JOURNAL OF VIROLOGY, Mar. 1985, p. 899–907
0022-538X/85/030899-09$02.00/0

Vol. 53, No. 3

A Deletion Mutation in the 5′ Part of the *pol* Gene of Moloney Murine Leukemia Virus Blocks Proteolytic Processing of the *gag* and *pol* Polyproteins

SARAH CRAWFORD AND STEPHEN P. GOFF*

Department of Biochemistry and Institute for Cancer Research, Columbia University College of Physicians and Surgeons, New York, New York 10032

Received 1 October 1984/Accepted 10 December 1984

Deletion mutations in the 5′ part of the *pol* gene of Moloney murine leukemia virus were generated by restriction enzyme site-directed mutagenesis of cloned proviral DNA. DNA sequence analysis indicated that one such deletion was localized entirely within the 5′ part of the *pol* gene, did not affect the region encoding reverse transcriptase, and preserved the translational reading frame downstream of the mutation. The major viral precursor polyproteins ($Pr65^{gag}$, $Pr200^{gag\text{-}pol}$, and $gPr80^{env}$) were synthesized at wild-type levels in cell lines carrying the mutant genome. These cell lines assembled and released wild-type levels of virion particles into the medium. Cleavage of both $Pr65^{gag}$ and $Pr200^{gag\text{-}pol}$ precursors to the mature proteins was completely blocked in the mutant virions. Surprisingly, these virions contained high levels of active reverse transcriptase; examination of the endogenous reverse transcription products synthesized by the mutant virions revealed normal amounts of minus-strand strong-stop DNA, indicating that the RNA genome was packaged and that reverse transcription in detergent-permeabilized virions was not significantly impaired. Processing of $gPr80^{env}$ to $gP70^{env}$ and P15E was not affected by the mutation, but cleavage of P15E to P12E was not observed. The mutant particles were poorly infectious; analysis indicated that infection was blocked at an early stage. The data are consistent with the idea that the 5′ part of the *pol* gene encodes a protease directly responsible for processing $Pr65^{gag}$, and possibly $Pr200^{gag\text{-}pol}$, to the structural virion proteins. It appears that cleavage of the *gag* gene product is not required for budding and release of virions and that complete processing of the *pol* gene product to the mature form of reverse transcriptase is not required for its functional activation.

Processing of precursor polyproteins to functional gene products plays a fundamental role in the assembly of many viruses, including phage (14, 23), poliovirus (18), and retroviruses (for review, see reference 22). All three genes of the replication-competent retroviruses (*gag*, *pol*, and *env*) encode polyproteins whose structural and functional domains are separated by proteolytic cleavages (2, 7, 22). In Moloney murine leukemia virus (M-MuLV), the *gag* polyprotein, $Pr65^{gag}$, is processed to yield four structural virion proteins: P15, P12, P30, and P10 (1, 35, 50). The *pol* gene is expressed as a large fusion protein, $Pr200^{gag\text{-}pol}$, that is processed to the mature reverse transcriptase enzyme, P80 (19, 31, 33), and to at least two additional proteins. Finally, the envelope precursor, $gPr80^{env}$, is cleaved to gP70, a glycoprotein on the virion surface, and P15E, a hydrophobic membrane protein attached to gP70 by disulphide bonds (8, 17, 20). All these cleavages are thought to be essential for activating the infectivity of the virus.

The extensive processing required to generate mature viral proteins from primary translation products may involve proteases of both host and viral origin. In the avian retroviruses, the protease responsible for processing the *gag* precursor has been purified and shown to be a viral protein of approximately 15,000 daltons, encoded by the 3′ part of the *gag* gene (5, 29, 52, 53). Much less is known about the mammalian systems. In contrast to the avian system, none of the *gag* gene products of the murine viruses has been shown to have proteolytic activity. Yoshinaka and Luftig have partially purified a proteolytic activity from virions of M-MuLV and Rauscher MuLV (57, 58, 60); the activity, which resides in a polypeptide of about 15,000 daltons, could be shown to process $Pr65^{gag}$ to the correct cleavage products in vitro (28, 59, 61). There is some recent evidence that this protease might be encoded by the *pol* gene. Oroszlan and co-workers (personal communication) have determined the N-terminal amino acid sequence of the mature reverse transcriptase of M-MuLV and aligned the protein with the residues predicted by the nucleic acid sequence of the *pol* gene. The placement leaves a region between the *gag* gene and the region encoding reverse transcriptase that has coding potential for a protein of approximately 15,000 daltons; the sequence of the protein encoded by this region has homology to known thiol proteases. These results suggested that the protease for cleavage of the *gag* precursor might be encoded in the 5′ portion of the *pol* gene. It could not be determined whether this protease cleaved the *pol* or *env* precursors or whether it might be required for other steps in the viral life cycle. The only report of a mutation in this region (29) showed that a single base-pair (bp) change did not affect replication or viability but prevented XC plaque formation.

To determine the functions of the 5′ part of the *pol* gene of M-MuLV, we introduced a series of deletion mutations into this region by in vitro mutagenesis of cloned proviral DNA. We then introduced the altered DNA into NIH/3T3 cells by cotransformation. Cell lines expressing the mutant genome released virions containing unprocessed $Pr65^{gag}$, demonstrating that the 5′ part of the *pol* gene was indeed required for *gag* cleavage and that cleavage was not necessary for virion assembly or release from the cell. Cleavage of the *gag-pol* precursor to mature reverse transcriptase also was blocked; surprisingly, this precursor showed full enzymatic activity in

* Corresponding author.

vitro. The infectivity of the mutant virions was dramatically reduced, implicating the processing of virion polyproteins in the activation of the particles.

MATERIALS AND METHODS

Cells and Viruses. NIH/3T3 and XC cells were grown in Dulbecco modified Eagle Medium (GIBCO Laboratories) supplemented with 10% calf serum. M-MuLV released by NIH/3T3 cells after transfection with plasmid clone pT11 (24) was the source of wild-type virus. Virus infections at high multiplicity were carried out in the presence of 8 μg of Polybrene (Aldrich Chemical Co.) per ml at 37°C for 2 h, and the infected cells were then maintained in 1.6 μg of Polybrene per ml overnight. Cotransformation and transfections of NIH/3T3 cells were as described previously (4, 45). Preparation of single-cell clones from cell populations was carried out as follows. Cells were trypsinized, diluted to a concentration of either 30 or 3 cells per ml, and distributed into cloning wells (0.2 ml). Virus-producing cell lines were identified by screening for release of reverse transcriptase (11).

