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Deletion Mutants of Moloney Murine Leukemia Virus Which Lack Glycosylated *gag* Protein Are Replication Competent

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A series of deletion mutations localized near the 5′ end of the Moloney murine leukemia virus genome was generated by site-specific mutagenesis of cloned viral DNA. The mutants recovered from such deleted DNAs failed to synthesize the normal glycosylated *gag* protein gPr80^{gag}. Two of the mutants made no detectable protein, and a third mutant, containing a 66-base pair deletion, synthesized an altered *gag* protein which was not glycosylated. All the mutants made normal amounts of the internal Pr65^{gag} protein. The viruses were XC positive and replicated normally in NIH/3T3 cells as well as in lymphoid cell lines. These results indicate that the additional peptides of the glycosylated *gag* protein are encoded near the 5′ end, that the glycosylated and internal *gag* proteins are synthesized independently, and that the glycosylated *gag* protein is not required during the normal replication cycle. In addition, the region deleted in these mutants apparently encodes no *cis*-acting function needed for replication. Thus, all essential sequences, including those for packaging viral RNA, must lie outside this area.

The structure of the Moloney murine leukemia virus (M-MuLV) genome is known in great detail; indeed, the complete nucleotide sequence has been determined (32). The detailed functions carried out by the products of the *gag*, *pol*, and *env* genes, however, are largely unknown (5). For example, although it is known that the *gag* proteins are structural elements in the virion particle, the actual role of each mature protein (termed P15, P12, P30, and P10) is uncertain. We have begun a genetic analysis of the genome of M-MuLV in an attempt to determine the various functions of the proteins and regulatory sequences required in the viral life cycle.

The first 620 nucleotides of the viral RNA (extending from the capped 5′ end to the AUG codon utilized for the synthesis of the Pr65^{gag} protein) are required for several functions important to the replication of the virus. Contained in this region are (i) the binding site for tRNA^{Pro}, which primes DNA synthesis (26); (ii) the splice donor site for the formation of *env* mRNA; (32; J. Champoux, personal communication); (iii) signals for packaging of viral RNA into virion particles (30); and (iv) a region of unknown size encoding N-terminal peptides of the glycosylated *gag* protein termed gPr80^{gag} (7). The region from the splice donor site (at nucleotide 205) to the start of Pr65^{gag} (at nucleotide 621) constitutes the largest region of the virus with uncertain function. Work on avian retrovirus mutants (30) has shown that part of the region is essential for RNA packaging, but the extent of the putative recognition site is unknown. In the case of M-MuLV, a large part of this RNA must also encode the glycosylated *gag* protein gPr80^{gag}. This protein contains essentially all of the peptides of Pr65^{gag} and approximately 4,000 to 10,000 daltons of additional protein at the N terminus (7); additional amino acids may be utilized as a signal peptide directing the protein to the endoplasmic reticulum for transport and glycosylation. Mannose-rich core oligosaccharide chains are added to the protein, probably in the P15 and P30 regions (7), and are then modified further by the addition of complex oligosaccharides. The protein is transported to the cell surface, and a portion may be shed from the cells (6). The AUG sequence at which protein translation starts is unknown. The function of this protein is equally unclear, although it is known to constitute the GCSA antigen on the surface of infected cells; a role in virion maturation has also been proposed.

Many laboratories have generated mutants of leukemia viruses which were blocked at various stages of the life cycle (1, 9, 13, 20–22, 27, 31, 36, 40, 43); these mutants have been extremely useful in defining the various functions carried out by the virus. The availability of fully infectious DNA clones of the M-MuLV genome makes possible the construction of defined dele-

tion mutations at known sites within genes. Thus, it should be possible to mutate any gene by modifying cloned DNA, to recover virus from cells transfected with the altered DNA, and to observe the effect of the mutation on the life cycle of the virus. To define more precisely the extent and position of the sequences needed for viral genomic packaging and for synthesis of $gPr80^{gag}$, we have carried out site-specific mutagenesis of a cloned DNA copy of the M-MuLV RNA genome. Surprisingly, several of the mutant DNAs gave rise to fully infectious replication-competent virus, even though no glycosylated *gag* protein was made.

MATERIALS AND METHODS

Cells and virus. NIH/3T3 fibroblasts were grown in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 10% calf serum (Flow Laboratories, Inc.). M-MuLV released by NIH/3T3 clone 4 was the source of infectious wild-type virus. This cell line was derived as a single-cell clone of NIH/3T3 cells infected at low multiplicity (<0.05) with the clone 1 strain of M-MuLV (11). Viral 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. Lymphocyte cell lines SWR/4 (34) and 70Z (25) were grown in RPMI 1640 medium (GIBCO Laboratories) supplemented with 10% fetal calf serum.

Cloned DNAs. Plasmid p8.2 contains a full-length permuted copy of M-MuLV circular viral DNA, joined at the unique *Hin*dIII site in the center of the genome and containing only one copy of the long terminal repeat (LTR). The clone was prepared by subcloning the *Hin*dIII viral DNA fragment of lambda 8.2 (33) into the *Hin*dIII site of plasmid pBR322 by standard methods.

Plasmid pZAP contains a full-length proviral copy of M-MuLV plus approximately 8 kilobases of flanking rat sequences subcloned from phage lambda into the *Eco*RI site of plasmid pBR322 (15). Plasmid pSV2GPT (gift from R. Mulligan) contains the *Escherichia coli gpt* gene linked to a simian virus 40 promoter (24).

