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Matrix Protein of Akv Murine Leukemia Virus: Genetic Mapping of Regions Essential for Particle Formation

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Type C retroviruses assemble at the plasma membrane of the infected cell. Attachment of myristic acid to the N terminus of the Gag precursor polyprotein has been shown to be essential for membrane localization and virus morphogenesis. Here, we report that the matrix (MA) protein contains regions that in conjunction with myristylation are important for Gag protein stability and the assembly of murine leukemia viruses. We identified these domains by generating a series of Akv murine leukemia virus mutants carrying small in-frame deletions within the coding region of the MA protein encompassing 129 amino acids. Studies show that mutants with deletions within the segment encoding the first 102 amino acids were all replication defective, whereas the C-terminal residues 103 to 124 seem not to have any critical function in virus maturation. Cells expressing the replication-defective genomes did not release any detectable Gag proteins. In one mutant, deletion of 3 amino acids in the N terminus resulted in an inefficiently myristylated, stable Gag polyprotein. The remaining defect genomes encoded unstable Gag proteins, although they were modified with myristic acid. The results suggest that the matrix domain plays an important role in stabilizing the Gag polyprotein.

Retroviruses are single-stranded RNA viruses with a lipoprotein envelope acquired during budding from the infected cell. The envelope, containing viral *env* glycoproteins, surrounds an icosahedral capsid composed of structural proteins encoded by the viral *gag* gene (9).

The genomes of replication-competent retroviruses comprise the major genes, *gag*, *pol*, and *env*, encoding three polyprotein precursors, which are cleaved posttranslationally into mature subunits with distinctive biochemical properties important for virus replication (8, 9). For murine leukemia virus (MuLV), the Gag polyprotein $Pr65^{gag}$ is proteolytically cleaved during virus maturation to the four Gag proteins (2, 8, 9, 29) designated, according to Leis et al. (24), NH_2-p15 (matrix protein [MA]), pp12 (unknown function), p30 (capsid protein [CA]), and p10-COOH (nucleocapsid protein [NC]). The MuLV enzymes are incorporated into virions as a $Pr200^{gag\text{-}pol}$ fusion protein (7, 39, 40, 46, 51). The *gag* gene product appears to play a critical role in the process of virus assembly. Expression of $Pr65^{gag}$ alone is sufficient for the assembly and release of noninfectious virus particles (42), and experiments have indicated that only two regions, MA and CA, are required for MuLV assembly (6, 14, 18, 22, 26, 31, 40).

MuLVs are classified as type C retroviruses, which assemble at the plasma membrane of the infected cell. The intracellular processes involved in the correct assembly of viral components prior to release from the cell have been only partly described previously. The Gag precursor proteins are translated on free polysomes and myristylated before being localized at the plasma membrane (8, 16, 37). It has been proposed that the MA domain of the Gag protein provides functions for stable membrane binding in that it has a hydrophobic character (1, 27) and is localized to the submembrane portion of the virion (12). Mutational analysis has demonstrated that attachment of myristate to the amino-terminal glycine of the matrix domain is required for plasma membrane association and particle formation (13, 22, 31, 32). However, the facts that not all Gag proteins are myristylated and that a variety of known myristylated proteins are not membrane bound (36, 41, 47) suggest that additional amino acid sequences within the MA domain are required for plasma membrane localization. Consistent with this, it was recently found that deletions within both the MA domain of Rous sarcoma virus (50) and the myristylated Gag protein of Mason-Pfizer monkey virus blocked virion formation (33). The deletion of 42 amino acids at the MA-p12 border of Moloney MuLV (Mo-MuLV) did not affect virus release. The virions were, however, impaired in the early steps of infection, presumably because of the blocked proteolytic cleavage of the P15-P12 fusion protein (6). Five amino acids from each site of the scissile bond seem to be required at the retroviral protease target site (44). The importance of the MuLV matrix domain in particle assembly was recently emphasized by studies of Gag–β-galactosidase fusion proteins showing a requirement of at least 99 amino acids from the amino terminus for plasma membrane localization and release from cells (14, 21). Here, we present a molecular genetic analysis of the matrix protein of Akv-MuLV. By using complete viral genomes, intact except for a series of deletions in the $p15^{gag}$ gene, we mapped a region within the matrix protein critical for virus assembly and maturation. Biochemical studies indicated that this region is important for Gag protein stability.

MATERIALS AND METHODS

Antisera. Goat antisera to purified Rauscher MuLV $p30^{gag}$ was obtained from K. Ulrich, The Fibiger Institute, Copenhagen, Denmark.

Oligonucleotide-directed mutagenesis. Oligonucleotides were synthesized as described previously (22). $p15^{gag}$ deletion mutants were constructed from the 3.7-kb *Pst*I-*Sac*I fragment of pAKR-59 (25) subcloned into the polylinker of M13mp18. This fragment contains most of the long terminal repeat, the 5′ untranslated region, the *gag* gene, and the

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TABLE 1. Oligonucleotides used to introduce in-frame deletions in the p15^{gag} gene