Cloned DNAs. Plasmid pT11 contains a complete infectious proviral copy of the M-MuLV genome (24) derived from the clone pMOV9 (3). Plasmid pSV2neo contains the kanamycin resistance gene of Tn*5* linked to a simian virus 40 promoter (45). Plasmid pA8.2 contains a permuted, infectious copy of M-MuLV with one long terminal repeat (43), cloned into the *Hin*dIII site of the pACYC177 vector (39).

Enzymatic manipulations of DNA. Partial restriction enzyme digests of plasmid DNAs with *Bst*EII were carried out at 25°C for various times, ranging from 1 to 60 min, and the digestion products were separated by electrophoresis on 0.8% agarose gels. Maximal yields of full-length linear DNAs were obtained after approximately 10 to 15 min of digestion with 5 U of enzyme per μg of DNA. The linear DNA was treated with BAL 31, separated by electrophoresis, eluted (51), circularized, and used to transform *Escherichia coli* HB101 as previously described (26). Plasmids isolated from colonies (16) were screened for deletions by digestion with *Hin*dIII plus *Bst*EII, followed by electrophoresis on 1.2% agarose gels.

Bacterial cultures. Growth of bacteria, transformations, and plasmid preparations (21) were as described before (4).

DNA sequence analysis. Mutant DNAs (10 μg) were cleaved with *Sac*I (New England Biolabs), and the terminal 5′ phosphate was removed by treatment with calf intestinal phosphatase (Boehringer Mannheim Biochemicals). The DNA was labeled with T4 polynucleotide kinase (Bethesda Research Laboratories) and [γ-^{32}P]ATP and then cleaved with *Xho*I. The end-labeled fragment was isolated and sequenced (27) as described previously (4).

Analysis of viral proteins. Monolayers of transformants at approximately three-fourths confluence in 10-cm dishes were starved for 30 min in medium lacking methionine and then were labeled with 150 μCi of [^{35}S]methionine (Amersham Corp.) in 2 ml of medium for 45 min. The cells were then lysed in 3 ml of buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.1 M NaCl, 10 mM sodium phosphate [pH 7.5]), and the extract was incubated with 15 μl of normal goat serum for 3 h at 4°C and then with 150 μl of fixed, washed *Staphlococcus aureus* (Pansorbin; Calbiochem-Behring) for an additional 3 h. The extract was clarified (35,000 rpm at 4°C for 3 h), and the labeled proteins were immunoprecipitated with goat anti-Moloney serum (NCI #77S-591) or anti-pol serum (NCI #77S-424) as described before (4). Proteins were analyzed by electrophoresis on 8 or 10% polyacrylamide gels (23), followed by fluorography. Proteins in virion particles were examined as follows. For detection of *gag* proteins, cells in one 10-cm dish were incubated in 5 ml of medium lacking leucine, and approximately 120 μCi of [^{3}H]leucine (Amersham Corp.) was added for 12 to 15 h. For analysis of P15E and P12E proteins, cells were incubated in medium lacking methionine, and 250 μCi of [^{35}S]methionine was added for 24 h. Labeled virion particles in the medium were harvested, passed through Swinnex acrodisk filters (pore size, 0.22 μm; Gelman Sciences, Inc.), and isolated on sucrose gradients (41). Virions were suspended in sample buffer (60 mM Tris-chloride [pH 6.8], 6 mM EDTA, 120 mM beta-mercaptoethanol, 1% SDS, 10% glycerol, 0.005% bromophenol blue), and the proteins were analyzed by electrophoresis on 12.5 or 16% polyacrylamide gels, followed by fluorography. In some cases, after disruption of the virions the proteins were immunoprecipitated with goat anti-Rauscher P30 (NCI #77S-102), anti-pol (NCI #77S-424), or anti-gP70 (NCI #79S-771) serum.

Analysis of viral DNAs. DNA synthesized in infected cells was prepared (16) and analyzed by blot hybridization (44) with a labeled (36) DNA probe as described before (4, 10). Endogenous reverse transcription reactions were used to detect the synthesis of minus-strand strong-stop DNA (13).

RESULTS

Construction of deletion mutations in the 5′ part of the *pol* gene. To generate deletions localized to the 5′ part of the *pol* gene, we scanned the nucleotide sequence of M-MuLV (42) to identify a restriction enzyme that would cleave in this region and make a minimum of cleavages elsewhere. The enzyme *Bst*EII recognizes a site in this region and only three other sites elsewhere in the viral genome. A small plasmid containing a proviral copy of the genome, pT11 (24), contained two additional *Bst*EII sites in the vector. The circular DNA of this plasmid was linearized by limited digestion with *Bst*EII, and approximately 10 to 200 nucleotides at the termini were removed by digestion with BAL 31 exonuclease. The linear DNA that was close to full size (13 to 14 kilobases [kb]) was purified by agarose gel electrophoresis, recircularized with T4 DNA ligase, and used to transform *E. coli* to ampicillin resistance. Plasmid DNAs isolated from the resulting colonies were screened for the presence of deletions by electrophoresis of the DNA fragments produced by complete digestion with *Bst*EII plus *Hin*dIII. A total of 77 plasmid DNAs of 174 screened contained a single deletion at one of the six *Bst*EII sites in the original DNA (Table 1). Nine of these were mapped to the *Bst*EII site in the 5′ portion of the *pol* gene. The approximate sizes of the deletions were estimated to range from 25 to 150 bp (Table 2).

Of the nine deletion mutations generated, only one-third would be expected to maintain the correct translational reading frame downstream from the mutation. The other two-thirds would be frameshift mutations and could not express most of the *pol* gene. To determine whether the mutations altered the reading frame and to establish the exact size and position of the deletions, the DNA sequence of five of the altered DNAs was determined by the method of Maxam and Gilbert (27). Four of the DNAs contained frameshift deletions and were not characterized further. Only one, mutant *dl*2905, preserved the translational reading frame. The 126-bp deletion was localized entirely within the 5′ region of the *pol* gene, beginning 183 bp downstream from the 3′ end of the *gag* gene and ending 51 bp upstream from

TABLE 1. Number of deletion mutations generated at various *Bst*EII sites of plasmid pT11

Mutations	Region affected[a]	Map position (kb) on viral genome	No. of plasmids
Single-site mutations in M-MuLV genome	P15^{gag}	1.2	5
	5′ part of *pol*	2.9	9
	Middle of *pol*	4.7	28
	5′ part of *env*	6.4	15
Other mutations	Vector		20
	Multiple regions	Multiple sites	46
	None	None	51

[a] The position of deletion was determined by loss of a specific *Bst*EII site as judged by electrophoretic analysis of complete digests with *Bst*EII plus *Hin*dIII. All single deletions ranged in size from 25 to 200 bp based on the electrophoretic mobility of fragments spanning the deleted *Bst*EII sites in plasmid pT11.

the portion of the *pol* gene encoding reverse transcriptase (Fig. 1).

