in the presence of pactamycin, a drug which blocks the initiation of protein synthesis, thus arguing for the addition of amino acids to the C terminus of $Pr63^{gag}$ as the mechanism behind the amber tRNA effect. Moloney murine sarcoma virus 124 30S RNA was translated into four major polypeptides, $Pr63^{gag}$, P42, P38, and P23. In the presence of amber tRNA, a new polypeptide, $Pr67^{gag}$, appeared, whereas $Pr63^{gag}$ synthesis was decreased. Quantitative estimates indicated that for every 1 mol of $Pr67^{gag}$ which appeared, 1 mol of $Pr63^{gag}$ was lost.

Murine retrovirus protein synthesis in infected cells appears to involve several mRNA's. The viral core proteins (*gag*) are synthesized from full-length (35S) subunit RNA (2, 14, 21, 24, 34). The viral reverse transcriptase is also translated from 35S RNA through the synthesis and subsequent cleavage of a 200,000-dalton (200K) precursor that contains both the *gag* and *pol* gene products (14, 19, 22, 24, 27). In the synthesis of envelope proteins, some of the viral 35S RNA transcript is apparently processed in the nucleus (32) to yield a 22S RNA, a step that involves splicing a stretch of several hundred nucleotides from the extreme 5′ end of the 35S RNA onto the nucleotide sequence coding for the *env* gene product in order to yield a functional *env* mRNA (30). In avian sarcoma viruses, the *src* gene product, $pp60^{src}$, is synthesized from a 21S RNA derived from the 3′ one-third of the genome (5, 13, 28, 29).

With the exception of the *pol* precursor, it appears that each viral RNA species serves as an mRNA for the gene at its 5′ end (21, 25, 29, 35). The *pol* precursor in the murine system is synthesized at about 0.025 the rate of the *gag* precursor, apparently from the same mRNA, suggesting that some kind of translational control must take place. It has been hypothesized that the mechanism which controls the relative molar amounts of *gag* and *pol* precursors depends on the infrequent suppression of a termination codon at the *gag* gene-*pol* gene junction (12, 19, 22), just as the synthesis of the Qβ A1 protein depends on the suppression of a normal termination codon (36). Alternatively, the *gag* and *gag-pol* mRNA's, although the same length, could be different in base sequence at the proposed termination codon.

Recently, Philipson et al. (27) reported that the use of an amber suppressor tRNA during

cell-free synthesis of clone 2 Moloney murine leukemia virus (Mo-MuLV) proteins enhanced the production of $Pr180^{gag\text{-}pol}$ at the expense of their $Pr78^{gag}$ polypeptide. In contrast, Weiss et al. (37) reported that amber suppressor tRNA did not augment the synthesis of the avian sarcoma virus *pol* precursor, $Pr180^{gag\text{-}pol}$.

We undertook the present study in order to elucidate further the steps involved in the synthesis of the reverse transcriptase precursor $Pr200^{gag\text{-}pol}$. Our results indicate that amber suppression of polypeptide termination can lead to enhanced synthesis of the joint *gag-pol* polypeptide, confirming the initial report by Philipson et al. (27). However, our results differ in that enhanced syntheses of Rauscher MuLV (R-MuLV) and Mo-MuLV clone 1 $Pr200^{gag\text{-}pol}$ occurred at the expense of $Pr65^{gag}$ and $Pr63^{gag}$, respectively, and not the larger *gag* gene product. Some of our results raise the question of whether virion 35S RNA may consist of more than one chemically different species.

MATERIALS AND METHODS

Cells and viruses. For the production of R-MuLV, virus-infected National Institutes of Health Swiss mouse cells (JLS-V16) were grown in a modified Eagle medium in 2-quart (ca. 1.89-liter) roller bottles as described previously (33). Both Mo-MuLV clone 1-infected cells (a generous gift from Karen Beemon) and Moloney murine sarcoma virus 124 (Mo-MuSV 124)-infected cells (a generous gift from Peter Duesberg and also Judy Ball) were grown in roller cultures in McCoy 5A medium containing 15% fetal calf serum. The Mo-MuSV 124-infected cells (clone G8-124) produce sarcoma virus against a low background of helper leukemia virus (1, 4, 8). Virus was harvested from the culture fluids by polyethylene glycol precipitation and was purified by isopycnic banding in 15 to 60% linear sucrose gradients (33).

Uninfected JLS-V16 cells grown in a modified Eagle medium (33) were the source of the S-10 extracts used in the cell-free protein synthesis assays.

Isolation of viral nucleic acids. Viral RNA was isolated from purified virus by sodium dodecyl sulfate-phenol-chloroform extraction as described previously (19, 22), except that the virus was exposed to 100 μg of proteinase K per ml in 10 mM Tris (pH 7.5)-100 mM LiCl-1 mM EDTA-0.5% sodium dodecyl sulfate for 15 min at 37°C before the organic extractions. Genome sized (70S) RNA was isolated from the R-MuLV and Mo-MuLV nucleic acids by centrifugation in a 5 to 25% sucrose gradient containing 10 mM sodium acetate (pH 5.1), 100 mM LiCl, 1 mM EDTA, and 0.1% sodium dodecyl sulfate for 18 h at 15,000 rpm and 40°C. To obtain genome size RNA from Mo-MuSV 124, the centrifugation speed was increased to 18,000 rpm. Subunit size (35S for R-MuLV and Mo-MuLV, 30S for Mo-MuSV 124) RNA was isolated by sucrose gradient centrifugation as described previously (19, 22). Mengovirus 35S RNA was a generous gift from George Thornton, Abilene Christian University.

