Mutations in *gag* Proteins P12 and P15 of Moloney Murine Leukemia Virus Block Early Stages of Infection

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A collection of mutants of Moloney murine leukemia virus with deletions in the *gag* gene was generated by restriction enzyme site-directed mutagenesis of a cloned proviral DNA. The mutants all contained deletions of the *NarI* site in the P12 region, and some contained deletions extending into the adjacent P15 region. The deletions did not significantly affect the assembly or release of viral particles. Examination of endogenous reverse transcription products demonstrated normal synthesis of minus- and plus-strand strong-stop DNAs, indicating that the RNA genome was packaged and that reverse transcription in detergent-permeabilized virions was not impaired. The virion particles contained high levels of an abnormal protein which corresponded to a P15-P12 fusion protein; proteolytic processing of this abnormal protein was completely blocked by all the mutations. The infectivity of the particles was dramatically reduced. Analysis of the low-molecular-weight DNA in infected NIH/3T3 cells indicated that the mutant virions could not carry out viral DNA synthesis. These data suggest that the P12 and P15 proteins may not be critical for virion assembly but do play an important role in early steps of viral infection.

The primary product of the *gag* gene of mammalian retroviruses is a precursor protein that is ultimately cleaved to yield four proteins found in the mature virion (1, 20). In the case of Moloney murine leukemia virus (M-MuLV), the chemical properties and the complete amino acid sequences of these proteins have been determined, but the function of these gene products has not yet been elucidated (16, 28). By introducing small deletions at specific sites in an infectious, cloned proviral DNA, it should be possible to analyze the function of a specific gene in the life cycle of the virus.

In this study, we have initiated a genetic characterization of P15, a hydrophobic protein encoded by the N-terminal domain of the *gag* gene, and of P12, an acidic phosphoprotein (17, 18) encoded by the second domain of the gene. Within the chronically infected cell, the proteins are initially synthesized as part of a precursor polypeptide termed Pr65^{gag} (1, 20). The precursor is cleaved to yield four polypeptides: P15, P12, P30, and P10. The P15 protein is thought to be involved in binding to the cell membrane during virion assembly (2, 13, 19); a role in the packaging of virion RNA during particle assembly has been suggested for P12 (24-26).

We have begun a genetic analysis of the functions of these proteins by generating a series of small deletions of the appropriate region of the *gag* gene in a cloned DNA copy of the viral genome. Biochemical analyses of the mutant viruses showed that the P12 and P15 proteins may not carry out essential roles in the formation of virion particles, packaging of the RNA genome, or the release of virions from the cell surface. Instead, the mutational analysis implicates these proteins in steps occurring early in the infection cycle, before reverse transcription of the viral RNA into DNA can occur.

MATERIALS AND METHODS

Cells and viruses. NIH/3T3 fibroblasts, XC cells (22), and M23 cells (27) were grown in Dulbecco modified Eagle

medium (GIBCO Laboratories) supplemented with 10% calf serum at 37°C in an atmosphere supplemented with 5% CO₂. M-MuLV released by NIH/3T3 clone 4 was the source of wild-type virus (23). Virus infections at high multiplicity were carried out in the presence of 8 µg of Polybrene per ml at 37°C for 2 h, and the infected cells were then maintained in 1.6 µg of Polybrene per ml overnight.

Cloned DNAs. Plasmid pT11 contains a complete and infectious copy of proviral M-MuLV DNA. This plasmid was derived by transfer of the insert DNA from clone pMOV9 (3) into the *HaeII* fragment of the plasmid vector pACYC177 by polydeoxyguanylic acid-polydeoxycytidylic acid tailing. The details of the construction will be described elsewhere (L. I. Lobel and S. P. Goff, manuscript in preparation). This plasmid gives rise to infectious virus when applied to NIH/3T3 cells in appropriate transfection assays. Plasmid pSV2neo contains the *Escherichia coli* kanamycin resistance gene linked to a simian virus 40 promoter (32). Plasmid pA8.2 contains a permuted, infectious copy of M-MuLV with one long terminal repeat (29) cloned into the *HindIII* site of the pACYC177 vector.

Enzymatic manipulations of DNA. Partial restriction enzyme digests of plasmid DNAs with *NarI* (New England Biolabs) were carried out at 37°C for various times (0 to 80 min), and the digestion products were separated by electrophoresis on 0.8% agarose gels (Sigma Chemical Co.) in TEA buffer (50 mM Tris-hydrochloride, 1 mM EDTA, acetic acid to pH 8.0) containing 1 µg of ethidium bromide per ml. Maximal yields of full-length linear DNAs were obtained after 60 min of digestion with approximately 3 U of enzyme per 10 µg of DNA.