DNA enzymology. Partial restriction enzyme digests of plasmid DNAs were carried out at 20°C over a range of time periods (5 min to 1 h). Full-length linear molecules were isolated by electrophoresis in 0.6% agarose (Sigma Chemical Co.) gel cast in TEA buffer (50 mM Tris-hydrochloride, 1 mM EDTA, acetic acid to pH 8.0) containing 1 μg of ethidium bromide per ml and were purified by the glass powder method (41). This linear DNA was digested with *Bal* 31 exonuclease at 25°C for 20 s in buffer (0.3 M NaCl, 6 mM $CaCl_2$, 6 mM $MgCl_2$, 10 mM Tris-hydrochloride [pH 8.0], 0.5 mM EDTA). The reaction was quenched with the addition of EDTA to a final concentration of 20 mM, and the DNA was purified by successive extractions with phenol and ether and precipitation with ethanol. The DNA was circularized with T4 ligase (New England BioLabs) at a DNA concentration of 1 μg/ml in buffer (50 mM Tris-hydrochloride [pH 7.5], 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP).

Bacterial cultures. *E. coli* HB101 (4) 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, 1 mM NaOH) and was transformed to ampicillin resistance by the $CaCl_2$ method (4). Selective plates and media contained 50 μg of ampicillin per ml. Plasmid DNA was isolated from small cultures (2 ml) by the rapid preparation of Holms and Quigley (16) and from large cultures (200 ml) by equilibrium centrifugation in cesium chloride-ethidium bromide (17).

DNA sequence analysis. Mutant DNAs (1 to 5 μg) were cleaved with *Bst*EII (New England BioLabs), and the terminal 5′ phosphate was removed by treatment with calf intestinal phosphatase (Boehringer Manheim Corp.) in buffer (10 mM Tris [pH 8.3], 10 mM $MgCl_2$) for 1 h at 37°C. The DNA was labeled with polynucleotide kinase (Bethesda Research Laboratories) and [γ-, ^{32}P]ATP (Amersham Corp.) and was recleaved with *Sac*I (New England BioLabs). The end-labeled DNA fragment containing the mutation was isolated by electrophoresis on a 10% polyacrylamide gel and subjected to the chemical degradation protocol of Maxam and Gilbert (23). Cleavage products were displayed on 8% polyacrylamide–8 M urea gels (34 by 14 cm) and exposed to X-ray film (Kodak XR5, Eastman Kodak Co.) with an intensifying screen (Lighting Plus, Du Pont Co.) at −70°C.

Mammalian cellular transfections and transformations. Cloned mutated viral DNAs were excised from the pBR322 vector plasmid by cleavage with *Hin*dIII (Bethesda Research Laboratories) and were religated at a concentration of 100 to 500 μg/ml to ensure extensive concatemerization. Plasmid DNAs pZAP and pSV2GPT were used as circular DNAs. NIH/3T3 cells were transfected with the DNA by the calcium phosphate precipitation method (14, 35) with salmon sperm DNA as the carrier. Transfections for XC plaque assays were performed by precipitating 0.5 μg of cloned DNA and 6 μg of carrier in a volume of 0.5 ml and applying them to 6-cm dishes containing 10^5 to 1.5×10^5 cells per dish for 4 to 6 h. At 3 days after transfection, cells were split 1:10, replated in 10-cm dishes, and allowed to grow to confluency. The presence of viable virus was determined by overlaying with XC cells (29) after UV irradiation of the monolayer.

Cotransfections with plasmid pSV2GPT DNA were carried out essentially in the same fashion. A mixture of 1 μg of viral DNA, 1 μg of pSV2GPT, and 12 μg of carrier was applied to 3×10^5 to 5×10^5 cells on a 10-cm plate. At 2 days after transfection, cells were fed with XAT medium (15 mM hypoxanthine, 0.2 mM aminopterin, 5 mM thymidine, 250 mM xanthine, 150 mM glutamine, 5 mM glycine, 25 mM mycophenolic acid). Cells were refed every 5 days, and colonies were cloned 3 weeks later. The presence of reverse transcriptase activity in the media was assayed by the method of Goff et al. (13).

Viral protein analysis. Cells (2×10^5 per 6-cm dish) were infected with filtered virus harvested from clones of transfected mutants. After 1 day, cells were passed 1:10 into 10-cm dishes. When monolayers were half confluent, the cells were starved for 0.5 h in Dulbecco modified Eagle medium minus methionine, and [^{35}S]methionine (200 μCi; Amersham Corp.) was added for 15 min. The cells were lysed, and the proteins were immunoprecipitated with goat anti-M-MuLV serum or rabbit anti-*gag* serum 507 (gift of D. Baltimore) by standard procedures. Immunoprecipitated proteins

were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography as described previously (18, 39). The proteins were treated with endo-β-*N*-acetylglucosaminidase (endo H; gift of P. Robbins) as described (38).

RESULTS

Construction of deletion mutants in cloned M-MuLV DNA. The DNA used as the parent molecule for all the mutagenesis described in this work was plasmid p8.2, an infectious full-length copy of the M-MuLV genome in plasmid pBR322. This clone was prepared by cleavage of proviral circular DNA at the single *Hin*dIII site before cloning; thus, the M-MuLV insert was circularly permuted relative to the usual linear form of the genome. A map of the insert is shown in Fig. 1.

Mutants were constructed by deleting particular restriction endonuclease recognition sites and part of the flanking DNA. To direct these deletions to the region encoding the 5′ end of the viral RNA, recognition sites for the enzymes *Pst*I and *Pvu*I were selected as the initial targets. These enzymes made cleavages in the area of interest, although they also cleave elsewhere in the M-MuLV genome and in the plasmid pBR322 vector DNA. Plasmid p8.2 DNA was partially digested with either *Pst*I or *Pvu*I, and full-length linears (cleaved only once) were isolated from an agarose gel. This DNA was treated with *Bal* 31 exonuclease to remove a few base pairs from each end. It was next purified and recircularized by ligation at a low concentration. The modified DNA was used to transform *E. coli* HB101 to ampicillin resistance, and 12 resistant colonies were picked and grown into small cultures for each starting enzyme. Plasmid DNA isolated from each culture was analyzed by agarose gel electrophoresis after cleavage with *Pvu*II and *Sac*I to determine the position and approximate size of the deletion.