Cell-free protein synthesis. The cell-free protein synthesis system and its preparation have been described previously (19). In essence, it contained micrococcal nuclease-treated (26) extract from uninfected JLS-V16 cells supplemented with a 0.5 M KCl wash of rabbit reticulocyte polyribosomes (10). Viral RNA was usually added at 40 μg/ml, and protein synthesis was allowed to proceed for 150 min at 30°C. When necessary, crude yeast suppressor-minus tRNA, amber suppressor tRNA, or ochre suppressor tRNA (a generous gift from Ray Gesteland) was added to a concentration of 20 to 40% μg/ml in the assay. The suppressor RNAs were isolated from total yeast RNA and enriched for serine tRNA by sequential benzoyl-DEAE-cellulose chromatography and Sepharose 4B chromatography (8, 9). The final pool was about 50% pure with respect to serine acceptor activity. However, the actual amount of suppressor tRNA was probably only a small fraction of the total RNA in the pool.

Immunoprecipitation. The cell-free mixture was adjusted to 0.5% Nonidet P-40 and 0.5% deoxycholate in buffer containing 20 mM Tris (pH 7.5) and 50 mM NaCl. The specific immunoprecipitation procedures and "clearing" reactions done to reduce nonspecific immunoprecipitation have been described previously (21).

Gel electrophoresis. Polyacrylamide gel electrophoresis in 6 to 12% linear gradient gel slabs (or 11.25% slabs) in 0.1% sodium dodecyl sulfate was performed by using the buffer system described by Laemmli (15). Fluorography was performed on the dried gels by the method of Bonner and Laskey (3). A linear response to radioactivity was obtained by preflashing the X-ray film (16). In some experiments, EN^{3}HANCE (New England Nuclear Corp.) was used instead of dimethyl sulfoxide-2,5-diphenyloxazole (PPO) to impregnate the gels with fluor.

Quantitative estimates of the amount of radioactivity in individual polypeptides were obtained by excising gel slices containing these polypeptides from the gel slabs. Each slice was hydrated with 100 μl of water and incubated in 5 ml of NCS counting fluor (10 ml of NCS solubilizer, 4.4 ml of Scinti-prep (Fisher Scientific Co.), 85.6 ml of toluene) at 37°C for 16 h. After chilling, the radioactivity in each slice was determined and divided by the apparent molecular weight of the polypeptide to give an estimate of approximate molar yield.

RESULTS

Translation of R-MuLV, Mo-MuLV clone 1, and Mo-MuSV 124 RNAs. Subunit size RNA from each of the virions was isolated as described previously (19, 22) and translated in vitro. As we have shown previously, R-MuLV 35S RNA is translated (Fig. 1, lane A) into major polypeptides of 65K ($Pr65^{gag}$), 75K ($Pr75^{gag}$), and 200K ($Pr200^{gag\text{-}pol}$), which are immunologically and structurally identical to their intracellular counterparts (19, 20).

Mo-MuLV clone 1 RNA was translated into polypeptides of 63K, 70K, and 200K; these are designated $Pr63^{gag}$, $Pr70^{gag}$, and $Pr200^{gag\text{-}pol}$, re-