To generate deletions at the sites of cleavage, the linear DNA was digested with *Bal* 31 (New England Biolabs) with 3 U of enzyme per µg of DNA at 25°C for 20 s in buffer (0.3 M NaCl, 6 mM CaCl₂, 6 mM MgCl₂, 0.5 mM EDTA, 10 mM Tris-hydrochloride [pH 8.0]). The reaction was quenched with electrophoresis sample buffer (10% glycerol, 0.1% sodium dodecyl sulfate [SDS], 20 mM EDTA), and the digestion products were purified by electrophoresis and recovered by the glass powder method (34). The DNA was

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recircularized by using T4 DNA ligase (gift of J. van Oostrom) at a low DNA concentration (1 µg/ml) and used to transform *E. coli* HB101 to ampicillin resistance (12). Plasmids isolated from colonies (9) were screened for deletions by digestion with *Pvu*II and *Bst*EII (New England Biolabs) or with *Hha*I. Reaction products were electrophoresed on 7.5% polyacrylamide gels. *Nar*I was not used to screen for deletions since complete digests could not be obtained.

Bacterial cultures. *E. coli* HB101 was grown in L broth (10 g of tryptone [Difco Laboratories] per liter, 5 g of yeast extract [Difco] per liter, 5 g of NaCl per liter, 10 mM NaOH) and was transformed to ampicillin resistance by the CaCl_2 method (12). Selective plates and media contained 50 µg of ampicillin per ml. Plasmid DNA was isolated from small cultures (2 ml) by the method of Holmes and Quigley (9) and from large cultures (200 ml) by equilibrium density gradient centrifugation in cesium chloride-ethidium bromide (10).

DNA sequence analysis. Mutant DNAs (5 to 10 µg) were cleaved with *Tth*111I (New England Biolabs), and the terminal 5' phosphate was removed by treatment with calf intestinal phosphatase (Boehringer Mannheim Corp.). The DNA was labeled with T4 polynucleotide kinase (Bethesda Research Laboratories) and [γ - ^{32}P]ATP (ICN Pharmaceuticals, Inc.) and was then cleaved with *Xho*I. The end-labeled fragment spanning the mutation was isolated by electrophoresis on a 7.5% polyacrylamide gel and was subjected to chemical degradation by the method of Maxam and Gilbert (14). Cleavage products were electrophoresed on 8% polyacrylamide-8 M urea gels (900 by 170 by 0.5 mm) and exposed to X-ray film (Kodak XAR5) with an intensifying screen (Du Pont Lightning Plus) at -70°C .

Mammalian cell transfections and transformations. Transfections of viral DNA to test for infectivity were performed by the calcium phosphate method (6). Approximately 0.5 µg of plasmid DNA and 6 µg of salmon sperm carrier DNA were coprecipitated in a volume of 0.5 ml of buffer (20 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 0.7 mM phosphate [pH 7.05]) by the addition of CaCl_2 to a final concentration of 125 mM. The DNAs were then added for approximately 6 h to 6-cm dishes containing 1×10^5 to 1.5×10^5 cells in 5 ml of medium. Three days after transfection, the cells were transferred with a 10-fold dilution to 10-cm dishes and were allowed to grow to confluency (3 to 5 days). The presence of viable virus was detected by XC cell overlay (22) after UV irradiation of the monolayers.

Cotransformations of cells were performed similarly. A mixture of 1 µg of viral DNA, 1 µg of pSV2neo DNA (31), and 12 µg of carrier DNA was precipitated as before and was applied to 3×10^5 to 5×10^5 cells on a 10-cm dish. Two days after transformation, the cells were fed with Dulbecco modified Eagle medium supplemented with the antibiotic G418 (GIBCO) at a concentration of 400 µg/ml. Cells were fed every 5 days, and the resulting colonies were pooled and maintained in 100 µg of G418 per ml.

Analysis of viral proteins. Monolayers of transformants at approximately half confluence were starved for 30 min in Dulbecco modified Eagle medium lacking methionine and were then labeled with 150 µCi of [^{35}S]methionine in 2 ml of medium for 45 min. The cells were then lysed in buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 M NaCl, 10 mM sodium phosphate [pH 7.5]), and the labeled proteins were immunoprecipitated with goat anti-Moloney serum (77S-591) as before (23). Proteins were analyzed by electrophoresis on 10% polyacrylamide gels (11), followed by fluorography.

To examine virion proteins, monolayers of producer cells

were incubated with 5 ml of Dulbecco modified Eagle medium lacking leucine, and 100 to 120 µCi of [^3H]leucine (Amersham Corp.) was added for 12 h. Labeled virus particles in the medium were harvested, passed through Swinnex Acrodisc filters (pore size, 0.22 µm; Gelman Sciences, Inc.), and isolated on sucrose gradients (27). The virions were suspended in sample buffer (0.5% SDS, 0.3 mM mercaptoethanol, 10% glycerol, 25 mM Tris-hydrochloride [pH 6.8]), and the proteins were fractionated by electrophoresis on 15% SDS-polyacrylamide gels (11). Proteins were detected by fluorography.