Approximately one-third to one-half of the plasmids examined contained deletions in M-MuLV sequences. Two of the DNAs mutagenized by cleavage with *Pst*I contained deletions of the entire 176-base pair (bp) *Pst*I fragment between 1.0 and 1.15 map units and were not examined further. A few mutants had deletions at the *Pst*I and *Pvu*I sites in plasmid pBR322, but nevertheless they must have encoded functional ampicillinase enzymes. Those plasmids containing small deletions mapping at either the *Pvu*I or *Pst*I site near the 5′ end of the M-MuLV genome were amplified in *E. coli* HB101 and purified on CsCl gradients for further analysis.

Mapping and sequencing of mutants. Three mutants containing small deletions in the sites of interest were selected for further study. Two of these mutants, termed *dl*902 and *dl*904, were prepared by cleavage with *Pvu*I; mapping showed that they had lost the *Pvu*I site at nucleotide position 421 and approximately 75 bp of flanking DNA. The third mutant, *dl*1003, was prepared from *Pst*I linears and had lost the *Pst*I site at position 565 as well as approximately 50 bp nearby. In all three cases, the remaining *Pvu*I and *Pst*I sites were intact.

The nucleotide sequences of the mutated regions were determined as described above. Briefly, each mutant DNA was cleaved with *Bst*EII, the 5′ ends were labeled with ^{32}P, and the DNAs were recut with *Sac*I. Maxam-Gilbert cleavage reactions (23) were performed on the fragment containing the site of the deletion, and the sequence was determined by electrophoresis of the DNA fragments. The numbers and positions of the bases missing in each mutant are shown in Fig. 1. Mutants *dl*902 and *dl*904 contained deletions of 56 and 83 bp, respectively, and mutant *dl*1003 contained a 66-bp deletion very close to the start of $Pr65^{gag}$. The sequences obtained in this region agree with the data of Shinnick et al. (32), except for one base substitution (a G to A change at nucleotide 617) near the AUG for $Pr65^{gag}$ (Fig. 1).

Biological activity. Plasmid p8.2 is a permuted clone of M-MuLV containing only one LTR. To study the infectivity of the mutagenized clones, each DNA was cleaved with *Hin*dIII and ligated into polymers to reconstruct a complete proviral structure. DNAs were applied to sensitive NIH/3T3 fibroblasts in a calcium phosphate precipitate, and the cells were tested for release of viable virus by overlay with XC cells. All three deletion mutants, *dl*902, *dl*904, and *dl*1003, formed XC syncytia similar in appearance and number to those formed by the wild-type viral clone p8.2. Media from these plates contained measurable reverse transcriptase activity (data not shown).

Clonal NIH/3T3 cell lines producing virus were isolated by cotransfection with two DNAs, namely plasmid pSV2GPT DNA carrying the selectable marker of resistance to mycophenolic acid, and each of the polymerized mutant DNAs. Cells expressing the *gpt* gene were selected in XAT medium (see above), and several mycophenolic acid-resistant colonies were cultured and tested for reverse transcriptase activity. Between one-third and all of the colonies exposed to DNAs of the mutants were positive in this assay (Fig. 2). To test the transmissibility of these apparently viable viruses, medium from the reverse transcriptase-positive clones was filtered and used to infect new NIH/3T3 cells. Cells infected in this manner also produced virus as measured by the XC assay, with titers in the range of 10^4 to 10^5 PFU/ml (data not shown).