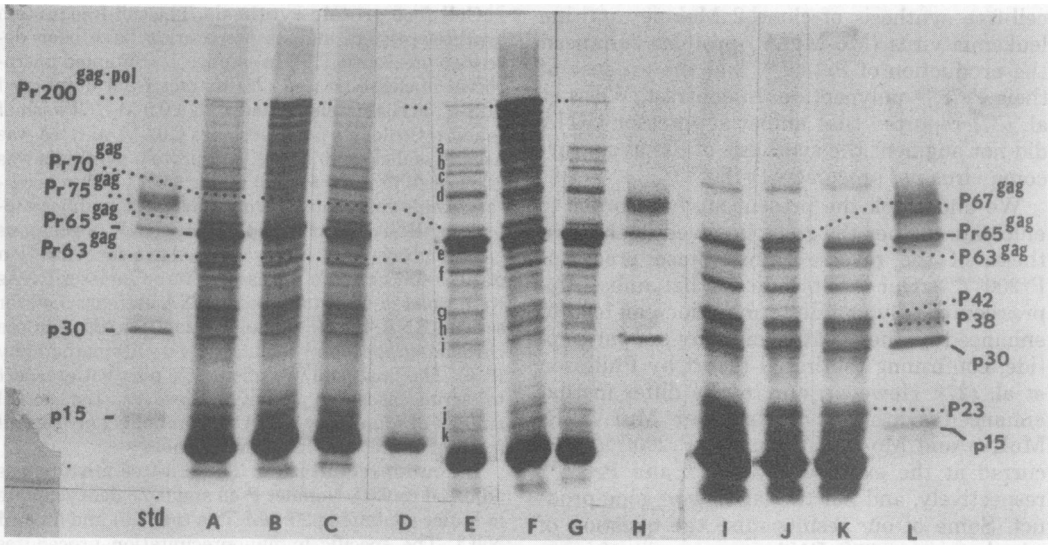

FIG. 1. *Cell-free translation of R-MuLV 35S RNA, Mo-MuLV clone 1 35S RNA, and Mo-MuSV 124 30S RNA in the presence and absence of suppressor tRNA's. Cell-free products of virion RNAs, which were synthesized in the presence and absence of suppressor RNAs, were analyzed on 6 to 12% polyacrylamide gels. Lane A, R-MuLV 35S RNA, suppressor-minus tRNA; lane B, R-MuLV 35S RNA, amber suppressor; lane C, R-MuLV 35S RNA, ochre suppressor; lane D, no RNA, suppressor-minus tRNA; lane E, Mo-MuLV 35S RNA, suppressor-minus tRNA; lane F, Mo-MuLV 35S RNA, amber suppressor; lane G, Mo-MuLV 35S RNA, ochre suppressor; lane I, Mo-MuSV 124 30S RNA, suppressor-minus tRNA; lane J, Mo-MuSV 124 30S RNA, amber suppressor; lane K, Mo-MuSV 124 30S RNA, ochre suppressor; lanes H and L, standards of R-MuLV-specific polypeptides prepared by exposure of a culture of R-MuLV-infected JLS-V16 cells to 50 μCi of ^{14}C-amino acids per ml for 20 min, followed by a chase incubation for 40 min and then by immunoprecipitation of the viral polypeptides from the cell extract by anti-R-MuLV.*

spectively (Fig. 1, lane E). Each of these polypeptides was immunoprecipitable with anti-R-MuLV p30, p15, p12, and p10 (Fig. 2A, lanes B through E). In addition, the Mo-MuLV Pr200$^{gag\text{-}pol}$ polypeptide was also precipitable with antibody against the R-MuLV reverse transcriptase (Fig. 2B, lane B). Additional polypeptides of 130K, 120K, 110K, 95K, 60K, 52K, 37K, 32K, 29K, 18K, and 16K were also observed (Fig. 1, lane E). The 95K to 130K polypeptides were precipitable with anti-p30 and anti-*pol* (Fig. 2B, lanes A and B), whereas the 16K to 60K series were *gag* but not *pol* related (Fig. 2A and Fig. 2B, lane B).

Mo-MuSV 124 30S RNA was also translated. Among the polypeptides synthesized (Fig. 1, lane K) were P63^{gag}, P42, P38, and P23. As its designation suggests, in vitro P63^{gag} appears to be identical to P63^{gag} found in Mo-MuSV 124-infected cells in that it contains antigenic determinants and tryptic peptides characteristic of p30, p15, and p12 (D. D. Lyons, E. C. Murphy, S.-M. Mong, and R. B. Arlinghaus, Virology, in press). The Mo-MuSV 124 P42 and P38 polypeptides synthesized in vitro are *gag* related, but they may also be related to P23. P23, however, does not appear to be related to either the *gag*, *pol*, or *env* gene product (Lyons et al., in press).

Effect of suppressor tRNA's on the translation of R-MuLV, Mo-MuLV clone 1, and Mo-MuSV 124 RNAs. In our translation system, R-MuLV and Mo-MuLV RNAs responded in a qualitatively similar way to amber tRNA. The magnitude of the response was dose dependent: 0.5 to 1.0 μg of amber tRNA per 25-μl assay was optimal (data not shown). Figure 1, lanes B and F, show the cell-free products of R-MuLV and Mo-MuLV clone 1 35S RNAs, respectively, when they were synthesized under conditions of amber suppression. Quantitative estimates (Table 1) showed that amber tRNA stimulated the synthesis of R-MuLV Pr200$^{gag\text{-}pol}$ by four- to fivefold, depressed the synthesis of R-MuLV Pr75^{gag} slightly, and depressed the synthesis of Pr65^{gag} by about 50%. Amber tRNA also increased the intensity of the numerous polypeptides found in the 75K to 200K region (Fig. 1, lane B). Translation of Mo-MuLV 35S in amber tRNA (Fig. 1, lane F) resulted in a 45-fold stimulation of Pr200$^{gag\text{-}pol}$, a significant decrease in Pr63^{gag} accumulation, and an increase in Pr70^{gag}

(Table 1). In contrast to these results, ochre suppressor tRNA was ineffective in stimulating R-MuLV or Mo-MuLV Pr200$^{gag\text{-}pol}$ synthesis. When ochre tRNA was used, the accumulations of R-MuLV Pr200$^{gag\text{-}pol}$ (Fig. 1, lane C) and Mo-MuLV Pr200$^{gag\text{-}pol}$ (Fig. 1, lane G) were identical to the accumulations found in translations done in the presence of suppressor-minus tRNA (Fig.