Virion proteins were also immunoprecipitated by using goat anti-Gross P12 (75S-711) or goat anti-Rauscher P30 (77S-102) sera.

Analysis of viral DNA synthesized in infected cells. Low-molecular-weight DNA was isolated from cells by the Hirt extraction procedure (8) 12 to 36 h after infection. The DNA was purified by phenol extraction, displayed by electrophoresis on a 0.6% agarose gel, blotted to nitrocellulose (30), and then hybridized with a labeled (21) viral DNA probe as described (4). The filter was dried and exposed to X-ray film with an intensifying screen as described above.

Analysis of viral DNA synthesized by virions in vitro. Endogenous reverse transcription reactions were used to detect the synthesis of minus-strand strong-stop DNA (7). Virus particles were harvested from cells at 12-h intervals and stored at 0°C . Purified virions were incubated in buffer (50 mM NaCl, 6 mM MgCl_2 , 1 mM dithiothreitol, 0.01% Nonidet P-40, 50 mM Tris-hydrochloride [pH 8.3]) containing three unlabeled deoxyribonucleoside triphosphates (1 mM each) and [^{32}P]dCTP (4 µM, 125 Ci/mmol) at 37°C for 30 min. The labeled DNA was purified by phenol extraction, ethanol precipitation, and RNAase treatment and was analyzed on 10% polyacrylamide gels.

Modified procedures were used to detect synthesis of plus-strand strong-stop DNA (15). The medium containing virions was clarified (1,000 rpm, 10 min, 4°C), and the virus was pelleted (15,000 rpm, 5 h, 4°C in the type 19 rotor) and then suspended in 0.2 ml of buffer (100 mM Tris-hydrochloride [pH 8.3], 100 mM NaCl, 1 mM EDTA). The virions were concentrated further by centrifugation (35,000 rpm, 12 h, 4°C) through 3 ml of 25% (wt/vol) sucrose in TNE buffer (50 mM Tris-hydrochloride [pH 8.3], 100 mM NaCl, 1 mM EDTA). Virions were suspended in TNE buffer (total volume, 50 µl) and stored at -70°C . This procedure effected a 3,000-fold concentration of the virus particles released into the medium; the yield from 90 ml of medium was used in a 50-µl reaction cocktail (15) with all four deoxyribonucleotides (2 mM each) and containing 100 µCi of [^{32}P]dTTP (Amersham) which was incubated at 40°C for 2 h. The product DNA was analyzed on 4% gels as for minus-strand strong-stop DNA.

Reverse transcriptase activity on exogenous templates was assayed by the rapid dot method (5).

RESULTS

Construction of deletions in the P12 region of the *gag* gene. The DNA sequence of the M-MuLV genome (28) predicts the existence of four recognition sites for the restriction enzyme *Nar*I: one in each copy of the long terminal repeat, one in P12^{gag}, and one in P30^{gag} (Fig. 1). Only the last two sites were found to be present in our cloned viral DNAs (L. Lobel, unpublished observations). To generate deletion mutations at these sites, a cloned proviral copy of the M-MuLV genome was linearized by limited digestion with *Nar*I, and a

FIG. 1. Structure of *gag* mutants of M-MuLV. (A) Complete genome of M-MuLV as a linear provirus. The regions encoding the *gag*, *pol*, and *env* precursors are shown in boxes. LTR, long terminal repeat. (B) Enlargement of the N-terminal domains of the *gag* gene. The positions of the recognition sites for cleavage by the enzyme *Nar*I in the P12 and P30 domains are indicated. (C) DNA sequence flanking the *Nar*I site in the P12 domain of wild-type M-MuLV. The boundary between domains P15 and P12, as well as the *Nar*I recognition site, are indicated. The filled boxes represent the DNA sequences found to have been deleted from each of the four indicated mutants. The first three have suffered loss of a number of bases equal to a multiple of three and therefore preserve the translational reading frame. The fourth mutation is a frame-shift mutation.

few nucleotides at the termini were removed by digestion with *Bal* 31 exonuclease. The full-length linear DNA was purified by agarose gel electrophoresis, recircularized with T4 DNA ligase, and used to transform *E. coli* to ampicillin resistance. Plasmid DNAs isolated from 40 ampicillin-resistant colonies were screened for the presence of deletions by digesting each DNA to completion with *Bst*EII and *Pvu*II, or else with *Hha*I. The reaction products were examined by agarose gel electrophoresis.