In summary, these viruses rendered NIH/3T3

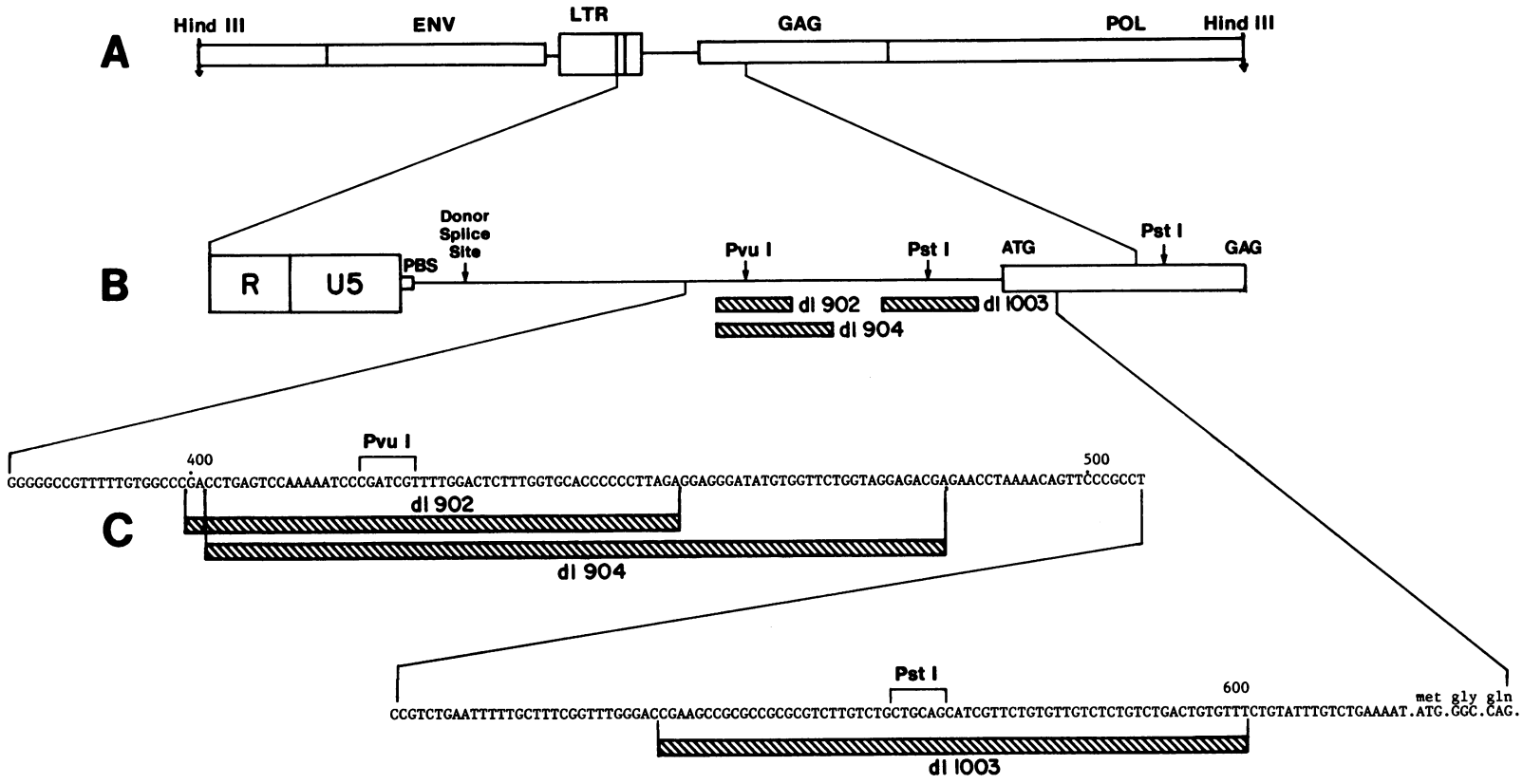

FIG. 1. Sizes and positions of deletions in the M-MuLV genome. (A) Structure of the DNA insert in plasmid p8.2. The DNA consists of a permuted copy of the entire genome of M-MuLV. The positions of the *env*, *gag*, and *pol* genes and of the viral LTR sequence are indicated by the boxed areas. (B) Enlargement of the region from the LTR to the site of initiation of $Pr65^{gag}$. The regions deleted in mutants *dl*902, *dl*904, and *dl*1002 are indicated by the hatched boxes. (C) Nucleotide sequence of the parent DNA in the region altered by mutagenesis. The nucleotides missing in each deletion are indicated by the hatched boxes; the sites for cleavage by *Pvu*I and *Pst*I are also shown.

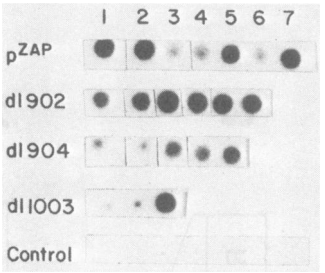

FIG. 2. Reverse transcriptase assays of supernatant fluids from clones of cells transfected by various DNAs. Each viral DNA was oligomerized, mixed with plasmid pSV2GPT DNA, and applied to NIH/3T3 cells. Individual clones resistant to mycophenolic acid were isolated, and the supernatant fluid was assayed for reverse transcriptase on an exogenous template (13). Results for several independent clones derived from each transfection are shown.

cells XC positive upon transfection, caused them to release reverse transcriptase-containing virions, and were transmissible to fibroblasts.

Growth of mutant viruses in lymphocytes. A number of strains of murine leukemia viruses, including the Gross virus and a BALB/c endogenous strain, replicate poorly in lymphocyte cell lines and also act as poor helpers for the stable transmission of Abelson MuLV into such cells (28). To examine whether these deletions could cause such a phenotype, two lymphocyte lines were infected with wild-type or mutant virus at a multiplicity of approximately one, and the virus released by these cells was monitored over several days. In both 70Z, a B-cell leukemia cell line, and SWR/4, an Abelson MuLV-induced (pre–B-cell) leukemia cell line, the mutant viruses replicated normally and were secreted into the medium at least as well as wild-type virus (Fig. 3). Thus, the region deleted is not essential for replication in these lymphocytes.

Analysis of *gag* proteins. Cells infected with the mutant viruses were pulse-labeled for 15 min with [^{35}S]methionine, and extracts of protein were prepared for analysis. Viral proteins were immunoprecipitated with a variety of sera, and the immune complexes were collected by binding to Formalin-fixed *Staphlococcus aureus*. Proteins were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by fluorography (Fig. 4). Wild-type virus (in NIH/3T3 clone 4 cells) clearly directed the synthesis of two *gag*-specific proteins, the internal Pr65^{gag} and the glycosylated gPr80^{gag} (which migrated slightly faster than the envelope protein gPr80^{env}). Cells containing mutants *dl*902 and *dl*904 showed only the internal *gag* protein and no detectable larger species even upon long overexposure of the fluorograph. The cells infected with mutant *dl*1003, however, synthesized Pr65^{gag} and a large amount of a novel protein migrating at approximately 71,000 daltons, detected by *gag*-specific sera (Fig. 4). The difference in apparent molecular mass between the wild-type gPr80^{gag} and the *dl*1003 mutant

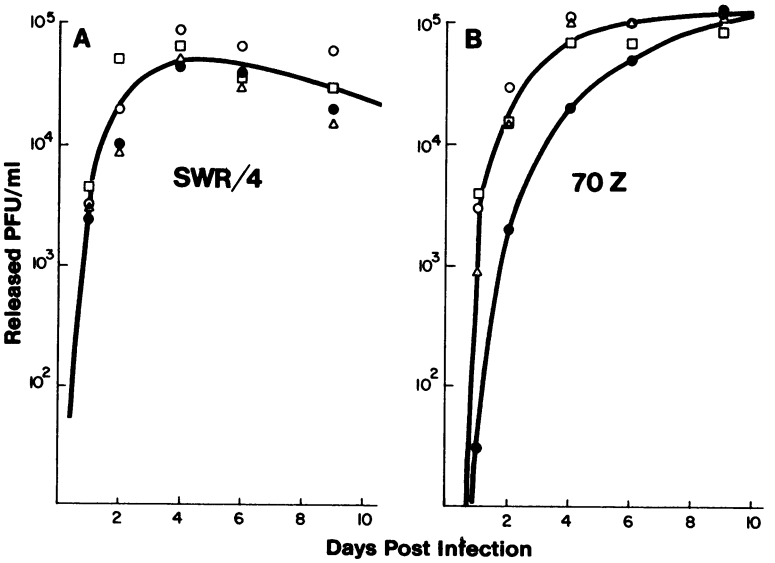

FIG. 3. Growth curves of various M-MuLV isolates in lymphocyte cell lines SWR/4 (A) and 70Z (B). The indicated cell lines were infected with virus on day zero, and supernatant fluids were collected on successive days. PFU in these supernatants were titrated by XC assay in NIH/3T3 cells. Mock-infected cultures released no PFU. Virus isolates used were clone 4 virus (●), *dl*902 virus (○), *dl*904 virus (□), and *dl*1003 virus (△).