Isolation of NIH/3T3 producer cell lines by cotransformation. To construct cell lines expressing the mutant *dl*2905 provirus, NIH/3T3 cells were cotransformed with a mixture of the mutant plasmid DNA and pSV2neo DNA, which encodes resistance to the antibiotic G418, in a 5:1 ratio. Cells taking up DNA were selected in medium containing G418, and the resulting colonies were pooled and grown into large-scale cultures. In similar experiments we have found that one-third to one-half of the cells stably incorporate and express the viral DNA (4, 38, 40).

The recipient cells were tested for viral gene expression by a rapid assay for release of reverse transcriptase activity into the growth medium (11). Examination of the medium from the recipient cells revealed high levels of the enzyme, indicating that the mutant genome was expressed and could direct the formation of at least some reverse transcriptase activity. Single-cell clones were isolated from these populations of G418-resistant cells, and the individual cloned cell lines were assayed for the production of reverse transcriptase activity as before. Of 76 cloned cell lines tested, 25 were found to release detectable levels of the enzyme (data not shown). Three of these lines were chosen for further analysis. A crude quantitation of the level of enzyme released by these cells indicated that they produced between one-third and one-half of the activity produced by a cloned cell line containing wild-type virus (Fig. 2). Thus, the mutation in the 5′ part of the *pol* gene did not prevent the formation and release of reverse transcriptase that was active on a synthetic template.

Analysis of viral precursor proteins synthesized in mutant *dl*2905. The viral gene products are first made in the form of large precursor proteins. To test for the ability of the mutant genome to direct the synthesis of these proteins, the transformed producer cell lines were starved for methionine and then pulse-labeled for 45 min with [^{35}S]methionine. Solubilized cell extracts were prepared, and the viral proteins were then immunoprecipitated with either goat anti-M-MuLV serum or rabbit anti-*pol* serum. The proteins were collected on fixed *S. aureus* and displayed by SDS-polyacrylamide gel electrophoresis, followed by fluorography (Fig. 3). Wild-type levels of Pr65^{gag}, Pr200$^{gag\text{-}pol}$, and gPr80^{env} polyproteins were synthesized in the cell lines containing the mutant genome. Thus, the mutation did not affect the expression of any of the major viral precursor proteins.

Analysis of Pr200$^{gag\text{-}pol}$ on 8% polyacrylamide gels showed that the mutant protein migrated with a slightly higher mobility than did the wild-type protein. The change was consistent with a loss of about 5 kilodaltons from the precursor, in good agreement with the change predicted for the loss of 42 amino acids caused by the deletion. In addition, low levels of a processed *gag-pol* protein of approximately 160,000 daltons was immunoprecipitated with the anti-*pol* serum from cells carrying a wild-type M-MuLV genome; this protein could not be detected in cells carrying the mutant *dl*2905 provirus.

Processing of the *gag* and *pol* precursors is blocked in *dl*2905. To examine the processing of the *gag* precursor protein to the mature structural virion proteins, cells were labeled continuously over 16 h with [^{3}H]leucine. Virions were then harvested from the supernatant medium and purified by centrifugation on sucrose gradients, and the labeled virion proteins were displayed by SDS-polyacrylamide gel electrophoresis, followed by fluorography (Fig. 4). Under these conditions the precursor proteins of wild-type virus could no longer be detected; the Pr65^{gag} precursor was completely processed to the four cleavage products P15, P12, P30, and P10. In contrast, mutant *dl*2905 virions contained a major protein of 65,000 daltons and no detectable cleavage products. Serology was used to show that the large protein was indeed the unprocessed Pr65^{gag}; the virion proteins were immunoprecipitated with rabbit anti-P30^{gag} serum and analyzed as before. This serum immunoprecipitated the mature P30 protein from wild-type virus and only bound the larger Pr65^{gag} from the mutant. No processed proteins reactive with this serum could be detected in the mutant, demonstrating that *gag* cleavage was completely blocked. It should be noted that the *gag* precursor protein of *dl*2905 is normal in structure because the *gag* gene has not been altered by the mutagenesis. These results indicate that virion assembly, budding, and release from the cell can all occur in the absence of *gag* cleavage.

Processing of the Pr200$^{gag\text{-}pol}$ precursor was examined by analysis of proteins after immunoprecipitation with rabbit anti-*pol* sera. Figure 5 shows that in wild-type virions, the precursor was completely processed to the mature reverse transcriptase enzyme, P80, during the 16-h labeling period. In contrast, only the unprocessed Pr200$^{gag\text{-}pol}$ was immunoprecipitated by this serum from the *dl*2905 virions. We estimate that less than 5% of the precursor was cleaved to the mature enzyme. Since the level of reverse transcriptase activity present in these virions was close to that of wild-type virus, this result suggests that the uncleaved *gag-pol*

TABLE 2. Structure of M-MuLV mutants with deletions at map position 2.9 kb

Mutant	Estimated deletion size[a]	Bases deleted[b]
*dl*2901	50–100	28
*dl*2902	50–100	116
*dl*2903	50–100	109
*dl*2904	25–50	53
*dl*2905	50–100	126
*dl*2906	75–150	
*dl*2907	50–100	
*dl*2908	50–100	
*dl*2909	50–100	

[a] The size of the deletion was estimated from the observed change in electrophoretic mobility of a 3.5-kb fragment spanning the *Bst*EII site in wild-type M-MuLV.

[b] Determined by DNA sequence analysis.