A total of 16 DNAs of the original 40 were found to contain a deletion at one of the *Nar*I sites. Surprisingly, all 16 mapped at the *Nar*I site in P12^{gag} (Table 1). This result indicated that *Nar*I showed a strong preference for cleavage at this site under conditions of limited digestion and very rarely, if ever, cleaved other sites. Similar results have been obtained in other laboratories (R. Roberts, personal communication), suggesting that some *Nar*I sites are readily cleaved but that others are refractory. The approximate sizes of the deletions recovered by this procedure were estimated to range from 10 to 200 base pairs (bp) (Table 1).

Complementation analysis: identification of mutations which preserve the translational reading frame. To test the resulting mutants for their biological activity, each of the altered DNAs was introduced into NIH/3T3 cells by transfection. After several days, the cultures were overlaid with XC cells as an indicator for virus production. None of the mutants was capable of inducing XC syncytia, whereas the parent DNA reproducibly generated 100 to 1,000 plaques per μ g (Table 1). All of the mutants were apparently unconditionally defective and could not induce syncytia without helper virus.

Of the 16 deletion mutations generated, only one-third would be expected to preserve the same translational reading frame that is present in the wild-type parent in the region downstream of the deletion. The other two-thirds would be frame-shift mutations and would not be able to express the *pol* gene encoded downstream. To determine whether the mutations altered the translational reading frame, the mutant DNAs were each used in a complementation test for *pol* gene function. Each DNA was introduced into M23 cells (27) by transfection. This cell line contains a defective M-MuLV provirus and expresses only the *gag* polypeptide. Any mutant DNA which can provide the missing *pol* and *env* functions can replicate in this host by complementation and can give rise to XC syncytial plaques (5, 23a).

When all of the 16 DNAs were tested, 5 proved capable of generating XC plaques in M23 cells, and 11 did not (Table 1). Complementation was not restricted to the very small deletion mutants since the five positive mutants included both very small and very large deletions. The ability of these five mutants to complement M23 demonstrated that they were capable of synthesizing a functional *gag-pol* precursor and that this protein could be incorporated productively into virions. These deletions in P12^{gag} must therefore have preserved the translational reading frame.

DNA sequence analysis. To confirm the predictions of the above genetic analysis, the DNA sequence of the P12 region of three complementing mutants (*dl*1506, *dl*1510, and *dl*1524) was determined by the method of Maxam and Gilbert (14). All three were found to have lost a number of bases equal to a multiple of three, consistent with the results of complementation assay (Table 1). In addition, one mutant which did not complement M23 cells (*dl*1503) was similarly analyzed. As predicted, the sequence data showed that this mutant contained a frame-shift deletion.

The nucleotides deleted in these four mutants are shown in

Fig. 1. Mutant *dl*1506 contained the smallest deletion (18 bp), which resulted in the loss of seven amino acids and the insertion of a proline residue. This deletion lay entirely within the P12 region of the *gag* gene, very close to the 5' end of this region. Mutant *dl*1524 contained a 48-bp deletion almost entirely within P12; it extended one amino acid across the boundary into the P15 region. Mutant *dl*1510 contained the largest deletion, 144 bp. This deletion removed a substantial portion of both the P15 and P12 regions. Finally, mutant *dl*1503 lost 31 bp; its sequence predicted termination of translation 112 amino acids downstream.

Isolation of producer cell lines: virion release. To construct cell lines carrying the mutant proviruses, NIH/3T3 cells were cotransformed with pSV2neo DNA and each of the three mutant DNAs that contained in-phase deletions. The resulting colonies were pooled and grown into large cultures. Two separate cell lines were established for each mutant. Experiments in this laboratory (23, 23a; unpublished observations) have shown that approximately one-half of the cell lines generated in this way contain appreciable quantities of integrated viral DNA. The frequency of cotransformation of the viral DNAs with the unlinked selectable DNA was quite variable, and often very few cells in the population retained viral sequences. Duplicate transformations were always performed to increase the odds of isolating at least one producer cell line.

To determine whether virion particles were released by these cell lines, the level of reverse transcriptase activity in the medium was measured with an exogenous synthetic template (5). Each mutant was found capable of releasing high levels of reverse transcriptase (Fig. 2A). The levels produced by mutants *dl*1506 and *dl*1510 were often approximately as high as those produced by the wild type, and those released by mutant *dl*1524 were only a fewfold (two- to fivefold) lower. Independent cotransformations of the same DNAs showed considerable quantitative variability in the level of enzyme, probably owing to the variable efficiency of cotransformation. The data show that substantial deletions of both P15 and P12 did not block particle formation or