Oligonucleotide	Oligonucleotide sequence[a]
518(Δ1)	5′ CCGCTTCTAAAAACATGGGACAGACCGTA-CTGAGTCTGACCCTAG 3′
519(Δ2)	5′ GGACAGACCGTAACCACCCCT-GAACACTGGGAAGATGTCCAGCGC 3′
545(Δ3)	5′ CCCCTCTGAGTCTGACCCTA-AATCAGTCCGTAGATGTCAAG 3′
546(Δ4)	5′ GTCCAGCGCATCGCGTCC-GTCACCTTCTGCTCTGCCG 3′
547(Δ5)	5′ GATGTCAAGAAGAGACGCTGG-GCCGAGTGGCCAACTTTCGG 3′
520(Δ6)	5′ GAGACGCTGGGTCACCTTCTGCTCT-TGGCCACAAGATGGTACTTTTAATTTGG 3′
521(Δ7)	5′ GGCCAACTTTCGGTGTAGGG-TTTAATTTGGACATTATTCTACAGG 3′
548(Δ8)	5′ GGGTGGCCACAAGATGGTACT-TCTAAGGTGTTCTCTCCTGG 3′
549(Δ9)	5′ GGACATTATTCTACAGGTTAAA-CCCCACGGACACCC 3′
550(Δ10)	5′ CTAAGGTGTTCTCTCCTGGT-ACCTGGGAGGCTATTGCCTATG 3′
551(Δ11)	5′ GGATCAGGTCCCATATATTGTC-CCCCCTCCGTGGGTCAAACC 3′
552(Δ12)	5′ GGGAGGCTATTGCCTATGAA-CCCAAACTCTCCCCCTCTCC 3′
553(Δ13)	5′ GGGTCAAACCTTTTGTCTCT-CTCCCATCCGGTCCTTCG 3′
554(Δ14)	5′ CCCTCTCCAACCGCTCCCATC-CGATCTGCCCTTTACCCTG 3′

[a] Dashes indicate the positions of deleted nucleotides.

N-terminal part of the *pol* gene. Fourteen oligonucleotides (Table 1) complementary to various nucleotide sequences within the p15^{gag} gene but each missing the nucleotides to be deleted, were used to create in-frame deletions on single-stranded M13 viral DNA by using the in vitro mutagenesis system from Amersham Corp. Mutated 1.25-kb *Bss*HII-*Sac*II fragments were excised from the replicative form of M13 and substituted for the wild-type fragment in pAKR-59. The deletions were confirmed by DNA sequence analysis (15). The plasmids carrying the various mutant viral DNAs were designated pΔ1 to pΔ14 (Fig. 1).

Transfection. Cotransfection and transfections were performed as described previously (22) but with omission of the glycerol shock. The calcium phosphate-DNA coprecipitate was added to the NIH 3T3 cell culture medium (5 ml) and allowed to remain in contact with the cells for 8 h at 37°C before the addition of fresh medium (10 ml).

Complementation assay. Five micrograms of *Pst*I-digested, concatenated viral DNAs and 10 μg of salmon sperm carrier DNA were transfected into monolayers of M23 cells (42) (kindly provided by S. Goff, Columbia University, New York, N.Y.) in T25 flasks by using the calcium phosphate precipitate method. At confluency (3 to 4 days), cells were split into 50-mm-diameter petri dishes and T25 flasks. When confluent (another 3 days), cells on the petri dishes were UV treated and overlaid with XC cells (35). Plaques were counted 4 days later. Cells in T25 flasks were monitored for a total of 14 days, and medium was harvested every third to fourth day for reverse transcriptase (RT) and reinfectivity tests. For reinfectivity tests, culture medium (6 and 9 days posttransfection) was passed through a 0.45-μm-pore-size filter, and volumes containing equal levels of RT activity were used to infect semiconfluent NIH 3T3 cells overnight in the presence of 8 μg of Polybrene per ml. Successful infection was detected by measuring the release of RT activity into the medium at various times postinfection.

RT assay. RT activity on exogenous templates was assayed as described previously (22) except that enzyme reactions were carried out for 30 min in a mixture of 50 mM Tris-HCl (pH 8.0), 0.2 mM $MnCl_2$, 60 mM NaCl, 0.05% Nonidet P-40, 10 μg of oligo(dT) per ml, 20 μg of poly(rA) per ml, 20 mM dithiothreitol, 1.9 μCi of [^{3}H]dTTP (30 Ci/mmol; Amersham Corp.), and 0.3 μM dTTP.

Cell labeling, immunoprecipitation, and SDS-PAGE analysis of virus proteins. Subconfluent monolayers of cells were labeled with 80 μCi of [^{35}S]methionine-cysteine per ml (Tran^{35}S-label, 1,136 Ci/mmol; ICN Pharmaceuticals, Inc., Irvine, Calif.) or 0.4 mCi of [^{3}H]myristic acid per ml {[9,10(*n*)-^{3}H]myristic acid, 60 Ci/mmol; Amersham Corp.} and lysed in lysis buffer with 30 μg of aprotinin per ml as previously described (22). Immunoprecipitates were collected by means of goat antiserum to the p30^{gag} domain and protein A-Sepharose and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (6 to 12% polyacrylamide gradient), fluorography, and scanning densitometry as described previously (22).

Analysis of viral protein release from G418-resistant cell cultures was carried out by harvesting the culture medium after labeling with [^{35}S]methionine-cysteine (80 μCi/ml) for 16 h (1.2 ml per 10^6 cells; total, 2 ml). Medium was passaged through a 0.45-μm-pore-size filter, and 5× lysis buffer (22) was added to a concentration identical to that of cell lysates.

Northern (RNA) blot. Total RNA was extracted from the cotransfected cells by using the method of Chomczynski and Sacchi (4), fractionated on denaturing agarose-formaldehyde gels, and transferred to Zeta-Probe blotting membranes (Bio-Rad). Filters were prehybridized at 65°C for 1 h in hybridization buffer (0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 4× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1% SDS, 160 μg of denatured *Escherichia coli* DNA per ml). Hybridizations were carried out for 16 h in the same buffer with 10^6 cpm of a ^{32}P-labeled randomly primed ecotropic virus-specific DNA probe per ml. To reprobe filters, labeled probe was removed by heating twice for 20 min at 95°C in 0.1× SSC-0.5% SDS. Filters were then prehybridized again before rehybridization.