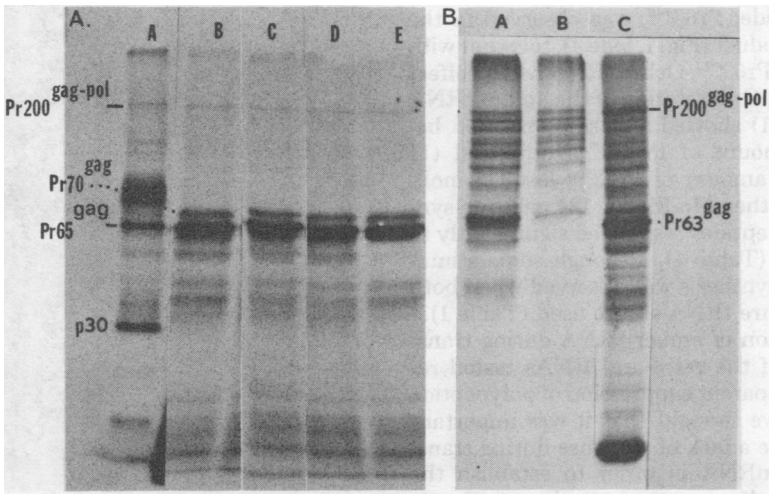

FIG. 2. *(A) Immunoprecipitation of the cell-free product of Mo-MuLV clone 1 RNA. The cell-free product of Mo-MuLV clone 1 35S RNA was immunoprecipitated with various antisera directed against viral polypeptides, and the immunoprecipitates were analyzed on 6 to 12% polyacrylamide gels. Lane A, R-MuLV polypeptide standard, as described in the legend to Fig. 1; lane B, anti-p30; lane C, anti-p15; lane D, anti-p12; lane E, anti-p10. (B) Immunoprecipitation of the Mo-MuLV 35S RNA cell-free product synthesized in the presence of amber suppressor with anti-reverse transcriptase serum. The cell-free product of Mo-MuLV 35S RNA synthesized in the presence of amber suppressor was immunoprecipitated with anti-R-MuLV p30 and anti-R-MuLV reverse transcriptase. Lane A, the Mo-MuLV cell-free product immunoprecipitated with anti-p30; lane B, Mo-MuLV cell-free product immunoprecipitated with anti-reverse transcriptase serum; lane C, original Mo-MuLV cell-free product.*

TABLE 1. *Quantitation of the effect of suppressor tRNA's on cell-free translation of R-MuLV, Mo-MuLV, and Mo-MuSV 124 RNAs*

Polypeptide	Amt of polypeptide synthesized (mol) under the following conditions:				
	Suppressor-minus tRNA (su$^-$)	Amber suppressor tRNA (amber)	Ochre suppressor tRNA (ochre)	Amber/su$^-$	Ochre/su$^-$
R-MuLV Pr200$^{gag\text{-}pol}$	6.5[a,b]	29.3	4.8	4.5	0.8
R-MuLV Pr75^{gag}	75[b]	69	111	0.9	1.5
R-MuLV Pr65^{gag}	710[b]	372	722	0.5	1.00
Mo-MuLV Pr200$^{gag\text{-}pol}$[c]	2.0	90.0	1.4	45	0.7
Mo-MuLV Pr70^{gag}	27	87	29	3.2	1.0
Mo-MuLV Pr63^{gag}	547	431	512	0.7	0.9
Mo-MuSV Pr67^{gag}	6.6	18	5.2	2.7	0.8
Mo-MuSV Pr63^{gag}	33	18	32	0.6	1.0
Mo-MuSV P42/P38	87	86	118	1.0	1.3
Mo-MuSV P23	157	262	255	1.6	1.6

[a] These data were obtained by dividing the radioactivity in a gel slice by the apparent molecular weight of the polypeptide. For the purposes of this analysis, an even distribution of methionine residues in the polypeptides was assumed.

[b] The amount of radioactivity in 6.5 mol of Pr200$^{gag\text{-}pol}$ was 1,301 cpm, in 75 mol of Pr75^{gag} it was 5,600 cpm, and in 710 mol of Pr65^{gag} it was 46,173 cpm. Background radioactivity in a gel slice was about 200 cpm.

[c] In many experiments with amber suppressor tRNA, but not ochre tRNA, cell-free translation was stimulated by 10 to 25%. We presume that this increase was due to the more efficient translation of the *pol* gene under these conditions, which occurred at the same time that the efficiency of re-initiation was decreasing. The quantitative results presented in this table were obtained from the cells shown in Fig. 4A and B.

1, lanes A and E). Quantitative estimates (Table 1) confirmed the ineffectiveness of ochre tRNA.

Mo-MuSV 124 RNA translation was also responsive to amber tRNA and unresponsive to ochre tRNA. In the presence of amber tRNA, a new polypeptide, $Pr67^{gag}$, was observed in the translation product (Fig. 1, lane J), together with a loss of some $Pr63^{gag}$. Ochre tRNA had no effect (Fig. 1, lane I). Quantitation of the amber tRNA effect (Table 1) showed a close correlation between the amount of $Pr67^{gag}$ synthesized (11 mol) and the amount of $Pr63^{gag}$ lost (15 mol). None of the other Mo-MuSV 124 cell-free-synthesized polypeptides responded significantly to amber tRNA (Table 1), although some stimulation of P23 synthesis was observed when both amber and ochre tRNA's were used (Table 1).

Since addition of amber tRNA during translation of all of the retroviral RNAs tested resulted in an apparent suppression of polypeptide termination, we thought that it was important to demonstrate a lack of response during translation of an mRNA in order to establish the specificity of the response. As Fig. 3 shows, translation of mengoviral 35S RNA in the presence of amber tRNA (Fig. 3, lane C) resulted in a cell-free product no different than that produced in its absence (Fig. 3, lane D).