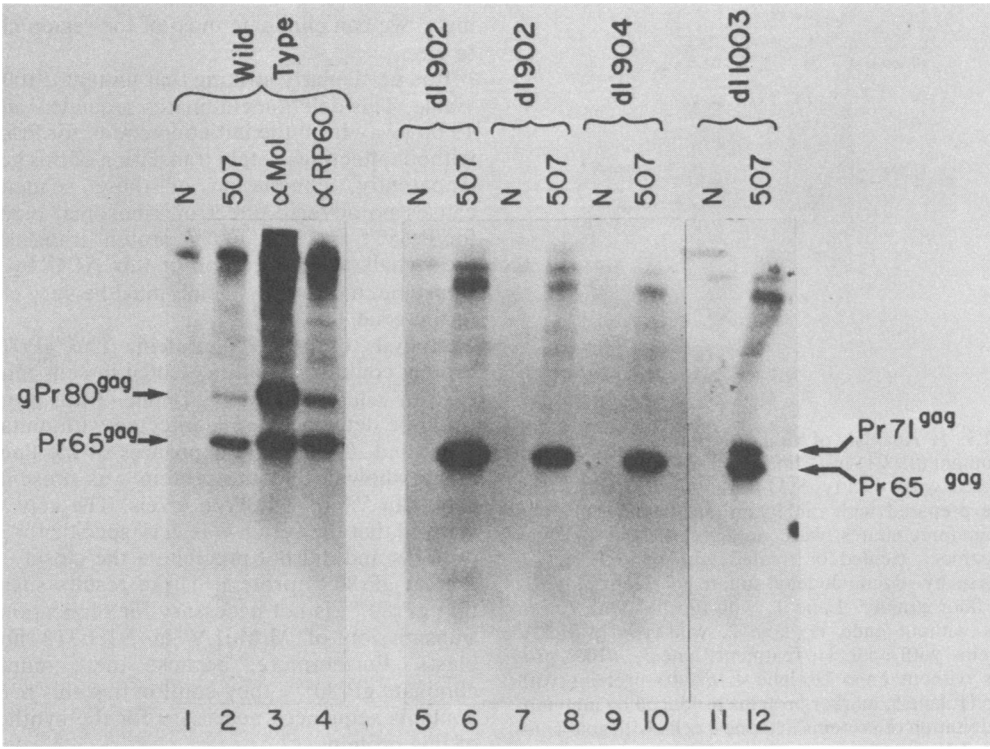

FIG. 4. Analysis of viral *gag* proteins synthesized in infected NIH/3T3 cells. Cell populations infected with various M-MuLV preparations were labeled with [^{35}S]methionine, and the labeled proteins were analyzed by immunoprecipitation, sodium dodecyl sulfate gel electrophoresis, and fluorography. Lanes 1 through 4: Proteins from cells infected with wild-type M-MuLV (clone 4 cells) were precipitated with normal goat serum (lane 1), rabbit anti-*gag* serum 507 (lane 2), goat anti-Moloney serum (lane 3), and goat anti-Rauscher Pr60^{gag} serum (lane 4). The positions of the wild-type proteins Pr65^{gag} and gPr80^{gag} are indicated. Lanes 5 through 12: Cells infected with mutants *dl*902 (two isolates), *dl*904, and *dl*1003, as indicated, were analyzed with normal goat serum (N) or rabbit anti-*gag* serum 507 (507). The positions of proteins Pr65^{gag} and Pr71^{gag} are indicated.

protein was approximately 9,000 daltons and was too great to be accounted for by the 66-bp deletion. One possible explanation for this discrepancy is that the deleted protein may also lack the carbohydrate found on normal gPr80^{gag}.

To determine whether the novel protein contained carbohydrate, the immunoprecipitated proteins were digested with endo H, which cleaves mannose-rich carbohydrate side chains, and the treated protein was analyzed as before (Fig. 5). Wild-type gPr80^{gag} was reduced in size by endo H treatment, migrating as a protein of approximately 73,000 daltons in these gels. The mutant *dl*1003 protein was completely unchanged by endo H treatment (Fig. 5). Thus, the mutant protein appeared to lack the normal glycosylation found on the wild-type large membrane-bound *gag* protein.

All these protein phenotypes were completely stable for many cell generations (data not shown). Thus, reversion or recombination with endogenous viruses did not interfere with these assays.

DISCUSSION

The site-specific mutations described in this work were constructed by *Bal* 31 exonuclease treatment of cloned viral DNA linearized by restriction enzymes. This method provided a rapid and reliable means for introducing mutations at selected sites throughout the viral genome: approximately 80% of plasmids isolated after the mutagenesis contained deletions. From such treatment of clone p8.2 DNA, linearized with *Pst*I and *Pvu*I, we obtained three mutants containing small deletions in the region between the 5′ LTR and the initiation AUG for the major *gag* and *gag-pol* precursor proteins. Several larger deletions which were not viable were also obtained (data not shown).