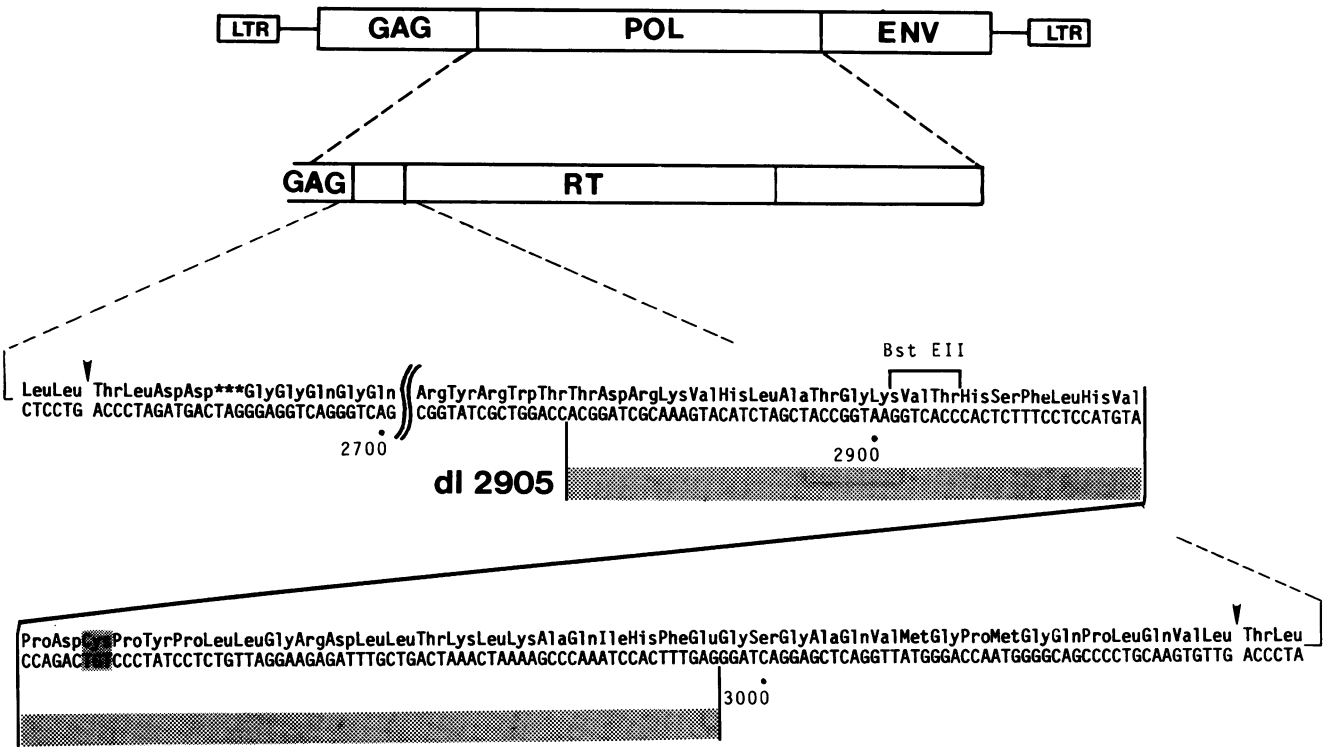

FIG. 1. Top: complete genome of M-MuLV as a linear provirus. The regions encoding the *gag*, *pol*, and *env* precursors are shown in boxes. Middle: enlargement of the *pol* gene. The position of the region encoding reverse transcriptase (RT), and the two additional regions to the 5′ and 3′, are shown, Bottom: DNA and amino acid sequences flanking the *Bst*EII site in the 5′ part of the *pol* gene of wild-type M-MuLV. Nucleotides are numbered from the 5′ edge of the 5′ long terminal repeat. The recognition site for cleavage by the enzyme *Bst*EII in the 5′ portion of the *pol* gene is indicated. The boundary between the C-terminal domain of *gag* and the N-terminal domain of *pol* is marked by asterisks, and the cleavage sites at the C terminus of *gag* and at the N terminus of reverse transcriptase are marked with arrowheads. The bases deleted in mutant *dl*2905 are indicated by the shaded area. The 126-bp deletion is positioned 183 bp from the 3′ end of the *gag* gene and 51 bp 5′ to the region encoding reverse transcriptase. Since the number of bp deleted was equal to a multiple of three, the mutation preserves the downstream translational reading frame in *pol*. The single cysteine residue in the region, proposed to be required for proteolytic activity (Oroszlan, personal communication), is shaded.

precursor is enzymatically active at least on synthetic substrates.

Processing of the *env* precursor of mutant *dl*2905. The *env* precursor protein of M-MuLV is normally cleaved soon after its synthesis to form the two products gP70^{env} and P15E (8, 17). The smaller protein is partially processed at very late times to generate P12E by removal of a carboxyterminal peptide (20). To examine the processing of gPr80^{env} in the mutant, virions were labeled with [^{35}S]methionine over a 24-h period and purified as before. Immunoprecipitation of the virion proteins with anti-gp70 serum, followed by electrophoresis, indicated that the mutant virions did contain the processed glycoprotein (Fig. 6A and B), migrating very close to the position of the unprocessed Pr65^{gag}. It should be noted that the anti-gP70 serum also precipitated the Pr65^{gag} in the mutant virions, while showing no cross-reactivity to any of the mature *gag* proteins in wild-type M-MuLV virions. This observation suggests that unprocessed Pr65^{gag} may be tightly associated with envelope proteins in the mutant virions and that *gag* cleavage reduces this association in wild-type virions.

The other product of *env* cleavage, P15E, was readily detected in total virion proteins (Fig. 6C). In wild-type M-MuLV, three low-molecular-weight proteins were labeled: the envelope products P15E and P12E and the *gag* product P12 (neither P15^{gag} nor P10^{gag} contains methionine). In contrast, in mutant *dl*2905 virions, only one low-molecular-weight protein migrating at the position of P15E was detected (Fig. 6C). No P12E was detected. This result suggests that cleavage of the *env* precursor to P15E occurred normally but that under conditions in which p15E is partially processed (at least 50%) to P12E in wild-type virions no processing of P15E to P12E occurs in the mutant.