RESULTS

Construction of mutants with deletions in the matrix protein of Akv. To map regions within the matrix protein involved in the assembly and maturation of MuLV, we have constructed a series of Akv mutants carrying in-frame deletions in the p15^{gag} gene (Fig. 1). The very N-terminal amino acids of the Gag precursor protein are considered essential for the addition of myristic acid (36, 47). Therefore, we left the 5 N-terminal amino acid residues unaffected. The carboxy-terminal 5 amino acid residues of p15^{gag} were also retained to preserve the cleavage site for the viral protease. The MA protein encompasses in total 129 amino acids, and the deletions introduced were distributed tandemly over the remaining 119 amino acids of the protein.

The plasmid pAKR-59 contains an 8.2-kb *Pst*I fragment of

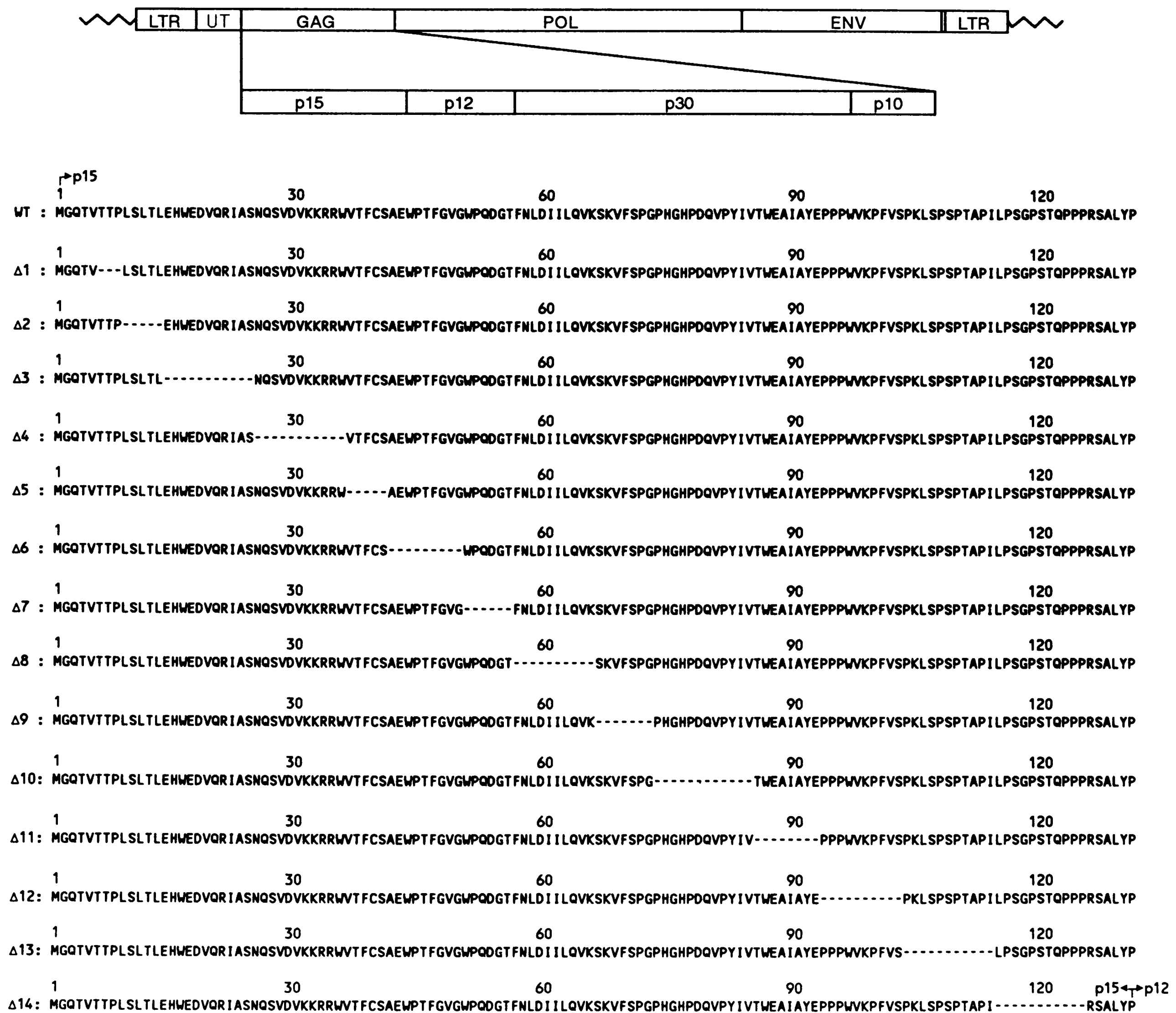

FIG. 1. Structure of p15^{gag} deletion mutants of Akv-MuLV. (A) Complete genome of an ecotropic MuLV, with enlargement of the *gag* region with the four encoded peptides (p15, p12, p30, and p10). LTR, long terminal repeat; UT, the region between the LTR and the Pr65^{gag} reading frame; GAG, group-specific antigens; POL, RT gene; ENV, envelope gene. (B) Amino acid sequence of the wild-type p15 matrix protein (MA). A series of 14 in-phase deletion mutants (Δ1 to Δ14) were generated by site-directed mutagenesis as described in Materials and Methods. The deleted amino acids are represented by broken lines.

the infectious λAKR(623) proviral genome cloned in pBR322 (25). Deletions were generated in a subcloned fragment by using oligonucleotide-directed mutagenesis as described in Materials and Methods. The individual mutated fragments were recloned into pAKR-59, which resulted in 14 plasmids carrying viral genomes with consecutive deletions within the p15^{gag} gene (Fig. 1, Δ1 to Δ14). DNA sequencing confirmed the presence of the deletions.