TABLE 1. Structure and biological activity of mutants of M-MuLV

Mutant ^a	Estimated deletion size (bp) ^b	Bases deleted ^c	XC plaques on cells	
			NIH/3T3	M23
<i>dl</i> 1503	50	31	—	—
<i>dl</i> 1506	<30	18	—	+
<i>dl</i> 1509	70	—	—	—
<i>dl</i> 1510	150	144	—	+
<i>dl</i> 1512	50	—	—	—
<i>dl</i> 1517	50	—	—	—
<i>dl</i> 1524	80	48	—	+
<i>dl</i> 1525	30	—	—	—
<i>dl</i> 1528	40	—	—	—
<i>dl</i> 1530	200	—	—	—
<i>dl</i> 1531	70	—	—	+
<i>dl</i> 1535	50	—	—	—
<i>dl</i> 1536	70	—	—	+
<i>dl</i> 1537	40	—	—	—
<i>dl</i> 1539	30	—	—	—
<i>dl</i> 1540	30	—	—	—

^a All mutants had deletions at map position 1.5 kilobases.

^b Size of deletion was estimated from the observed change in electrophoretic mobility of a 709-bp fragment spanning the *Nar*I site in wild-type M-MuLV.

^c Determined by DNA sequence analysis (see Fig. 1).

release. These results also indicate that reverse transcriptase was incorporated successfully into these particles and was proteolytically cleaved to yield enzyme that was active on at least an exogenous synthetic template.

Viral DNA synthesis in vitro. To determine whether the mutant virions contained RNA and could copy this RNA into DNA, an endogenous reverse transcription reaction (7) was carried out for two of the mutants. Multiple harvests were collected from the cells releasing the mutant virions, and the virus in these harvests was purified and concentrated. The virus was permeabilized with detergents and incubated with labeled triphosphates, and the viral DNA thus synthesized was analyzed by electrophoresis on polyacrylamide gels followed by autoradiography.

Both mutants tested (*dl*/1506 and *dl*/1510) were found to synthesize minus-strand strong-stop DNA at wild-type levels (Fig. 3A) after correction for the number of particles used in the assay. Thus, the deletions did not appreciably affect any assembly processes such as packaging of the viral RNA genome into particles, tRNA primer binding, or incorporation of functional reverse transcriptase into these particles.

To test the virions further for their ability to carry out DNA synthesis, virions of one mutant (*dl*/1506) were examined under different conditions. Virions were incubated to allow initiation of plus-strand DNA and formation of plus-strand strong-stop DNA (15), and the labeled DNA was then analyzed as before. Figure 3B shows that this mutant was capable of synthesizing the plus-strand intermediate in amounts comparable to those of the wild type. Thus, permeabilized virions were capable of extensive synthesis. Both strands were initiated, and at least minus-strand strong-stop DNA jumped normally to allow for the observed synthesis of the plus-strand strong-stop DNA.

Infectivity of mutant viruses. Despite the nearly wild-type levels of virus released by these cell lines, the mutant viruses produced were not biologically transmissible at normal efficiency. Supernatants from each producer cell line were harvested and used to infect fresh NIH/3T3 cells. The supernatants used in the infections contained nearly wild-type levels of virus as judged by reverse transcriptase assays and also by direct analysis for virion proteins (data not shown). The medium above the infected cells was then

FIG. 2. Reverse transcriptase activity released into the medium by independent pairs of cell lines. (A) Activity in cell lines which expressed viral genes after cotransformation of NIH/3T3 cells with a mixture of proviral DNA and pSV2neo DNA. After 3 weeks, supernatant fluid was collected and assayed by the rapid dot method (5). The two spots shown for each mutant represent assays of two independent cell lines generated by two independent cotransformations of each viral DNA. 1, Mutant *dl*/1506 DNA; 2, mutant *dl*/1510 DNA; 3, mutant *dl*/1524 DNA; 4, wild-type pT11 DNA. (B) Release of reverse transcriptase by cells infected with the virus shown in (A). Mutants are labeled as in (A). Virus harvests were collected from each of the cell lines shown in (A) and were used to infect fresh NIH/3T3 cells. Supernatants were taken from these cells and assayed as before. Whereas virus collected from producers of wild-type M-MuLV was highly infectious, virus collected from producers of the three mutants showed no or very little infectivity.