Kinetics of suppression of polypeptide termination by amber tRNA. The rates of accumulation of the various Mo-MuLV polypeptides during amber suppression were examined in an effort to determine more precisely the role and fate of each viral polypeptide. Additional interest was taken in this experiment because of the possibility of determining, under conditions where $Pr200^{gag\text{-}pol}$ synthesis was stimulated 20- to 40-fold, whether any post-translational processing of $Pr200^{gag\text{-}pol}$ was occurring. Figures 4 and 5 show the results of an experiment in which samples of a translation mixture programmed with Mo-MuLV 35S RNA and suppressor-minus tRNA (Fig. 4, panel B) or amber tRNA (Fig. 4, panel A) were removed at varying times and analyzed by gel electrophoresis. $Pr63^{gag}$ synthesis was first evident after about 15 min of translation, and $Pr200^{gag\text{-}pol}$ synthesis was evident after 60 min, thus giving the approximate ribosome transit time for each and the rate of translation in the system (about 45 amino acids per min). $Pr63^{gag}$ and $Pr70^{gag}$ first appeared about 45 min ahead of $Pr200^{gag\text{-}pol}$, strongly arguing that they are not products of post-translational processing of $Pr200^{gag\text{-}pol}$. Similarly, since all of the polypeptides in the 75K to 200K range appeared before $Pr200^{gag\text{-}pol}$, it also appears unlikely that these arise by processing of $Pr200^{gag\text{-}pol}$.

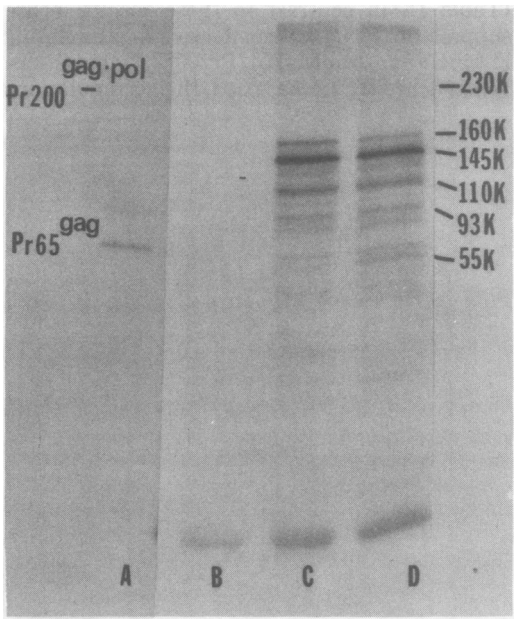

FIG. 3. *Translation of mengoviral 35S RNA in the presence of amber suppressor tRNA. The cell-free product of mengoviral 35S RNA, which was synthesized in the presence and absence of amber suppressor tRNA, was analyzed on a 6 to 12% polyacrylamide slab gel. Lane A, R-MuLV polypeptide standard as described in the legend to Fig. 1; lane B, no RNA, suppressor-minus tRNA; lane C, mengoviral RNA, suppressor-minus tRNA; lane D, mengoviral RNA, amber suppressor tRNA.*

Quantitative data on the accumulation of Mo-MuLV polypeptides during amber suppression were obtained by determining the radioactivity in gel slices containing individual polypeptides. Assuming an even distribution of methionine residues, estimates of relative molar amounts of these polypeptides were made by dividing the radioactivity in a polypeptide by its apparent molecular weight. Figure 5 shows the results of this kind of analysis on the polypeptide accumulation pattern shown in Fig. 4A and B. Under suppressor-minus conditions $Pr63^{gag}$ accumulated in a nearly linear fashion for 2.5 h (Fig. 4A). In contrast, during amber suppression $Pr63^{gag}$ accumulated in a linear fashion only during the first hour of translation. During the remaining 90 min, the level of $Pr63^{gag}$ remained nearly constant (Fig. 5). This result suggests that suppression is more effective late in translation than at earlier times. $Pr70^{gag}$ synthesis was stimulated by amber tRNA during the first hour of translation but declined to suppressor-minus levels at later times (Fig. 5). As shown above, $Pr200^{gag\text{-}pol}$ synthesis was greatly stimulated by amber suppression (Fig. 5).