Analysis for replication competence of the MA deletion mutants. In the parental plasmid, pAKR-59, and in the derived mutant plasmids, the long terminal repeat sequences are present in a permuted form. When excised from the vector by *Pst*I, concatemers generated by ligation containing complete viral genomes may cause the production of infectious virus upon transfection into NIH 3T3 cells (22). Cells were exposed to the mutated viral DNAs in parallel with wild-type DNA, and infectivity was assessed by assays for RT activity in the culture fluid at different time points after transfection. Wild-type-, Δ13-, and Δ14-transfected cells were positive 7 days posttransfection, while mutants Δ1 to Δ12 were completely negative even after 3 weeks (Table 2). Thus, we conclude that deletions in the N-terminal and central regions of the p15^{gag} gene render the viral genome noninfectious, whereas deletions in the C terminus have no obvious deleterious effect.

Since very few of the primary transfected NIH 3T3 fibroblasts become virus producing, this assay detects only infectious viruses transmissible in cell culture. Analysis of the replication-defective constructs thus requires establishment of the DNAs in cells by cotransfection with a selectable marker gene. Cells were cotransfected with 10 μg of concatenates of DNA from pAKR-59 or from each of the mutated

TABLE 2. Phenotypes of matrix protein deletion mutants of Akv-MuLV

Mutant	Residues deleted in p15[gag]	Level of: RT activity[a] in: NIH 3T3 cells[d]	Fluid of G418-resistant cells	Complementation[b]: UV-XC plaque assay[e]	Reinfection of NIH 3T3 cells[f]	mRNA[c]: 35S	21S
Δ1	6–8	–	–	12	+	0.7	0.3
Δ2	9–13	–	–	–	–	0.8	0.5
Δ3	14–24	–	–	–	–	0.9	0.2
Δ4	25–35	–	–	–	–	0.7	0.4
Δ5	36–40	–	–	–	–	1.2	0.2
Δ6	41–49	–	–	–	–	1.0	0.5
Δ7	50–55	–	–	–	–	0.2	0.1
Δ8	56–65	–	–	–	–	0.4	0.2
Δ9	66–72	–	–	–	–	0.9	0.5
Δ10	73–84	–	–	–	–	1.4	0.6
Δ11	85–92	–	–	–	–	0.6	0.4
Δ12	93–102	–	–/+	2	–	0.5	0.3
Δ13	103–113	+++	+++	≥150	+++	99	108
Δ14	114–124	+++	+++	≥150	+++	66	77
Akv	WT[g]	+++	+++	≥150	+++	100	100

[a] +++, wild-type level; –/+, low activity, not always above the background level; –, background level.
[b] Production of infectious virions by transfected M23 cells was determined by the methods indicated.
[c] Scanning data from Northern blot analysis. The measurements were normalized to that of glyceraldehyde phosphate dehydrogenase and are given relative to the value of the wild type (Akv), which was set to 100.
[d] RT activities in culture medium of transfected-cell populations were determined 7, 14, and 21 days after transfection.
[e] Numbers of plaques per 10^6 cells are given. –, no plaques observed.
[f] +++, fully infectious; +, less infectious; –, noninfectious.
[g] WT, wild type.

plasmids (pΔ1 to pΔ14), and 1 μg of pSV2*neo* (45) and recipient cells were selected with G418 (500 μg/ml). Surviving colonies were pooled and grown into large cultures in medium containing 100 μg of G418 per ml and then were tested for the release of RT-containing particles. As expected, cells cotransfected with wild-type, Δ13, or Δ14 DNA were positive. Each of the mutant Δ1 to Δ11 DNAs were found not to cause production of virions, while the release of low levels of RT activity was found occasionally for Δ12-transfected cells by this assay (Table 2). These results confirm that the region of the MA protein spanning amino acids 6 through 102 (Δ1 to Δ12) is essential for the proper assembly of virions.

Complementation test for *gag-pol*. The *pol* protein is synthesized as a *gag-pol* fusion protein, Pr200[gag-pol] (28, 43, 51), which in the presence of Pr65[gag] (11) is incorporated into assembling particles, presumably by means of the *gag* domain (14, 21, 40). Therefore, mutations in *gag* may also influence *pol* functions. To determine whether the mutated virus DNAs could provide *gag-pol* and *env* functions in *trans*, the M23 cell line was used in a complementation analysis. M23 cells express Pr65[gag] but no detectable *gag-pol* or *env* proteins and assemble and release noninfectious virions (42). The defective MuLV provirus in M23 cells has been shown to complement various *gag* mutants to yield infectious virus (6, 11, 40).

DNA from wild-type and each of the 14 mutants was transfected into M23 cells. Transfected cells were tested for the release of complemented infectious virions by UV-XC plaque assay (35) and by cell-free reinfection of NIH 3T3 cells (Table 2). The results of the two assays showed that deletion mutants Δ13 and Δ14 each had the level of the wild-type virus, whereas mutants Δ1 through Δ12 in general were low or negative in both UV-XC and reinfection tests. M23 cells transfected with Δ1 or Δ12 gave rise to a few XC plaques indicative of complementation; however, only the rescued virions of Δ1 could be transferred to NIH 3T3 cells. The results suggest that mutants with deletions in the N-terminal and central parts of the p15[gag] gene (Δ1 through Δ12) render their *gag-pol* molecules low in function or nonfunctional in the process of viral particle assembly, even when assisted in *trans* by a functional Pr65[gag].