FIG. 3. Synthesis of viral DNA intermediates by mutants of M-MuLV. Virions were harvested from producer cell lines and incubated in an endogenous reverse transcription reaction. The reaction products were displayed by electrophoresis on acrylamide gels and exposed to autoradiography. (A) Reactions were carried out in limiting concentrations of deoxyribonucleotide precursors to cause accumulation of minus-strand strong-stop DNA, the first product of reverse transcription. Lane 1, Mutant *dl*/1506; lane 2, mutant *dl*/1510; lane 3, wild-type virus pT11. The mutants synthesized minus-strand strong-stop DNA at levels comparable to the quantity of virus harvested. (B) Reactions were carried out in high deoxyribonucleotide concentrations to allow extensive synthesis. Lanes 1 and 2, Mutant *dl*/1506 virus harvested from two independent producers; lane 3, wild-type virus. Both minus- and plus-strand strong-stop DNAs were synthesized at wild-type levels by the mutant virions. The positions of marker DNAs of known sizes (pBR322 *Hinf*-I fragments) are indicated to the right.

assayed for reverse transcriptase activity. Cells infected with virus of the two deletion mutants containing the largest deletions (*dl*/1510 and *dl*/1524) did not themselves become producers of virus; no reverse transcriptase activity was released by these cells (Fig. 2B). The cells infected with the deletion mutant containing the smallest deletion (*dl*/1506) did become producers, but the level of activity was much lower (10- to 50-fold) than that of cells infected with wild-type virus. Apparently, all three mutants could induce transformed cells to assemble and shed virions, but the particles released were defective in some way so as to either abolish or reduce their infectivity in NIH/3T3 cells.

FIG. 4. Viral DNA synthesized after infection of NIH/3T3 cells by mutant viruses. Approximately 24 h after infection, low-molecular-weight DNA was isolated and fractionated by electrophoresis on an agarose gel. The DNA was transferred to nitrocellulose, and viral DNA was detected by hybridization with a ^{32}P -labeled viral DNA probe. Three different autoradiographic exposures of the same filter are shown to emphasize the ratios between the levels of DNA synthesized by the mutants and by wild-type viruses. (A) A 2-h exposure; (B) a 1-day exposure; (C) a 7-day exposure. Lanes 1, Uninfected NIH/3T3 cells; lanes 2 and 3, mutant *d/1506* collected from two independent producers; lanes 4, mutant *d/1510* collected from a producer; lanes 5 and 6, wild-type virus collected from two producers of pT11 virus; lanes 7, wild-type virus collected from clone 4 cells. The positions of the host cell chromosomal DNA, 8.8-kilobase (kb) linear proviral DNA, and the two circular proviral DNAs are indicated. Whereas the DNA synthesized by the wild-type viruses is readily detectable in a 2-h exposure, no or very little DNA in the mutant-infected cells can be detected, even after a 1-week exposure.

Synthesis of viral DNA in infected cells. One of the first steps carried out by retroviruses is the reverse transcription of the viral genomic RNA into DNA. To determine whether the mutant virions could synthesize DNA *in vivo*, cells were acutely infected with undiluted harvests of producer cells, and after 30 h the low-molecular-weight DNA was isolated by the Hirt extraction method (8). The DNAs were purified, separated by gel electrophoresis, blotted to nitrocellulose (30), and hybridized with labeled M-MuLV DNA. Finally,

the blots were examined by autoradiography (Fig. 4). The levels of all three viral DNA forms—the full-length linears and the two circular forms (36)—were dramatically reduced after infection by mutant *d/1506*, and no viral DNA at all could be detected after infection with mutant *d/1510*. The course of the infection was apparently blocked at a very early stage: the virions could not even direct the synthesis of the linear DNA, the first full-length product of reverse transcription.

The levels of reverse transcriptase present in the virus preparations used to infect the cells was examined to ensure that virus was present at high concentrations. The levels were close to that of the wild type: harvests of both mutants *d/1506* and *d/1510* exhibited approximately one-half the activity of wild-type virus. The amount of viral DNA synthesized by these virus preparations, however, was more than 500-fold reduced relative to the wild type.

Analysis of viral proteins. The virus-specific proteins encoded by two of the mutants were examined in detail. Intracellular viral precursor proteins were labeled with [^{35}S]methionine, solubilized, immunoprecipitated from the extracts, and displayed by SDS-polyacrylamide gel electrophoresis (Fig. 5). Both mutants *d/1506* and *d/1510* synthesized *gag*-related proteins in amounts comparable to those of the wild type. *d/1506* synthesized a Pr65^{*gag*} protein whose mobility was indistinguishable from that of the wild type; mutant *d/1510* directed the synthesis of a protein of higher mobility than normal. The novel protein migrated at the position of a 60,000-dalton protein, consistent with the presence of a 48-amino-acid deletion.

To determine the nature of the proteolytic cleavages made in these proteins after particle maturation, virions released

FIG. 5. Intracellular proteins synthesized in cells cotransformed with viral DNAs. Proteins were metabolically labeled with [^{35}S]methionine, immunoprecipitated from lysates with goat anti-Moloney serum (NCI 77S-591), and analyzed by SDS-polyacrylamide gel electrophoresis. Lane 1, Mutant *d/1506*; lane 2, mutant *d/1510*; lane 3, clone 4 cells; lane 4, clone 4 cells immunoprecipitated with normal goat serum.