When these three DNAs were applied to NIH/3T3 cells in a calcium phosphate precipitate, viable virus was produced, as demonstrated by XC plaque assay and by the presence of reverse transcriptase activity. When NIH/3T3 cells were cotransfected with viral DNAs and plasmid

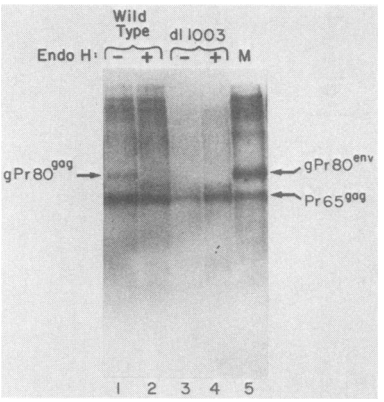

FIG. 5. Analysis of viral *gag* proteins synthesized by mutant *dl*1003 virus. Immunoprecipitates from cells infected with wild-type M-MuLV or mutant *dl*1003 were prepared with rabbit anti-*gag* serum 507. The immunoprecipitates were solubilized, and portions were mock treated or treated with endo H before analysis by sodium dodecyl sulfate gel electrophoresis and fluorography. Lane 1, wild-type M-MuLV proteins without endo H; lane 2, wild-type M-MuLV proteins with endo H treatment; lane 3, *dl*1003 proteins without endo H; lane 4, *dl*1003 proteins with endo H; lane 5, marker proteins produced by immunoprecipitation of proteins in clone 4 cells with goat anti-M-MuLV serum.

pSV2GPT, a majority of the clones surviving in XAT medium produced reverse transcriptase activity in the supernatant medium, indicating the presence of released viral particles. In this way, producer cell lines were readily isolated. It should be noted, however, that the percentage of clones cotransfected may be an overestimate, since we cannot rule out the possibility of virus spread on the original transfection plate. Each of these clones produced fully transmissible virus, which could be filtered and passed to new NIH/3T3 cells to render them XC positive.

Murine leukemia viruses contain an unusually long leader sequence (620 bp in the case of M-MuLV) between the 5′ end of the viral RNA and the initiation codon for the $Pr65^{gag}$ and $Pr200^{gag\text{-}pol}$ precursors (32). We have shown that deletions within an approximately 200-bp region apparently do not affect viral functions in NIH/3T3 fibroblasts. Thus, we conclude that the deleted regions contain no *cis*-acting sequences required for viral replication. R. Mann has recently isolated one M-MuLV mutant containing a 350-bp deletion in this region (personal communication); this mutant is defective and unable to transmit stably its own genome to infected cells. Because our mutants are not impaired in replication functions, we can further narrow the window in which these control sequences are located to a region close to the 5′ end; accordingly, we can eliminate most of the region close to *gag*.

It is particularly striking that mutant *dl*1003 is viable. This deletion eliminates sequences up to 19 bp away from the initiation codon for $Pr65^{gag}$ without affecting protein translation of this gene. Apparently, elimination of these sequences causes no adverse effect on ribosomal binding for $Pr65^{gag}$ and $Pr200^{gag\text{-}pol}$ protein translation; the signals for recognition of this AUG by the ribosome, if they exist at all, must lie very close to the gene.

Analysis of $gPr80^{gag}$ protein. The $gPr80^{gag}$ protein could be readily detected in cells infected with wild-type virus, but none of this protein could be detected in cells infected with mutants *dl*902 and *dl*904. Long exposures of the fluorograms showed that the protein was present at less than 5% of wild-type levels. The gels also showed that the serum was very specific for *gag* proteins and did not precipitate the closely migrating $gPr80^{env}$ protein. These results suggest that $gPr80^{gag}$ is not necessary for replication or transmission of M-MuLV in NIH/3T3 fibroblasts. Furthermore, because these mutants eliminate $gPr80^{gag}$, they confirm that this region contains sequences necessary for the synthesis of this protein.

Immunoprecipitation of *gag* proteins from mutant *dl*1003 revealed a protein migrating at approximately 71,000 daltons; we interpreted this protein to be a shortened $gPr80^{gag}$. The observed shift in mobility was too great, however, to be accounted for by a 22-amino-acid deletion. Since $gPr80^{gag}$ is a glycosylated protein, the large shift in mobility of the mutant protein might result from the combination of a lack of carbohydrate side chains and a shortened polypeptide. When the mutant *dl*1003 protein was treated with the enzyme endo H, which selectively removes the mannose-rich side chains, no change in mobility was detected. In contrast, wild-type $gPr80^{gag}$ was reduced in apparent size by endo H treatment to approximately 73,000 daltons, only slightly larger than the mutant protein. The difference in the apparent size of the deglycosylated wild-type protein and the *dl*1003 mutant protein is small: about 2,000 daltons, very close to the difference predicted from a 66-bp DNA deletion. This good agreement suggests that the polypeptide moiety of both proteins begins and ends at identical sequences and that the only difference is the internally deleted region of *dl*1003.

Previous studies (7) tentatively map the sites of glycosylation in wild-type $gPr80^{gag}$ to the region shared with the major *gag* proteins P30 and P15, far downstream from the position of the deletion in *dl*1003. This mutation must, therefore, eliminate glycosylation of sites at a considerable distance. We consider two possibilities

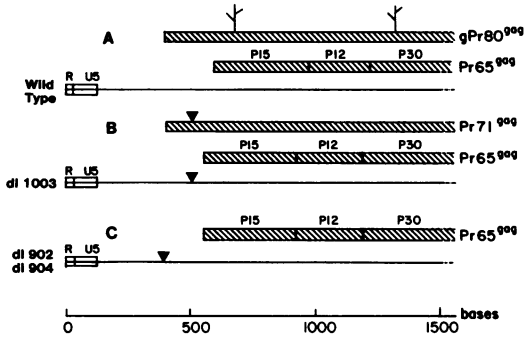

FIG. 6. Proposed structure of RNA genomes (lines) and protein products (hatched boxes) of various M-MuLV isolates. (A) Two *gag* proteins, $gPr80^{gag}$ and $Pr65^{gag}$, are synthesized by wild-type M-MuLV. The structure of the RNA template for the synthesis of $gPr80^{gag}$ is unknown. Carbohydrates are added to $gPr80^{gag}$ at portions corresponding to P15 and P30 in the mature cleavage products of $Pr65^{gag}$. (B) Proteins synthesized by mutant *dl*1003 are $Pr71^{gag}$ and $Pr65^{gag}$. The 66-bp deletion in this genome causes a shortening of the larger *gag* protein without affecting $Pr65^{gag}$. The deletion also abolishes the addition of the carbohydrate chains usually found at the downstream sites. (C) Only $Pr65^{gag}$ is synthesized by mutants *dl*902 and *dl*904; production of the larger protein is completely abolished by the deletions.