Transcription of mutant proviral genomes. To examine whether the constructed deletions influenced the level of viral RNA, a Northern blot analysis was carried out. Cells infected with MuLV express two major species of viral RNA, the genome-size 35S and the spliced 21S (49). 35S mRNA directs the synthesis of the Gag and Gag-*pol* polyproteins (28, 30), and the 21S mRNA directs synthesis of the *env* gene products. Transcription of the mutant viral sequences was analyzed by Northern blotting of total cellular RNA isolated from the G418-resistant cell populations by using a 400-bp *Sma*I ecotropic virus-specific *env* probe (20). For use as an internal standard, the filters were subsequently rehybridized to a glyceraldehyde phosphate dehydrogenase-specific probe. The autoradiographs were analyzed by scanning densitometry, and the results were normalized to glyceraldehyde phosphate dehydrogenase and wild-type data (Table 2). No proviral transcripts were detected in control cells. Viral RNA was detected for all of the deletion constructs. For the deletion mutants encoding replication-competent virus (Δ13 and Δ14), RNA levels reached that of the wild type (Akv), whereas lower levels of RNA were observed for the replication-incompetent constructs (Δ1 to Δ12). The high-level expression of genes from replication-competent virus has been explained by their entry into cells by infection, while genes of nonreplicative virus, entering the cell by transfection only, are less efficiently expressed (19).

Analysis of Gag proteins. Subcultures of the G418-resistant cell populations were tested for the presence of Gag polyproteins by labeling for 7 h with [^{35}S]methionine-cysteine.

Cells were lysed, and immunoprecipitated material was analyzed by SDS-PAGE and fluorography. In cells transfected with Δ1, Δ13, Δ14, and wild-type DNA, Pr65^{gag} was readily identified, whereas cells containing viral genomes with deletions in the central part of the p15^{gag} gene (Δ2 to Δ12) in several experiments showed a very small amount of the Gag precursor (data not shown). To test whether the very low-level accumulation of Gag proteins in cells carrying the mutant genomes could be explained by their export into the cell culture medium, G418-resistant cells were labeled with [^{35}S]methionine-cysteine for 16 h. Volumes of culture fluids corresponding to roughly four times the number of cells used for analysis of cell lysates were subjected to immunoprecipitation. No Gag proteins were detected in medium from cells harboring noninfectious viruses (Δ1 through Δ12), whereas, as expected, *gag*-related proteins were observed in supernatant from cells producing infectious virions (data not shown). Thus, the low-level intracellular accumulation of Δ2- to Δ12-encoded Gag polyproteins cannot be accounted for by export into the medium.

Myristylation of mutant Gag polyproteins. Modification with myristic acid is known to be essential for the function of the Gag precursor. In conjunction with the N-terminal glycine, the primary amino acid sequence near the N terminus of the protein possesses the signal required for myristylation (47). The shortening of the amino terminus of Pr65^{gag} in the deletion mutants may interfere with the myristylation process and cause the observed differences in Gag polyprotein accumulation. To test this possibility, G418-resistant cells expressing the viral genome Akv, Δ1, Δ2, or Δ3 were labeled, in parallel with control cells (Neo), for 1 h with [^{35}S]methionine-cysteine or [^{3}H]myristic acid. The cell lysates were immunoprecipitated with anti-p30^{gag} serum, and the precipitated material was subjected to SDS-PAGE and fluorography (Fig. 2).

In Akv- and Δ1-transfected cells, ^{35}S-labeled Gag polyproteins were clearly identified, whereas the Gag polyprotein in cells expressing the Δ2 or the Δ3 genome was hardly detectable. In parallel cultures labeled with [^{3}H]myristic acid, the Gag polyprotein immunoprecipitated from the Akv, Δ2, and Δ3 cell lines had incorporated radioactive label, whereas at the position of Δ1-Pr65^{gag} a very faint band was observed. Control cultures did not show any incorporation of either ^{35}S or ^{3}H radioactivity at the position of the Gag polyprotein. These data indicate that the Δ1-encoded Gag polyprotein is inefficiently myristylated but suggest a proper modification of Gag proteins carrying the Δ2 or Δ3 deletion.

The deletion in Δ1 leaves the 5 amino-terminal amino acids of p15^{gag} unaltered, whereas the Δ2 and Δ3 mutants retain 8 and 13 amino-terminal residues, respectively. We conclude that the 5 N-terminal amino acids of Pr65^{gag} are insufficient as a myristylation signal and that the 8 amino-terminal residues mediate proper myristylation, indicating that the context of amino acids 6, 7, and 8 is of importance for attachment of myristic acid. Thus, the deficiency of the Δ2 and Δ3 mutants cannot be explained by an abrogated myristylation of the Gag products.

Kinetics of Gag polyprotein turnover. The turnover of wild-type and mutant Gag precursor proteins was analyzed by pulse-chase labeling experiments. G418-resistant cells carrying the mutant genome Δ1, Δ3, Δ7, or Δ11 were, in parallel with Akv-transfected cells and control cells (Neo), pulse-labeled with [^{35}S]methionine-cysteine for 15 min and chased for 15 min, 45 min, or 4 h. Cell lysates were immunoprecipitated and analyzed by SDS-PAGE followed by fluorography (Fig. 3). In Akv-infected cells, bands of the