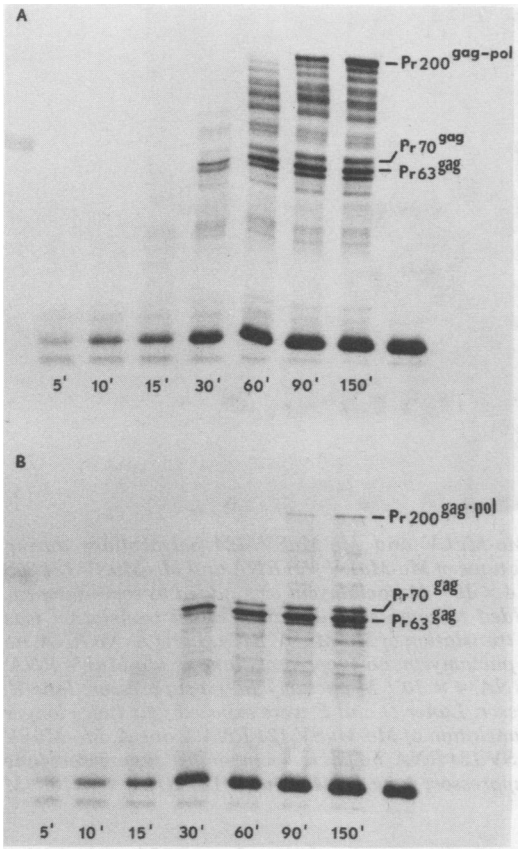

FIG. 4. *Time-dependent accumulation of Mo-MuLV polypeptides in the presence and absence of amber suppressor tRNA. Mo-MuLV 35S RNA was translated in a 50-μl assay in the presence and absence of amber suppressor tRNA. At the times indicated across the bottoms of the gels, 6 μl was removed for analysis by electrophoresis in 6 to 12% polyacrylamide gels. (A) Translation in the presence of amber suppressor tRNA. The cell-free product was analyzed by gel electrophoresis after 5, 10, 15, 30, 60, 90, and 150 min of translation. (B) Translation in the absence of suppressor tRNA. The cell-free product was analyzed by gel electrophoresis after 5, 10, 15, 30, 60, 90, and 150 min of translation.*

Amber suppressor during blockade of polypeptide chain initiation. We next performed experiments designed to test whether the effect of amber tRNA involved the addition of amino acids to the C terminus of Mo-MuLV $Pr63^{gag}$ to form both $Pr70^{gag}$ and $Pr200^{gag\text{-}pol}$. Since the *pol* gene is situated on the 3' side of the *gag* gene, which itself is at the extreme 5' end of the genome, it was considered very unlikely that amber suppression to yield a 200K polypeptide could involve anything but the addition of *pol* polypeptide sequences to $Pr63^{gag}$. Immunoprecipitation of the 200K polypeptide produced during amber suppression by both anti-p30 (Fig. 2B, lane A) and anti-*pol* (Fig. 2B, lane B) provided convincing evidence that it was, in fact, $Pr200^{gag\text{-}pol}$ produced by the addition of amino acids to the C terminus of $Pr63^{gag}$.

Analysis of the stimulation of $Pr70^{gag}$ synthesis by amber tRNA showed that $Pr70^{gag}$ was actually a closely spaced doublet (Fig. 4). Under suppressor-minus conditions, the lower band predominated (Fig. 4B), whereas in the presence of amber tRNA the upper band was stimulated (Fig. 4A). This effect is not easily visible in Fig. 4, but it was readily apparent in the X-ray film and was reproducible in all of our experiments. It seemed possible that amber tRNA was suppressing a termination site either just ahead or just beyond the *gag* gene to produce $Pr70^{gag}$. To distinguish between these possibilities, we used a drug that blocks polypeptide chain initiation within 2 min of its addition (17). In this experiment, 4×10^{-7} M pactamycin (17, 37) was added after 5 min of protein synthesis. After another 5 min, 1 μg of amber tRNA was added. Under these conditions, both $Pr200^{gag\text{-}pol}$ and the "upper" $Pr70^{gag}$ polypeptide can be formed only

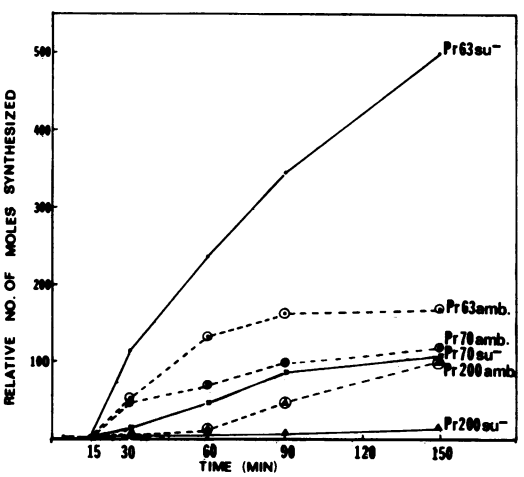

FIG. 5. *Quantitation of the accumulation of Mo-MuLV polypeptides in the presence and absence of amber suppressor tRNA. The accumulations of individual polypeptides in the fluorographs shown in Fig. 4 were quantitated by determining the radioactivity in each polypeptide at each time point. The radioactivity in each polypeptide was divided by its apparent molecular weight to give an estimate of the amount accumulated. The relative accumulations of $Pr63^{gag}$ in the presence (⊙) and absence (●) of amber suppressor are shown, as are similar values for $Pr70^{gag}$ in the presence (⊗) and absence of amber suppressor and for $Pr200^{gag\text{-}pol}$ in the presence (◉) and absence (▲) of amber suppressor.*