which could account for the effect of this mutation. One possibility is that the deletion in *dl*1003 alters the structure of this protein in a manner which decreases the accessibility of the glycosylation sites. On the average, only one-third of the classic tripeptide sites (-Asn-X-Thr/Ser) are found glycosylated in membrane-bound and secreted proteins, and thus it has been proposed (37) that the addition of carbohydrate side chains is dependent on the tertiary structure surrounding the glycosylation site. A deletion of 22 amino acids may well change the conformation of $gPr80^{gag}$ so as to eliminate glycosylation. Alternatively, the deletion in *dl*1003 may interfere with a signal sequence which normally directs this protein to the membrane. The mutant protein may not be translated on the rough endoplasmic reticulum, and according to current models for the synthesis of membrane proteins, it would not be glycosylated or transported to the cell membrane. We are currently testing the localization of the protein by surface protein iodination with lactoperoxidase and by surface immunofluorescent stains.

If the *dl*1003 protein has lost part of a signal sequence normally tagging it as a membrane protein, it must have one unusual property. Normally, short leader peptides of about 30 amino acids are cleaved from membrane proteins during secretion, and mutations in this leader block glycosylation and cleavage (2, 3, 10) to leave a larger translation product. The *dl*1003 protein, despite its deletion, would be larger than wild-type $gPr80^{gag}$ if a leader peptide of 30 amino acids were not removed. Thus, $gPr80^{gag}$ may resemble the case of ovalbumin, which contains an internal signal sequence needed for membrane transport and which does not undergo cleavage of a leader peptide (19).

Structure of the gene for $gPr80^{gag}$. The deletion mutants described in this paper help define the region which encodes the glycosylated *gag* protein of M-MuLV. The extra amino acids found in $gPr80^{gag}$ and not in $Pr65^{gag}$ have been localized by peptide analysis (7) to the amino terminal segment of the longer protein. The simplest interpretation of the structure of $gPr80^{gag}$ is that translation is initiated at a site in the leader RNA and continues in phase into the sequence of the regular $Pr65^{gag}$ (Fig. 6). Three AUG initiator methionine codons are found in the published sequence (32) upstream of the AUG for the $Pr65^{gag}$, but none of these codons is in the same translational reading frame as the $Pr65^{gag}$. It is possible that proper translational initiation or RNA splicing sites have not been identified. A less likely possibility is that this sequence may be translated starting from a valine GTG codon like that found in procaryotic proteins or from an entirely different codon.

Possible protein function. Our work provides the first example of mutants in the glycosylated membrane-bound *gag* protein. Previous efforts to isolate such mutants by immunoselection of infected cells with anti-*gag* sera and complement lysis have only resulted in isolation of cellular mutants defective in membrane protein processing (8, 12). The production of viable virus by some of these cell lines despite the lack of a glycosylated *gag* protein on their cell surfaces is in agreement with our findings.

The construction of viable deletion mutants of M-MuLV which lack $gPr80^{gag}$ provides conclusive evidence that this protein is not required for replication and transmission in NIH/3T3 fibroblasts or in lymphocytes. No known function has yet been associated with the glycosylated *gag* protein, but the protein may be involved in some little-understood function of leukemogenesis. The availability of mutants in $gPr80^{gag}$ will facilitate a critical evaluation of possible roles for this protein in the transformation of lymphoid cells in vivo.

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LITERATURE CITED

1. **Alevy, M. C., and P. K. Vogt.** 1978. Ts *pol* mutants of avian sarcoma viruses: mapping and demonstration of single-cycle recombinants. Virology 87:21–23.