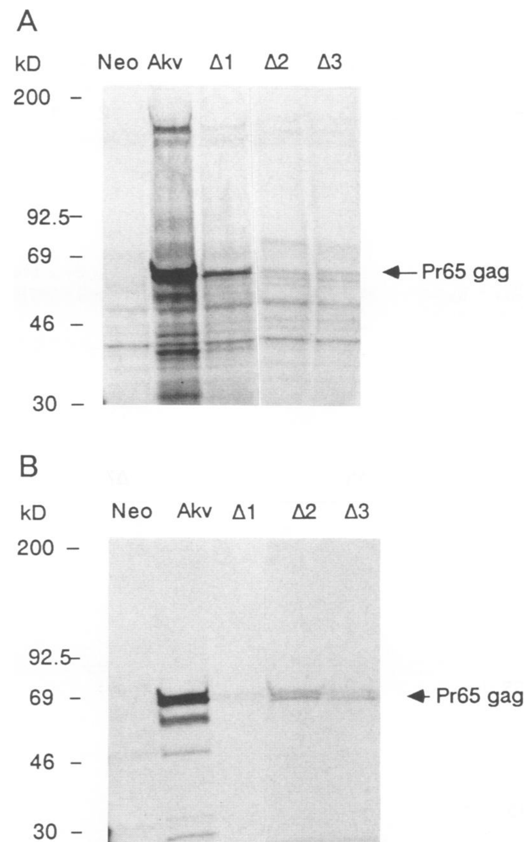

FIG. 2. Myristylation of mutant Gag polyproteins. Cotransfected NIH 3T3 cells carrying the Akv, Δ1, Δ2, or Δ3 viral genome and control cells (Neo) were labeled for 1 h with [^{35}S]methionine-cysteine (A) or [^{3}H]myristic acid (B). Gag proteins were immunoprecipitated from the cell lysates and fractionated by SDS–6 to 12% gradient PAGE and detected by fluorography. Preflashed films were exposed for 6 days (^{35}S) and 20 days (^{3}H). The positions of molecular size markers and Pr65^{gag} are indicated.

gag-related proteins Pr200$^{gag-pol}$, gPr80^{gag}, and Pr65^{gag} were identified after a labeling period of 15 min (Fig. 3A, Akv, lanes 1). The processing of Pr65^{gag} was observed as a reduction in the intensity of the polyprotein band and the simultaneous appearance of a band corresponding to p30^{gag}. After a 15-min chase, a faint band of p30 was observed (Fig. 3A, Akv, lanes 2), gradually increasing after a chase of 45 min and 4 h (Fig. 3A, Akv, lanes 3 and 4). Densitometry-scanning data suggested a processing half-life of approximately 5 h for the wild-type precursor protein. The Δ1-encoded Gag polyprotein had a level of roughly 40% compared with that of the wild-type protein and appeared to be very stable. No processing of this protein was observed even after a 4-h chase (Fig. 3A, Δ1, lanes 1 through 4). The Δ3-, Δ7-, and Δ11-encoded Gag precursors were more difficult to identify because of the expected low levels of accumulation and background bands (Fig. 3A, Neo, lanes + and −). However, visual analysis supported by densitometry scanning could detect distinct bands corresponding to Gag polyproteins after 15 min of pulse-labeling (Fig. 3A, Δ3, Δ7, and Δ11, lanes 1). These bands were absent or greatly reduced after chase periods as short as 15 min (Fig. 3A, Δ3, Δ7, and Δ11, lanes 2). Within the detection limit of our analysis, we estimated the half-lives of the Δ3, Δ7, and Δ11