FIG. 6. Virion proteins in particles isolated from various producer cell lines. (A) Producer cells were labeled with [3 H]leucine, virions were isolated, and total virion proteins were analyzed by electrophoresis and fluorography. Lanes 1 and 2, Virions of mutant *dl1506* harvested from two independent producer lines; lane 3, virions of mutant *dl1510*; lane 4, wild-type virions from producers of pT11. The positions of P30^{gag}, the novel fusion protein of 24,000 daltons in *dl1506* virions, and the smaller wild-type virion proteins are indicated. (B) Virion proteins prepared as in (A) were immunoprecipitated with either anti-P30 serum (NCI 77S-102; α P30) or anti-P12 serum (NCI 75S-711; α P12), as indicated, before electrophoresis. Lanes 1 and 2, virions from mutant *dl1506* producers, lanes 3, virions from producers of wild-type virus pT11. The positions of P30, the fusion protein P24, and wild-type P12 proteins are indicated. (C) Total virion proteins released by producer cells. Lane 1, Virions of mutant *dl1524*; lane 2, virions of mutant *dl1510*; lane 3, Virions of wild-type pT11. The positions of the novel fusion proteins of molecular weights 22,000 and 19,000 are indicated.

from the producer cell lines were labeled for 12 h in the presence of [3 H]leucine and then were purified on sucrose gradients (see above). SDS-gel electrophoresis showed that the *gag* precursor of the mutants did not give rise to the normal cleavage products (Fig. 6A). Wild-type virus contained P30; a cluster of smaller proteins, P15, P12, P10; and the envelope protein P15E. Mutant *dl1506* showed normal amounts of proteins comigrating with P30, P10, and the envelope protein P15E but lacked detectable P15 and P12. In their stead, a very abundant novel protein migrating at approximately 24,000 daltons was detected. This protein presumably was a fusion of P15 and P12.

Serology was used to confirm the identity of the proteins in mutant *dl1506* virions. Antisera specific to P30^{gag} immunoprecipitated the protein comigrating with authentic P30, and antisera specific to P12^{gag} immunoprecipitated the novel 24,000-dalton protein (Fig. 6B). Thus, mutant *dl1506* apparently cleaved the *gag* precursor to yield normal P30 and P10 proteins but failed to cleave P15 and P12. The mutant virions accumulated large amounts of the P15-P12 fusion protein, even though the amino acids at the site for cleavage were still present.

The other two mutants tested showed similar patterns of proteins. Mutant *dl1510* retained small amounts of a novel protein migrating at the position of a 19,000-dalton protein, whereas mutant *dl1524* showed a 22,000-dalton protein (Fig. 6C). The sizes of both new proteins were in good agreement with the sizes predicted for the fusion of P15 and P12 and the loss of the amino acids normally encoded by the deleted bases. In addition, the level of particles produced, as judged from the level of virion proteins synthesized, was in good agreement with the level of released reverse transcriptase originally detected (data not shown). Thus, the mutations did not appreciably affect the amount of reverse transcriptase incorporated into each virion.

DISCUSSION

The mutations which we have generated in the P12 and P15 regions of the *gag* gene have surprisingly little effect on the assembly and release of virions. Analysis of labeled proteins showed that the *gag* precursor is made in nearly normal amounts and is able to form virions which contain all of the known viral proteins, including *gag*, *pol*, and *env*. The virions were shown to contain wild-type levels both of proteins and of active reverse transcriptase. The data offer no support for possible roles of the P12 and P15 domains of the *gag* protein in virus particle assembly. These results should be contrasted to the behavior of mutants in the P30 domain (23a), which are completely defective in particle formation.

Reverse transcriptase assays revealed high levels of active enzyme in these virions. Thus, the *gag-pol* precursor is also functionally expressed; it is incorporated into these particles and activated appropriately by proteolysis to yield polymerase activity. The level of reverse transcriptase released by the producer cell lines was somewhat variable and often less than the level released by wild-type producer cells. (Much of this variability is probably attributable to variation in the efficiency of the cotransformation procedure used to generate the cell lines: only one-half to one-third of the cell lines actually received high levels of the mutant viral DNA. Wild-type virus can spread by infection to all the cells soon after transformation, whereas the mutant viruses cannot spread efficiently.) Further data indicating that the *gag-pol* precursor was fully functional were provided by the complementation tests: the mutants replicated normally in M23 cells, which are deficient in *gag-pol* function. These results are in contrast to those obtained with P30 mutants isolated previously (23a), which are unable to provide *pol* function in these assays. The *gag* region as a whole can be divided into two

functional domains: a domain in the P15-P12 region which is dispensable for particle formation and *pol* encapsidation but is needed early in infection, and a domain in the P30 region required for both particle formation and *pol* utilization.