Reverse transcription of the viral RNA by *dl*2905 virions. The results of the analysis of the virion proteins indicated that cleavage of *gag* and *pol* precursors was blocked by the deletion but that virion assembly, budding, and release from the cell surface could still occur. The analysis of reverse transcriptase activity further showed high levels of enzymatic activity. To determine whether viral RNA and primer tRNA were present and could be used as templates, endogenous reverse transcription reactions were carried out. Multiple harvests were collected from cells producing wild-type and mutant viruses, and the virions were purified and concentrated. After detergent permeabilization, the virus particles were incubated with labeled nucleotides, and the DNA synthesized was examined by electrophoresis on a 10% polyacrylamide gel and autoradiography (Fig. 7). Minus-strand strong-stop DNA, the first major intermediate of reverse transcription, was synthesized by the mutant virions. This experiment demonstrated that the RNA genome and primer were packaged into viral cores during formation of the mutant particles. The results suggest, since no mature reverse transcriptase could be detected in the mutant parti-

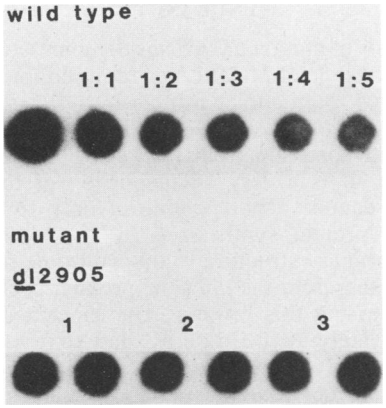

FIG. 2. Reverse transcriptase activity released into the medium by cell lines carrying mutant *dl*2905 or wild-type proviral DNA. Supernatant fluid was collected from producer cell lines and assayed by the rapid dot method (11). Upper row: the indicated dilutions of medium from wild-type producer cells were assayed. Bottom row: harvests from three independent mutant producer cell lines were assayed in duplicate. The level of activity released by each of the lines was approximately one-half to one-third that of the wild type.

cles, that the uncleaved *gag-pol* precursor was capable of initiating DNA synthesis correctly from the $tRNA^{Pro}$ primer and could at least direct the synthesis of the first DNA product. It is unlikely that small amounts of processed $P80^{pol}$ were responsible for these results since less than 5% of the precursor was processed and the level of reverse transcriptase activity observed was at most two- to three-fold lower than that of wild-type virus. We cannot, however, completely rule out the possibility that undetected processed enzyme carried out this synthesis, because only a small number of enzyme molecules (perhaps 1 in 100 per virion) might be sufficient.

Infectivity of mutant virus: DNA synthesis in infected NIH/3T3 cells. To determine whether mutant *dl*2905 might be replication competent, the proviral cloned DNA was applied to NIH/3T3 cells in a calcium phosphate precipitate (12). Under these conditions, wild-type virus can initiate an infection and spread throughout the culture, whereas any defective genome cannot (38, 40). Viral spread can be detected by overlaying the recipient cells with the XC indicator line (37). Analysis of *dl*2905 DNA in this way was reproducibly negative; there was no detectable virus.

To measure the infectivity of the mutant virions directly, virus was harvested from producer cell lines containing either wild-type or mutant proviral DNA, and an equal particle number (as judged both by levels of viral proteins and of reverse transcriptase activity) were used to infect fresh NIH/3T3 cells. Successful infection was detected by measuring the kinetics of release of reverse transcriptase activity into the medium at various times postinfection and by XC overlay. Wild-type virus induced release of enzyme activity within 2 days postinfection (Fig. 8), and XC overlay 8 days postinfection indicated high titers. In contrast, the mutant virions induced only a trace of reverse transcriptase activity even after 5 days (Fig. 8) and showed no plaques after XC overlay.

One of the earliest events that can be easily measured after infection is the process of reverse transcription of the RNA genome into full-length DNA. To determine whether viral DNA synthesis could be carried out by the mutant, cells were acutely infected with equal numbers of mutant or wild-type particles, and the low-molecular-weight DNA was isolated (15) 30 h postinfection. The DNA was purified, fractionated by agarose gel electrophoresis, blotted to nitrocellulose, and hybridized with a radioactive viral DNA

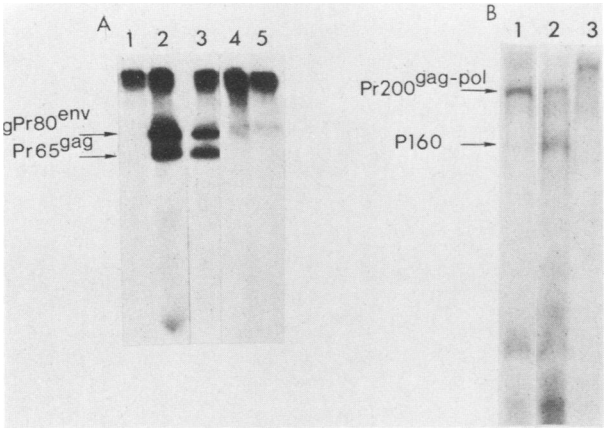

FIG. 3. Intracellular proteins synthesized by producer cell lines. Viral proteins were pulse labeled with [^{35}S]methionine, immunoprecipitated from lysates, and analyzed by SDS-polyacrylamide gel electrophoresis, followed by fluorography. (A) Detection of *gag* and *env* precursors. Proteins were immunoprecipitated with goat anti-Moloney serum (lanes 1 to 3) or with normal goat serum (lanes 4 and 5). Lanes: 1, control cells transformed with pSV2neo DNA alone; 2, cells carrying wild-type M-MuLV DNA (pT11); 3, mutant *dl*2905 DNA; 4, mutant *dl*2905 DNA; 5, wild-type M-MuLV DNA. The positions of the *env* and *gag* precursors are indicated. (B) Detection of *gag-pol* precursors. Proteins were immunoprecipitated with anti-*pol* serum (lanes 1 to 3). Lanes: 1, mutant *dl*2905 DNA; 2, wild-type M-MuLV DNA; 3, pSV2neo DNA alone. The positions of the *gag-pol* precursor and a prominent cleavage product are indicated.

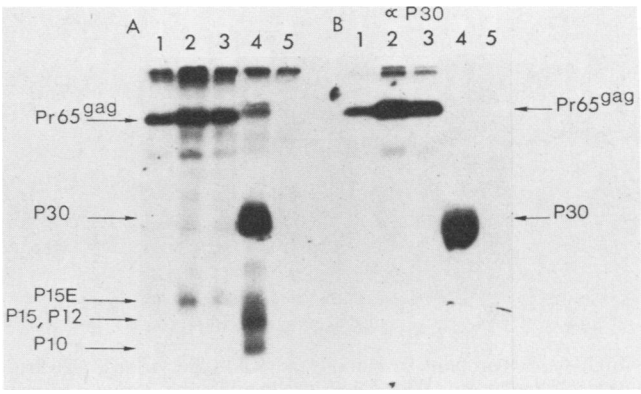

FIG. 4. *gag* proteins in virion particles purified from supernatants of cell lines carrying either wild-type or mutant DNAs. (A) Producer cell lines were labeled with [^{3}H]leucine for 16 h, virions were purified, and total virion proteins were displayed without immunoprecipitation. Lanes: 1 to 3, virions from three independent cell lines carrying mutant *dl*2905 DNA; 4, virions from a wild-type producer cell line; 5, harvests from control cells carrying pSV2neo DNA alone. (B) Virions prepared as in A were disrupted, and virion proteins were immunoprecipitated with anti-$P30^{gag}$ serum before electrophoresis. Lanes: 1 to 3, virions of mutant *dl*2905; 4, virions of wild-type M-MuLV; 5, harvests of control cells carrying only pSV2neo DNA. None of the *gag* cleavage products (P15, P12, P30, or P10) were present in mutant *dl*2905 virions. Instead, high levels of $Pr65^{gag}$ were present and were immunoprecipitated by the serum.