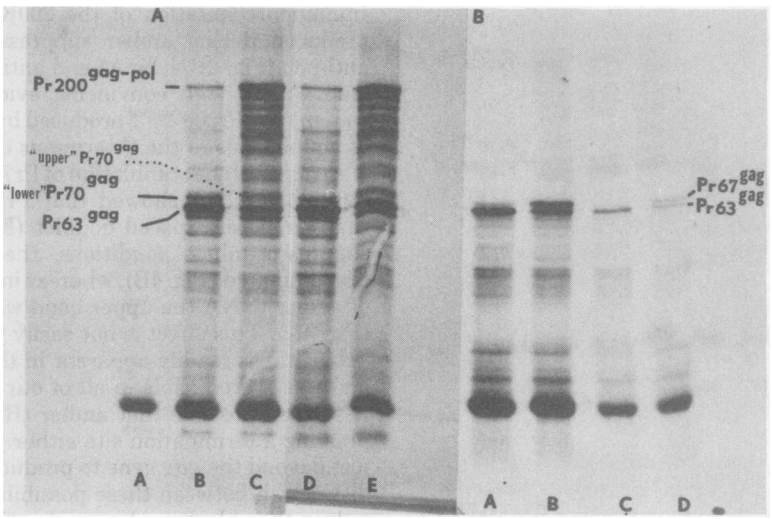

FIG. 6. *Amber suppression of the termination of Mo-MuLV and Mo-MuSV 124 polypeptides during blockade of polypeptide chain initiation. Cell-free translations of Mo-MuLV 35S RNA and Mo-MuSV 124 30S RNA were started as usual. After 5 min of translation, 4×10^{-7} M pactamycin was added to some samples. After another 5 min, amber suppressor tRNA was added to some samples, after which translation was allowed to proceed for 2.5 h. (A) Pactamycin effect of the translation of Mo-MuLV RNA. Lane A, No RNA, no pactamycin, no suppressor; lane B, Mo-MuLV RNA, no pactamycin, no suppressor; lane C, Mo-MuLV RNA, no pactamycin, amber suppressor; lane D, Mo-MuLV RNA, 4×10^{-7} M pactamycin, no suppressor; lane E, Mo-MuLV RNA, 4×10^{-7} M pactamycin, amber suppressor. Lanes D and E were exposed four times longer than lanes A through C. (B) Pactamycin effect on the translation of Mo-MuSV 124 RNA. Lane A, Mo-MuSV 124 RNA, no pactamycin, no suppressor; lane B, Mo-MuSV 124 RNA, no pactamycin, amber suppressor; lane C, Mo-MuSV 124 RNA, 4×10^{-7} M pactamycin, no suppressor; lane D, Mo-MuSV 124 RNA, 4×10^{-7} M pactamycin, amber suppressor.*

from previously initiated polypeptide chains. In addition, any initiation and polypeptide synthesis on the 5′ side of the *gag* gene which could have been responsible for the stimulation of the upper $Pr70^{gag}$ polypeptide would have already occurred and been terminated before the addition of amber tRNA (amber tRNA was added after 10 min of translation, whereas it would take less than 2 min to synthesize the 7K polypeptide sequence necessary to form $Pr70^{gag}$ from $Pr63^{gag}$). In this experiment, the control assays (Fig. 6A, lanes B and C) showed clearly the stimulation of $Pr200^{gag\text{-}pol}$ and the upper $Pr70^{gag}$ by amber tRNA. Figure 6A, lane E, also shows that $Pr200^{gag\text{-}pol}$ and the upper $Pr70^{gag}$ polypeptide are still enhanced by amber tRNA under conditions in which polypeptide chain initiation is blocked, arguing that the amber tRNA effect involves the addition of amino acids to the C terminus of $Pr63^{gag}$. As an alternative mechanism, our experiments do not exclude the possibility that the upper $Pr70^{gag}$ arises by suppression of the termination of the "lower" $Pr70^{gag}$. In Fig. 6A, lanes D and E were exposed to X-ray film four times longer than lanes A, B, and C to show clearly the continued enhancement of the upper $Pr70^{gag}$ polypeptide.

A pactamycin plus amber tRNA experiment identical to the one described above was performed during translation of Mo-MuSV 124 30S RNA. The results (Fig. 6B, lanes A through D) showed that the 67K polypeptide which was synthesized in amber tRNA (Fig. 6B, lane B) arose by the addition of amino acids to the C terminus of $Pr63^{gag}$ (Fig. 6B, lanes B and D).

DISCUSSION

Translation of Mo-MuLV clone 1 35S RNA in our cell-free protein synthesis system resulted in a spectrum of polypeptides similar to those described in another report (14). We observed polypeptides of 70K ($Pr70^{gag}$), 63K ($Pr63^{gag}$), 60K, 52K, 37K, 32K, and 18K, which contained only *gag* protein antigenic reactivity. By analogy with our own studies on the cell-free products of R-MuLV 35S RNA, it appears likely that the polypeptides of 60K and less are a result of premature termination of $Pr63^{gag}$ (20). We also observed polypeptides with both *gag* and *pol* immunoreactivity, the largest of which had an apparent molecular weight of 200,000 ($Pr200^{gag\text{-}pol}$) and is most probably analogous to the authentic *pol* precursor $Pr200^{gag\text{-}pol}$ found intracellularly (12, 22, 23, 24, 27). There was also

a spectrum of polypeptides in the 70K to 200K region, which contained anti-p30 and anti-*pol* immunoreactivity. These polypeptides most probably arise as a result of premature termination of $Pr200^{gag\text{-}pol}$, since tryptic mapping experiments (Murphy, unpublished data) show that they all contain tryptic peptides characteristic of the *gag* proteins, as well as progressively fewer *pol*-specific tryptic peptides, depending on their molecular weights.