A role for P12 in the packaging of the viral RNA genome into virions has been suggested previously; this suggestion was based on the observed specific binding of the P12 protein to genomic RNA (24–26). The results of the *in vitro* reverse transcription assays, however, clearly indicate that the RNA genome was packaged at normal levels in these mutants. Furthermore, the genome was packaged in a functional way, bound to primer tRNA properly and in an appropriate conformation to act as a template for reverse transcriptase. On the basis of these results, we believe that the 5' portion of P12^{gag} cannot be essential for recognition of the viral genomic RNA or for its encapsidation. A further examination of the course of the reverse transcription reaction in one of the mutants (*d/1506*) demonstrated that this mutant is also capable of synthesizing higher-molecular-weight DNA products, such as plus-strand strong-stop DNA, at levels comparable to wild-type virus. Thus, the structure of the RNA packaged appeared to have no defect which precluded the elongation of the minus-strand DNA chain or the strong-stop jump necessary for plus-strand DNA synthesis.

The particles synthesized by all the mutants, however, were unable to induce XC syncytia and were poorly infectious in NIH/3T3 cells. Only mutant *d/1506* could pass to new cells, and even for this mutant the efficiency of the process was significantly reduced relative to the wild type. Since DNA-mediated transfer of the viral DNAs into cells resulted in high-level release of particles and infection did not, we could surmise that the virions were blocked at some early stage of infection preceding formation of the integrated provirus. An examination of the preintegrative viral DNA synthesized after infection confirmed this surmise: levels of full-length double-stranded linear and circular forms were all dramatically reduced. The reduction in the levels of DNA synthesized was easily sufficient to account for the observed loss of infectivity.

The block caused by the mutations must occur at a very early stage of infection. The structural defect in the virions might prevent such processes as proper binding to the receptor on the cell surface, uptake into cellular endosomes, fusion of the virion membrane with that of the endosome, or uncoating of the virion core. Because the envelope glycoprotein is likely to mediate the earlier events, we believe that uncoating of the virion is the step most likely to be affected by these mutations. We cannot, however, rule out the possibility of an alteration in *env* structure being caused indirectly by the changes in *gag*. If the process of opening of the virion to allow reverse transcription is directly blocked by the mutations, the defect can apparently be bypassed *in vitro* by the detergents which are used in reverse transcription reactions to permeabilize the particles artificially.

Several temperature-sensitive mutants of MuLVs showing a similar phenotype have been described. Mutant ts1 of M-MuLV is unaffected in assembly and release of particles but cannot initiate an infection at the nonpermissive temperature (35). Mutants ts17, ts19, and ts29 of Rauscher MuLV were also shown to be unable to infect cells at the nonpermissive temperature (32) or to act as helpers for transfer of defective sarcoma viruses (33). The temperature-sensitive period occurred from 3 to 24 h postinfection and after adsorption and penetration of the cell. Because of their similarity to the deletion mutants constructed here, these temperature-sensi-

tive mutants may be affected in the P15 and P12 regions of the *gag* gene.

The structural alterations in our mutants which cause the observed phenotype affect both P15 and P12. Examination of labeled virion proteins from each of the mutants revealed an abnormal P15-P12 fusion protein in the range of 19,000 to 24,000 daltons. Proteolytic processing of this precursor to the mature products was completely blocked. In the case of mutant *d/1506*, the precursor was retained at a high molarity: its intensity on autoradiography suggested that it was roughly equimolar to P30.

It is surprising that mutant *d/1506* accumulated an uncleaved precursor since the amino acids at the site of cleavage were not changed. Presumably, a secondary structure required for the cleavage cannot form as a result of the mutation. The mutation in *d/1506* results in the deletion of the codons for seven amino acids and the insertion of a codon for the amino acid proline, two amino acids away from the site of proteolytic cleavage. In addition to the presence of the deletion itself, it is possible that the insertion of proline, an amino acid which strongly affects secondary structure, at this particular location may directly alter the protein conformation required for the cleavage. It should be noted, however, that the change may be subtle; there are several proline residues on both sides of the deletion, and one proline residue was present among the seven amino acids removed.

The observed effects on the virus life cycle may partially result from the inability of these mutants to cleave the *gag* precursor and generate the mature *gag* proteins P15 and P12. During particle formation the *gag* polyprotein may function as a single structural domain, and cleavage to the mature proteins may create four distinct and independent domains and be a prerequisite for viral infectivity. This notion is consistent with our observations that failure to separate P15 and P12 does not affect virion assembly or release but instead blocks infection at an early stage. If proteolytic cleavage itself is indeed an essential step in generating infectious virions, then mutations affecting the responsible protease might also affect early events rather than assembly. We are currently generating such mutants to test this hypothesis.

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