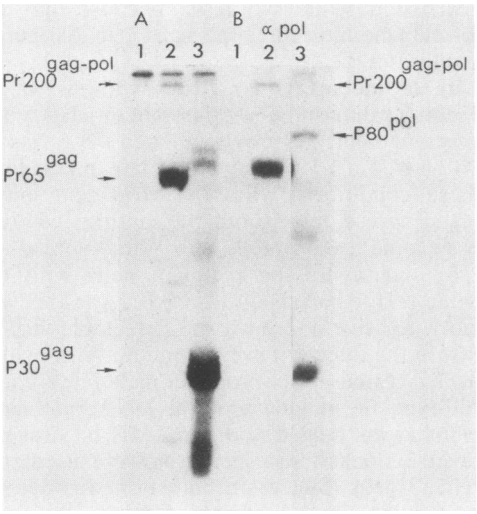

FIG. 5. *pol* proteins in virion particles isolated from supernatants of cell lines carrying wild-type or mutant DNAs. (A) Total virion proteins labeled with [^{3}H]leucine. Lanes: 1, harvests from control cells carrying pSV2neo alone; 2, mutant *dl*2905 virions; 3, wild-type virions. (B) Proteins were immunoprecipitated with anti-*pol* serum before electrophoresis. Lanes: 1, harvests from control cells; 2, mutant *dl*2905 virions; 3, wild-type virions. High levels of the Pr200$^{gag\text{-}pol}$ precursor were visible in the mutant virions and could be immunoprecipitated with the anti-*pol* serum. In the wild-type virions the precursor was processed to P80^{pol}. The serum also showed reactivity to P30^{gag} and immunoprecipitated both Pr65^{gag} and P30.

probe, and the viral species was detected by autoradiography (Fig. 9). The amount of viral DNA was reduced to undetectable level in the mutant. This result suggests that the course of infection was blocked at an early stage, before synthesis of full-length DNA could occur.

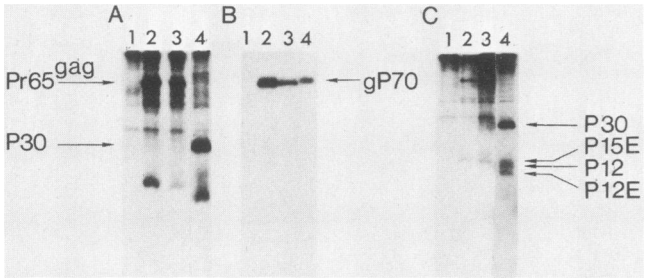

FIG. 6. *env* proteins in mutant and wild-type virions. (A) Producer cell lines were labeled with [^{35}S]methionine, virion particles were purified, and the total virion proteins were displayed by SDS-gel electrophoresis on a 12.5% polyacrylamide gel. Lanes: 1, supernatants from control cells; 2 and 3, virions from two independent cell lines carrying *dl*2905 DNA; 4, wild-type virions. (B) Samples shown in A were treated with detergents, and the *env* gene products were immunoprecipitated with anti-*env* serum before electrophoresis. Mutant and wild-type virions both show gP70^{env}, although the mutant protein nearly comigrates with the unprocessed Pr65^{gag}. (C) Producer cells were labeled for 24 h with [^{35}S]methionine, and the total virion proteins were analyzed on a 16% gel. Of the small virion proteins, only P15E, P12E, and P12 contain methionine and are detected in this experiment. The wild-type virions show all three proteins, whereas the mutant virions contain P15E but no P12E. The mutant virions do not show P12 because no *gag* cleavage occurs.

DISCUSSION

Our analysis of mutant *dl*2905 has demonstrated that the 5′ part of the *pol* gene of M-MuLV is required for the processing of the Pr65^{gag} precursor protein to the mature cleavage products. The region lies immediately downstream from the *gag* gene and upstream from the region encoding reverse transcriptase. It is a very small portion of the *pol* gene, sufficient to encode a polypeptide of only 16,000 daltons. The Pr65^{gag} protein synthesized by the mutant virus is identical in primary structure to the wild-type protein but is not cleaved; therefore the *pol* gene product is clearly essential for processing this protein. The block caused by the mutation is likely to be directly due to the loss of the proteolytic activity needed to make the cleavages, although we cannot rule out that the mutation leads to an altered virion structure indirectly rendering the *gag* protein resistant to cleavage. We feel that this latter possibility is remote since biochemical analyses identifying a protease of this size from virions of M-MuLV and Rauscher MuLV (28, 57–59, 61) are consistent with the genetics reported here and further suggest that the region encodes a protease directly responsible for the cleavages. These studies have demonstrated that the proteolytic activity could make all three cleavages in vitro and suggest that this single protease is responsible for the processing of Pr65^{gag}.

The protease of avian retroviruses is encoded by the 3′ proximal domain of the *gag* gene (5, 52, 53). The positioning

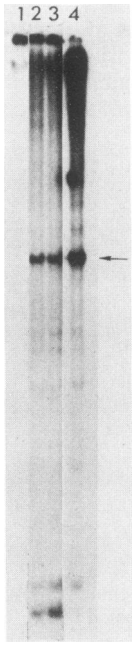

FIG. 7. Synthesis of minus-strand strong-stop DNA in mutant and wild-type virions. Virion particles were purified from supernatants of producer cell lines and incubated with labeled nucleotides to allow reverse transcription, and the reaction products were displayed by electrophoresis on 10% polyacrylamide gels, followed by autoradiography. Lanes: 1, harvests from cell lines transformed with pSV2neo DNA alone; 2 and 3, virions harvested from two independent cell lines expressing *dl*2905 DNA; 4, virions of wild-type M-MuLV. The length of the autoradiographic exposure for lanes 2 and 3 was twice that of lane 4. The mutant virions synthesized approximately as much strong-stop DNA per virion particle as did the wild type.

of the gene for the M-MuLV protease at the beginning of the *pol* gene thus indicates a significant difference between the avian and mammalian retroviruses. It should be noted, however, that the gene order in these systems is similar: in both cases the protease gene lies between the region encoding the major *gag* proteins and that encoding reverse transcriptase. The difference is in the position of the termination codon delineating the boundary between the *gag* and *pol* genes. Thus, the simplest change that could have occurred during the evolution of these retroviral families is a movement in the point of termination of the *gag* gene.