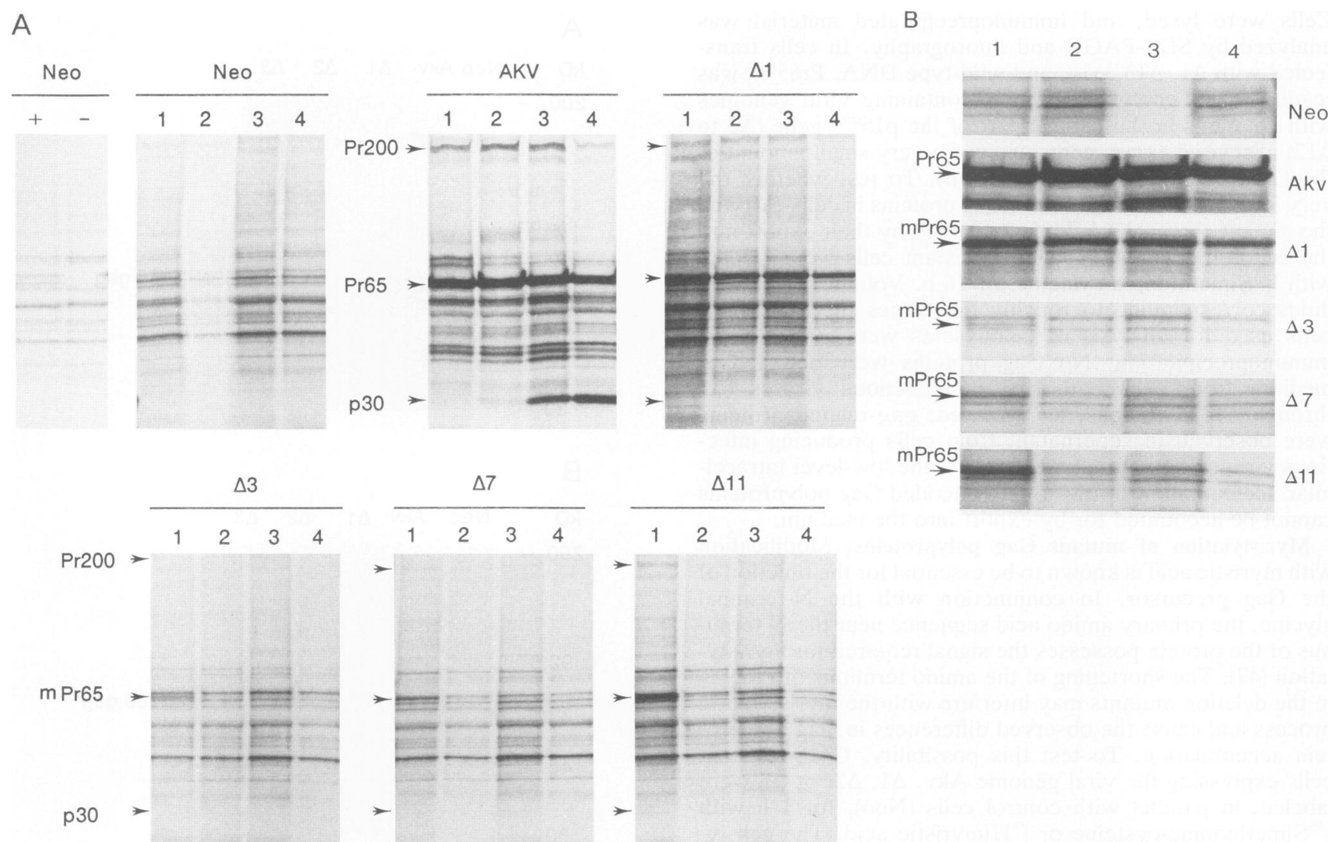

FIG. 3. Kinetics of intracellular Gag protein turnover. (A) G418-resistant cell lines expressing wild-type (AKV) or mutant genomes Δ1, Δ3, Δ7, and Δ11 and control cells (Neo) were pulse-labeled with [^{35}S]methionine-cysteine for 15 min (lanes 1) and chased for 15 min (lanes 2), 45 min (lanes 3), and 4 h (lanes 4). Cells were lysed, and viral antigens were immunoprecipitated with goat anti-p30^{gag} serum, fractionated by SDS-6 to 12% gradient PAGE, and detected by using fluorography. Preflashed films were exposed for 25 days. The positions of Pr200$^{gag\text{-}pol}$, Pr65^{gag}, and the p30^{gag} peptide are indicated. Lanes + and − indicate tests of cell lysates with and without the addition of p30^{gag} antiserum, respectively (film exposed for 13 days). Incubation of cell lysates with preimmune goat serum prior to immunoprecipitation (clearing) did not reduce immunocomplexing of background proteins at that particular position. (B) Enlargement of the Pr65^{gag} region of the fluorograms. The positions of mutant Gag polyprotein bands are below those of the upper background bands. mPr65, mutant Pr65^{gag}.

Gag precursors to be ≤5 min and thus much shorter than that of Akv-Pr65^{gag}. The results suggest that the low-level accumulation of Δ2- to Δ12-Pr65^{gag} can be explained by the instability of the Gag precursor protein. The accumulation of the Gag precursor in the cell is determined by the rate of synthesis and the rate of breakdown. We cannot exclude the hypothesis that a decreased rate of Gag protein synthesis contributed to the low level of mutant Gag proteins, but we emphasize that the observed levels are fully explained by the rapid rate of degradation.

DISCUSSION

The Gag polyprotein of MuLV is translated as a cytoplasmic protein, and the attachment of myristic acid to the N-terminal glycine of the precursor protein (16, 37) is required for cell membrane association and virus particle release (5, 13, 22, 31, 38). Since not all myristylated proteins are found to be membrane bound (36, 41, 47), we wanted to analyze the role of amino acids within the MA domain in virus assembly. The data reported here show that the MA protein of Akv-MuLV contains regions important for the stability of Gag proteins and thus for virus particle assembly.

We constructed a series of mutants with consecutive in-frame deletions in the p15^{gag} gene of Akv-MuLV. We have used DNA transfection into NIH 3T3 cells of otherwise intact MuLV genomes carrying these deletions to study the biological properties of the mutated virus DNAs in a host which supports their native replication. Deletions within the amino-terminal and central parts of the MA-coding region (Δ1 through Δ12) rendered the viral genome noninfectious, whereas deletions in the C-terminal region (Δ13 and Δ14) had no obvious effect on virus replication (Fig. 1 and Table 2). These results are in agreement with those of Crawford and Goff (6), who showed that MuLV *gag* mutants with a deletion at the border of the p15 and p12 *gag* genes permitted virus particle assembly and release. In addition, studies of the incorporation of *gag*-β-galactosidase fusion proteins into Mo-MuLV have indicated that the N-terminal two-thirds of the MA protein are essential for plasma membrane localization and assembly into virus particles (14, 21). Taken together, these and our results suggest that except for the 27 C-terminal amino acids (103 to 129), the entire MA structure is essential for the assembly of MuLV.

We determined a wild-type level of transcription of the infectious Δ13 and Δ14 genomes (Table 2), whereas lower transcription levels were determined for all of the noninfectious mutant genomes (Δ1 to Δ12). These differences in RNA

expression levels are most likely due to the fact that transfected DNA is transcribed much less efficiently than DNA transferred by infection (19). Moreover, the higher levels of viral RNA in Akv, Δ13, and Δ14 populations may reflect the fact that all cells harbor proviruses, while only the primary transfectants of Δ1 to Δ12 express viral genomes. For Akv and the constructs with wild-type properties (Δ13 and Δ14), the ratio between Gag protein and mRNA is significantly lower than that for Δ1. We cannot fully explain this observation. Possible explanations may involve mechanisms of translational control or compartmentalization; i.e., only a fraction of the viral RNA is available for translation. However, the mRNA level found in Δ1 supports the accumulation of the variant Pr65*gag* in almost wild-type quantities. This suggests that the mRNA amounts found in all of the Δ1- to Δ12-transfected cells are sufficient to support a level of Gag polyprotein expression comparable to that of the wild type and ascertain the general integrity of the mutated genomes relating to transcription.