Translation of Mo-MuSV 124 30S RNA resulted in the synthesis of *gag*-related polypeptides of 67K ($Pr67^{gag}$), 63K ($P63^{gag}$), 42K (P42), and 38K (P38). $P63^{gag}$ was the major *gag*-related polypeptide, containing antigenic determinants of p30, p15, p12, and p10 and tyrosine-containing tryptic peptides of all of the core proteins except p10 (Lyons et al., in press). $Pr67^{gag}$ was present in large amounts only when amber tRNA was used, and our evidence strongly suggests that it arises by suppression of the termination of $Pr63^{gag}$. It seems possible that $Pr67^{gag}$ is analogous to the $Pr70^{gag}$ observed in the cell-free product of Mo-MuLV RNA, except that deletions in the Mo-MuSV 124 genome (4, 6, 11) may limit its size to 67K instead of 70K. P42 and P38 have been found to be *gag* related when both immunological and structural criteria are used, but they may also be related to P23. The other cell-free product of Mo-MuSV 124 RNA is a 23K polypeptide (P23). Immunological and structural analyses suggest that P23 is not related to the products of the *gag, pol,* and *env* genes (Lyons et al., in press). The patterns and antigenic reactivities of the in-vitro synthesized Mo-MuSV 124 polypeptides are in general agreement with a previous report (27).

Our evidence strongly suggests that the syntheses of R-MuLV $Pr65^{gag}$, Mo-MuLV clone 1 $Pr63^{gag}$, and Mo-MuSV 124 $Pr63^{gag}$ are normally terminated by a UAG codon. However, the Mo-MuLV 35S RNA seemed to be much more readily suppressible than the R-MuLV 35S RNA. These results are in partial agreement with those of Philipson et al. (27), who observed that amber suppression of Mo-MuLV clone 2 $Pr78^{gag}$ led to the enhancement of $Pr180^{gag\text{-}pol}$ synthesis. In their experiments, $Pr65^{gag}$ was not affected by amber tRNA, arguing that release of this polypeptide occurred before the UAG codon was reached (27). In our experiments, however, $Pr63^{gag}$ seemed clearly to be the source of the increased amount of $Pr200^{gag\text{-}pol}$ under conditions of amber suppression.

The difference between our data and those of Philipson (27), although possibly trivial, leads to a consideration of whether more than one viral mRNA may exist. For both Mo-MuLV clone 1 and Mo-MuLV clone 2, $Pr63^{gag}$ ($Pr65^{gag}$) appears to be the precursor to the viral core proteins (38). In our experiments, translation of the mRNA for clone 1 $Pr63^{gag}$ appears to be suppressible and to allow the formation of $Pr200^{gag\text{-}pol}$. The larger *gag*-related polypeptide, $Pr70^{gag}$, is affected by inclusion of amber tRNA in the translation assays, but cannot be the source of the increased amount of $Pr200^{gag\text{-}pol}$. The experiments of Philipson et al. (27), however, showed clearly that clone 2 $Pr65^{gag}$ was not affected by amber tRNA. Instead, the mRNA coding for a polypeptide designated $Pr78^{gag}$ appeared to be the source of the increased amount of their $Pr180^{gag\text{-}pol}$ (equivalent to our $Pr200^{gag\text{-}pol}$). These differences between clonal isolates of Mo-MuLV suggest that in Mo-MuLV clone 1, a single mRNA may be the source of both $Pr63^{gag}$ and $Pr200^{gag\text{-}pol}$, whereas in Mo-MuLV clone 2 the mRNA for $Pr78^{gag}$ and $Pr180^{gag\text{-}pol}$ may exist independently of the mRNA for $Pr65^{gag}$. In Mo-MuLV clone 1, the mRNA coding for $Pr70^{gag}$ may be analogous to the mRNA in Mo-MuLV clone 2 which codes for $Pr78^{gag}$ and $Pr180^{gag\text{-}pol}$.

The possibility that at least two viral mRNA's may exist is made even more attractive since recent reports have made it clear that two types of *gag*-related polypeptides exist (6, 31). One type is the well-documented core protein precursor ($Pr63^{gag}$ in Mo-MuLV clone 1); the other is a glycosylated polypeptide ($gPr80^{gag}$ in Mo-MuLV clone 4A). It is not known whether these polypeptides arise from a common precursor or even from the same mRNA. Our evidence suggests, however, that translation of Mo-MuLV clone 1 RNA results in two different types of *gag* gene products, $Pr63^{gag}$ and $Pr70^{gag}$. $Pr63^{gag}$ is identical in its tryptic map to authentic intracellular $Pr63^{gag}$. $Pr70^{gag}$, although quite similar in its tryptic map to $Pr63^{gag}$, contains a tryptic peptide found in authentic glycosylated $gPr80^{gag}$ but not in $Pr63^{gag}$ (Murphy, unpublished data). This evidence, together with the different responses of $Pr63^{gag}$ and $Pr70^{gag}$ syntheses to amber tRNA, argues in favor of the existence of more than one functionally distinct mRNA species in Mo-MuLV clone 1 35S RNA. Interestingly, formation of the avian sarcoma virus $Pr180^{gag\text{-}pol}$ polypeptide is not stimulated by amber tRNA (37), suggesting that control of its synthesis is mediated in a way distinct from that of the murine virus $Pr200^{gag\text{-}pol}$.

The results presented here do not necessarily mean that $Pr200^{gag\text{-}pol}$ is normally synthesized in infected cells by a mechanism involving amber suppression. Among the several alternatives still open is the possibility that the normal low level of $Pr200^{gag\text{-}pol}$ synthesized is translated from an

mRNA whose terminator codon has been spliced out and that amber suppression simply augments that amount by allowing continued translation of the mRNA specifying $Pr63^{gag}$, an event which may not take place in vivo.

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