The complete block to proteolytic cleavage of the *gag* precursor was found to have remarkably little effect on the assembly and release of virions. Earlier studies of a temperature-sensitive mutant of M-MuLV termed *ts*3 (56) and of similar mutants of Rauscher MuLV termed *ts*24, *ts*25, and *ts*26 (46) have correlated *gag* cleavage with the process of virion assembly and release: these mutants were not able to process $Pr65^{gag}$ or to shed virions at the nonpermissive temperature (47–50, 54, 55). Election microscopic studies of one of these mutants (*ts*3) demonstrated that cleavage of $Pr65^{gag}$ to the structural virion proteins was correlated with a change in the particle morphology to the mature form (25). Our studies prove that proteolysis is not required for such steps as budding and release of virions, since wild-type levels of virions containing unprocessed $Pr65^{gag}$ were released by cells containing the *dl*2905 genome. Instead, we propose that a block to virion assembly is the primary lesion in the temperature-sensitive mutants and that this step is a prerequisite for proteolytic processing of *gag* products. Thus, the critical mutation in the temperature-sensitive mutants could be in the *gag* gene itself rather than in the protease gene. This suggestion is supported by previous results from our laboratory which showed that lesions in the P30 domain of *gag* blocked both virion assembly and cleavage of the altered *gag* protein (40). It also is supported by studies of Gazdar murine sarcoma virus (9), showing that virus particles could be assembled and released even though the $Pr65^{gag}$ of this virus was not cleaved (34).

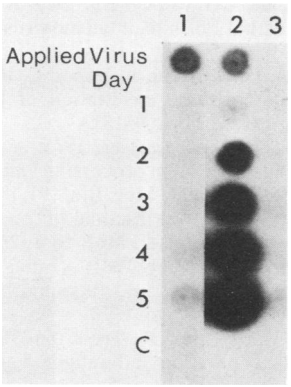

FIG. 8. Infectivity of mutant virions as assayed by release of reverse transcriptase activity from infected cultures. Virus harvests were collected from producer cell lines carrying mutant *dl*2905 DNA, wild-type M-MuLV DNA, or pSV2neo DNA alone. Top row: levels of reverse transcriptase in these harvests were determined by the rapid reverse transcriptase assay. Columns: 1, mutant *dl*2905 virus; 2, wild-type virus; 3, harvests from control cells. The levels of reverse transcriptase activity present in mutant and wild-type harvests were similar. These virus preparations were then used to infect fresh NIH/3T3 cells, and the supernatant medium above these cultures was harvested on successive days. The levels of reverse transcriptase in these harvests are shown in successive rows. The wild-type virus was highly infectious and induced the release of high levels of virus within 2 days. The mutant virus was much less infectious. Row C: control cocktail alone.

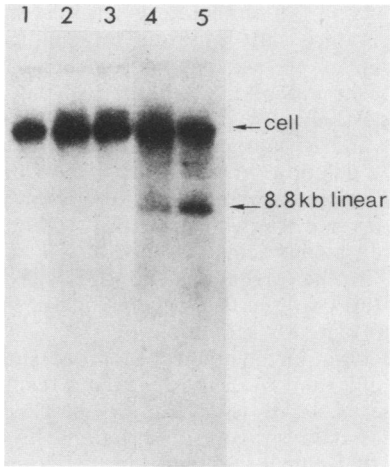

FIG. 9. Viral DNA synthesized after infection of NIH/3T3 cells with mutant or wild-type virions. Low-molecular-weight DNA was isolated from infected cells and fractionated by agarose gel electrophoresis. The DNA was transferred to nitrocellulose, and the viral DNA was detected by hybridization with a labeled DNA probe, followed by autoradiography. Lanes: 1, uninfected NIH/3T3 cells; 2 and 3, cells infected with mutant *dl*2905 virus; 4, cells infected with a 1:5 dilution of wild-type virus; 5, cells infected with a 1:2 dilution of wild-type virus. The positions of contaminating high-molecular-weight cell DNA and of 8.8-kb linear proviral DNA are indicated. No viral DNA could be detected in cells infected with the mutant virus.

A surprising result was the finding that the *dl*2905 deletion also blocked cleavage of the *gag-pol* precursor. High levels of unprocessed $Pr200^{gag\text{-}pol}$ were present in the mutant virions, demonstrating that all the cleavages normally occurring were blocked. In this case the mutation affects both the protease and the structure of the substrate for the protease; thus we cannot determine whether the primary cause of the block is the absence of the protease or an altered conformation of the precursor. In addition, it is possible that the inability to cleave $Pr65^{gag}$ to the mature proteins might indirectly interfere with the processing of the *gag-pol* precursor. We feel, however, that the simplest explanation is that the protease is directly responsible for the processing of $Pr200^{gag\text{-}pol}$ as well as $Pr65^{gag}$. Results in the avian system (30) have shown that the purified protease can make at least some of the cleavages in *pol* gene products.

It also was surprising that the mutant virions contained nearly normal levels of reverse transcriptase activity. Our results showed that the mutant precursor was active in vitro with either synthetic polynucleotides or the endogenous viral genome as substrates. The uncleaved enzyme was apparently capable of correct initiation from the $tRNA^{Pro}$ primer and could direct the synthesis of the first major intermediate of reverse transcription, minus-strand strong-stop DNA. The observed activity was close to wild-type levels and probably too high to be accounted for by any undetected trace of processed $P80^{pol}$. Previous studies of temperature-sensitive mutants of M-MuLV and Rauscher MuLV led to the suggestion that processing of $Pr200^{gag\text{-}pol}$ was required for activation of the enzymatic activity (48, 54). Our results show that removal of the *gag* portion and the C-terminal portion of the *gag-pol* precursor is not required for activation of the enzyme. It is possible that the appear-

ance of activity in the temperature-sensitive mutants soon after a temperature shift was due to renaturation of the mutant enzyme or its subsequent packaging into virions, rather than to its processing. Alternatively, it may be that the wild-type $Pr200^{gag\text{-}pol}$ is inactive and requires processing but that the *dl*2905 mutant precursor is active without cleavage. The deletion present in the *dl*2905 precursor lies very close (17 amino acids) to the site of cleavage at the N terminus of reverse transcriptase; thus, the deletion effectively removed amino acids attached to the N terminus of the enzyme in the precursor and thus may mimic the cleavage. In this case the wild-type precursor might require cleavage at this site for activation.