2. **Bedouelle, H., P. J. Bassford, Jr., A. V. Fowler, I. Zabin, J. Beckwith, and M. Hofnung.** 1980. Mutations which alter the function of the signal sequence of the maltose binding protein of *Escherichia coli*. Nature (London) **285**:78–81.
3. **Blobel, G., and B. Dobberstein.** 1975. Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. J. Cell Biol. **67**:835–851.
4. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *E. coli*. J. Mol. Biol. **41**:459–472.
5. **Dickson, C., R. Eisenman, H. Fan, E. Hunter, and N. Teich.** 1982. Protein biosynthesis and assembly, p. 513–648. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
6. **Edwards, S. A., and H. Fan.** 1979. *gag*-Related polyproteins of Moloney murine leukemia virus: evidence for independent synthesis of glycosylated and unglycosylated forms. J. Virol. **30**:551–563.
7. **Edwards, S. A., and H. Fan.** 1980. Sequence relationship of glycosylated and unglycosylated *gag* polyproteins of Moloney murine leukemia virus. J. Virol. **35**:41–51.
8. **Edwards, S. A., and H. Fan.** 1981. Immunoselection and characterization of Moloney murine leukemia virus-infected cell lines deficient in surface *gag* antigen expression. Virology **113**:95–108.
9. **Eisenman, R. N., W. E. Mason, and M. Linial.** 1980. Synthesis and processing of polymerase proteins of wild-type and mutant avian retroviruses. J. Virol. **36**:62–78.
10. **Emr, S. D., J. Hedgpeth, J. M. Clement, T. J. Silhavy, and M. Hofnung.** 1980. Sequence analysis of mutations that prevent export of lambda-receptor, an *Escherichia coli* outer membrane protein. Nature (London) **285**:82–85.
11. **Fan, H., and M. Paskind.** 1974. Measurement of the sequence complexity of cloned Moloney murine leukemia virus 60 to 70*S* RNA: evidence for a haploid genome. J. Virol. **14**:421–429.
12. **Fittig, T., M. Ruta, and D. Kabat.** 1981. Mutant cells that abnormally process plasma membrane glycoproteins encoded by murine leukemia virus. Cell **24**:847–858.
13. **Goff, S., P. Traktman, and D. Baltimore.** 1981. Isolation and properties of Moloney murine leukemia virus mutants: use of a rapid assay for release of virion reverse transcriptase. J. Virol. **38**:239–248.
14. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–471.
15. **Hoffmann, J. W., D. Steffen, J. Gusella, C. Tabin, S. Bird, D. Cowing, and R. A. Weinberg.** 1982. DNA methylation affecting the expression of murine leukemia proviruses. J. Virol. **44**:144–157.
16. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114**:193–197.
17. **Katz, L., D. T. Kingsbury, and D. R. Helinski.** 1973. Stimulation by cyclic adenosine monophosphate of plasmid deoxyribonucleic acid replication and catabolite repression of the plasmid deoxyribonucleic acid-protein relaxation complex. J. Bacteriol. **114**:557–591.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
19. **Lingappa, V. R., J. R. Lingappa, and G. Blobel.** 1979. Chicken ovalbumin gene contains an internal signal sequence. Nature (London) **281**:117–121.
20. **Linial, M., and W. S. Mason.** 1973. Characterization of two conditional early mutants of Rous sarcoma virus. Virology **53**:258–273.
21. **Mason, W., and C. Yeater.** 1977. A mutant of Rous sarcoma virus with a conditional defect in the determinants of viral host range. Virology **77**:443–456.
22. **Mason, W. S., C. Yeater, T. V. Bosch, J. A. Wyke, and R. R. Friis.** 1979. Fourteen temperature-sensitive replication mutants of Rous sacoma virus. Virology **99**:226–240.
23. **Maxam, A., and W. Gilbert.** 1979. Sequencing end-labeled DNA with base specific chemical cleavages. Methods Enzymol. **65**:499–599.
24. **Mulligan, R. C., and P. Berg.** 1981. Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase. Proc. Natl. Acad. Sci. U.S.A. **78**:2072–2076.
25. **Paige, C. J., P. W. Kincade, and P. Ralph.** 1978. Murine B cell leukemia line with inducible surface immunoglobulin expression. J. Immunol. **121**:641–647.
26. **Peters, G., and J. E. Dahlberg.** 1979. RNA-directed DNA synthesis in Moloney murine leukemia virus: interaction between the primer tRNA and the genome RNA. J. Virol. **31**:398–407.
27. **Rohrschneider, J. M., H. Diggelman, H. Ogura, R. R. Friis, and H. Bauer.** 1976. Defective cleavage of a precursor polypeptide in a temperature-sensitive mutant of avian sarcoma virus. Virology **75**:177–187.
28. **Rosenberg, N., and D. Baltimore.** 1978. The effect of helper virus on Abelson virus-induced transformation of lymphoid cells. J. Exp. Med. **147**:1126–1141.
29. **Rowe, W. P., W. E. Pugh, and J. W. Hartley.** 1970. Plaque assay techniques for murine leukemia viruses. Virology **42**:1136–1139.
30. **Shank, P. R., and M. Linial.** 1980. Avian oncornavirus mutant (SE21Q1b) deficient in genomic RNA: characterization of a deletion in the provirus. J. Virol. **36**:450–456.
31. **Shields, A., O. N. Witte, E. Rothenberg, and D. Baltimore.** 1978. High frequency of aberrant expression of Moloney murine leukemia virus in clonal infections. Cell **14**:601–609.
32. **Shinnick, T. M., R. A. Lerner, and J. G. Sutcliffe.** 1981. Nucleotide sequence of Moloney murine leukemia virus. Nature (London) **293**:543–548.
33. **Shoemaker, C., S. Goff, E. Gilboa, M. Paskind, S. W. Mitra, and D. Baltimore.** 1980. Structure of a cloned circular Moloney murine leukemia virus DNA molecule containing an inverted segment: implications for retrovirus integration. Proc. Natl. Acad. Sci. U.S.A. **77**:3932–3936.
34. **Silverstone, A. E., N. Rosenberg, V. L. Sato. M. P. Schneid, E. A. Boyse, and D. Baltimore.** 1978. Control of cell proliferation. vol. 5. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
35. **Smotkin, D., A. M. Gianni, S. Rozenblatt, and R. A. Weinberg.** 1975. Infectious viral DNA of murine leukemia virus. Proc. Natl. Acad. Sci. U.S.A. **72**:4910–4913.
36. **Stephenson, J. R., and S. A. Aaronson.** 1973. Characterization of temperature-sensitive mutants of murine leukemia virus. Virology **54**:53–59.
37. **Struck, D. K., and W. J. Lennarz.** 1980. The function of saccharide-lipids in synthesis of glycoproteins, p. 35–80. *In* W. Lennarz (ed.), The biochemistry of glycoproteins and proteoglycans. Plenum Publishing Corp., New York.
38. **Tarentino, A. L., and F. Maley.** 1974. Purification and properties of an endo B-*N*-acetyl-glucosaminidase from *Streptomyces griseus*. J. Biol. Chem. **249**:811–816.
39. **Traktman, P., and D. Baltimore.** 1982. Protease bypass of temperature-sensitive murine leukemia virus maturation mutants. J. Virol. **44**:1039–1046.
40. **Tronikc, S. R., J. R. Stephenson, I. M. Verma, and S. A. Aaronson.** 1975. Thermolabile reverse transcriptase of a mammalian leukemia virus mutant temperature sensitive in its replication and sarcoma virus helper functions. J. Virol. **16**:1476–1482.
41. **Vogelstein, B., and D. Gillespie.** 1979. Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. U.S.A. **76**:615–619.
42. **Wensink, P. C., D. J. Finnegan, J. E. Donelson, and D. S. Hogness.** 1974. A system for mapping DNA sequences in the chromosomes of *Drosophila melanogaster*. Cell **3**:315–325.
43. **Wong, P. K. Y., L. J. Russ, and J. A. McCarter.** 1973. Rapid selective procedure for isolation of spontaneous temperature-sensitive mutants of Moloney leukemia virus. Virology **51**:424–431.