The replication-defective viral construct showed two phenotypes: (i) deletion of 3 amino acids in the N terminus of the MA domain (Δ1) caused the accumulation of a stable Gag protein; however, neither processing into structural *gag* peptides nor virus production was observed, and (ii) mutants with deletions within the central coding region (Δ2 through Δ12) failed to accumulate Gag proteins. By pulse-chase experiments, we determined the half-lives of Δ3-, Δ7-, and Δ11-encoded Gag polyproteins to ≤5 min. Since no Gag protein appeared to be released from the cells harboring the Δ1 to Δ12 mutated genomes and viral RNA was determined in quantities sufficient for the synthesis of Gag proteins in almost wild-type amounts, as seen for Δ1-Pr65*gag*, the low-level accumulation of Δ2- to Δ12-Pr65*gag* can be explained by the instability of the Gag precursor. While we favor this interpretation, it is possible that deletions Δ2 to Δ12 have some quantitative effect on Gag expression as well or that the mutant myristylated Gag precursor could have toxic effects on the cell, resulting in the selection of cells with reduced levels of expression of viral genes. In accordance with our observations, it was recently reported that deletions (33) and certain point mutations (34) in the central region of the MA domain of Mason-Pfizer monkey virus caused instability of the capsid precursor protein.

The presumed loss of stability of Δ2- to Δ12-encoded Gag polyproteins is not explained by a lack of myristylation, since we determined proper myristylation of the Δ2- and Δ3-encoded Gag polyproteins retaining 8 and 13 amino acids, respectively, unaltered at the amino terminus. These results are consistent with those of previous studies locating the signal for protein myristylation within the N-terminal 6 to 7 amino acids (3, 23, 36, 47). The intracellularly stable Δ1-Pr65*gag* is very inefficiently myristylated, which explains the defective biological function of this mutant. In support of our results, it has been reported that unmyristylated Pr65*gag* is very stable (38). It might be suggested that the lack of myristylation contributes to the stability of Δ1-Pr65*gag*.

The Δ1 and Δ12 mutants were rescued at a low level by coexpression with Gag proteins in M23 cells that are competent for particle formation. Although Δ1-encoded Gag precursor polyproteins are inefficiently myristylated, the small amount of myristylated *gag-pol* proteins may contribute to the formation of few infectious virions. Our analysis cannot rule out the possibility that unmyristylated *gag-pol* molecules are incorporated to a low extent into virus particles assisted by the myristylated Gag proteins in M23 cells. The low rate of complementation of the Δ1 and Δ12 mutants

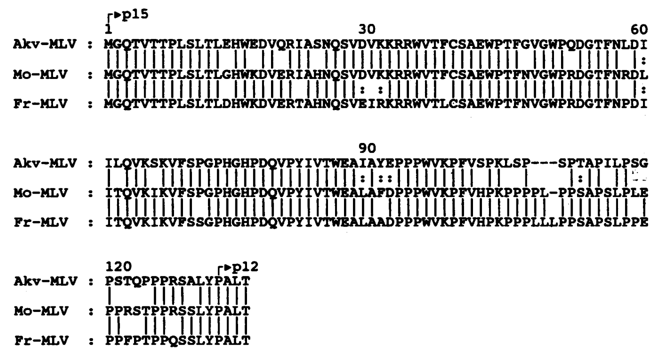

FIG. 4. Amino acid sequence comparison of MA proteins of Akv-MuLV, Mo-MuLV, and Friend MuLV (Fr-MLV). The predicted amino acid sequences of the MA proteins are aligned. Bars indicate identities; dots show conserved amino acid changes. Broken lines are introduced in the sequence to maximize homologies between the proteins. In the region of MA of Akv in which deletions Δ1 to Δ12 are located (residues 6 to 102), 89.7 and 86.6% are identical to or conserved relative to MA of Mo-MuLV and Friend MuLV, respectively. In the region of Akv in which Δ13 and Δ14 are introduced (residues 103 to 124), 54.5% of the residues between Akv-MuLV, Mo-MuLV, and Friend MuLV are identical or conserved.

may be due to an incorrect ratio of *gag* to *gag-pol* molecules. Nevertheless, the rescuing of the Δ1 and Δ12 mutants suggests that neither deletion totally interrupts the *gag-pol* conformation needed for interaction with wild-type proteins in the *gag*-to-*gag* and *gag*-to-*gag-pol* multimerization essential for the virus assembly process. Similar observations have also been reported for C-terminal MA mutants of Mo-MuLV (6) and human immunodeficiency virus type 1 MA mutants (48). It is noteworthy, however, that our deletions in the central region of the p15*gag* gene, as also observed for several mutations in P30 of Mo-MuLV (40), block virus assembly as well as incorporation of Pr200*gag-pol* into M23 particles (Table 2). The failure to rescue these mutants may be explained by the low level of functional Gag precursor proteins.

Our characterization of the panel of p15*gag* deletion mutants of Akv shows that the structural region encompassing amino acid residues 6 to 102 is essential for the function of the MA protein in the assembly and the maturation of MuLV particles. It is of interest that the region in which deletions Δ1 through Δ12 are located shows high sequence homology between the matrix proteins of Akv-MuLV, Mo-MuLV, and Friend MuLV (10, 11a, 17, 43) (Fig. 4). In contrast, a much higher level of sequence variation exists in the C-terminal part in which deletion mutants (Δ13 and Δ14) preserved wild-type properties. This sequence conservation supports our observations indicating the importance of the N-terminal three-fourths of the MA protein in virus assembly and maturation. The native structure of this region seems to contribute to the inherent stability of the precursor molecules.

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