The *dl*2905 mutation did not prevent processing of $gPr80^{env}$ precursor to gP70 and P15E; however, our results suggested that cleavage of P15E to P12E was blocked in mutant virions. These data support observations by Durbin and Manning (6) that clonal variability in the extent of $Pr65^{gag}$ cleavage correlated with the extent of cleavage of P15E to P12E. The inability to cleave P15E may result directly from inactivation of the responsible protease or indirectly by the inability to process $Pr65^{gag}$ in the mutant virions. Taken together, our results indicate that the gene product encoded by the 5′ part of *pol* is required for all posttranslational processing events which generate the mature viral proteins, except for the cleavage of the envelope precursor $gPr80^{env}$ to gP70 and P15E. A comparison of amino acid sequences at the cleavage sites separating the domains of the *gag* and *pol* products as well as at the site separating P15E from P12E shows that hydrophobic amino acids predominate (32; S. Oroszlan, personal communication). In contrast, the amino acids at the site of cleavage separating gP70 from P15E, whose cleavage is not affected by the *dl*2905 mutation, are highly polar (arginine, histidine, lysine, arginine, and glutamic acid). The hydrophobic nature of the amino acids composing the sites whose cleavages are blocked by the mutation may be a common feature required for recognition by the *pol* protease.

Although the mutant virions were released from the cell, contained viral genomic RNA, and could synthesize DNA in vitro, the virus was very poorly infectious when applied to NIH/3T3 cells. The infection was blocked at an early stage, before reverse transcription of the genome could occur. It may be that the cleavage of P15E to P12E is required for infectivity. We propose, however, that the block results from the failure to cleave the *gag* precursor and that virions made up of unprocessed *gag* precursors cannot correctly carry out such steps as entry into the cell, localization to the correct region in the cell, or uncoating to allow entry of the nucleotides needed for reverse transcription. We have previously described mutations in the $P15^{gag}$ and $P12^{gag}$ domains of M-MuLV which prevented cleavage between these domains (4). These mutations also blocked an early stage of infection, preceding reverse transcription of the viral RNA; these results thus are consistent with the notion that *gag* cleavage is essential for these early events. Cleavage of $Pr65^{gag}$ and $Pr200^{gag\text{-}pol}$ may generate distinct and independent domains whose functions are required for infection. Such cleavages are important for many viruses of diverse families and may be a common mechanism for activating the specialized functional proteins needed for infectivity.

ACKNOWLEDGMENTS

We thank Margaret Grimes for technical assistance.

This work was supported by Public Health Service grant CA 30488 awarded by the National Cancer Institute and by an award from the Chicago Community Trust/Searle Scholars Program. Salary support for S.P.G. was provided by the Irma T. Hirschl Trust.

LITERATURE CITED

1. **Arcement, L. J., W. I. Karshin, R. B. Naso, and R. B. Arlinghaus.** 1977. "*Gag*" polyprotein precursors of Rauscher murine leukemia virus. Virology **81**:284–297.
2. **Bishop, J. M.** 1978. Retroviruses. Annu. Rev. Biochem. **47**:35–88.
3. **Chumakov, I., H. Stuhlmann, K. Harbers, and R. Jaenisch.** 1982. Cloning of two genetically transmitted Moloney leukemia proviral genomes: correlation between biological activity of the cloned DNA and viral genome activation in the animal. J. Virol. **42**:1088–1098.
4. **Crawford, S., and S. P. Goff.** 1984. Mutations in *gag* proteins P12 and P15 of Moloney murine leukemia virus block early stages of infection. J. Virol. **49**:909–917.
5. **Dittmar, K. J., and K. Moelling.** 1978. Biochemical properties of p15-associated protease in an avian RNA tumor virus. J. Virol. **28**:106–118.
6. **Durbin, R. K., and J. S. Manning.** 1984. Coordination of cleavage of *gag* and *env* gene products of murine leukemia virus: implications regarding the mechanism of processing. Virology **134**:368–374.
7. **Eisenman, R. N., and V. M. Vogt.** 1978. The biosynthesis of oncovirus proteins. Biochim. Biophys. Acta **475**:187–259.
8. **Famulari, N. G., D. L. Buchhagen, H.-D. Klenk, and E. Fleissner.** 1976. Presence of murine leukemia virus envelope proteins gp70 and p15(E) in a common polyprotein of infected cells. J. Virol. **20**:501–508.
9. **Gazdar, A. F., L. A. Phillips, P. S. Sarma, P. T. Peeble, and H. C. Chopra.** 1971. Presence of sarcoma genome in a "non-infectious" mammalian virus. Nat. New Biol. **234**:69–72.
10. **Goff, S. P., E. Gilboa, O. N. Witte, and D. Baltimore.** 1980. Structure of the Abelson murine leukemia virus genome and the homologous cellular gene: studies with cloned viral DNA. Cell **22**:777–785.
11. **Goff, S. P., 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.
12. **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.
13. **Haseltine, W. A., D. G. Klein, A. Panet, E. Rothenberg, and D. Baltimore.** 1976. Ordered transcription of RNA tumor virus genomes. J. Mol. Biol. **106**:109–131.
14. **Hendrix, R. W., and S. R. Casjens.** 1975. Assembly of bacteriophage lambda heads: protein processing and its genetic control in petit lambda assembly. J. Mol. Biol. **91**:187–199.
15. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–371.
16. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114**:193–197.
17. **Ikeda, H., W. Hardy, Jr., E. Tress, and E. Fleissner.** 1975. Chromatographic separation and antigenic analysis of proteins of the oncornaviruses. V. Identification of a new murine viral protein, p15(E). J. Virol. **16**:53–61.
18. **Jacobson, M. F., and D. Baltimore.** 1968. Polypeptide cleavages in the formation of poliovirus proteins. Proc. Natl. Acad. Sci. U.S.A. **61**:77–84.
19. **Jamjoom, G. A., R. B. Naso, and R. B. Arlinghaus.** 1977. Further characterization of intracellular precursor polyproteins of Rauscher leukemia virus. Virology **78**:11–34.
20. **Karshin, W. L., L. J. Arcement, R. B. Naso, and R. B. Arlinghaus.** 1977. Common precursor for Rauscher leukemia virus gp69/71, p15(E) and p12(E). J. Virol. **23**:787–798.
21. **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**:477–485.

22. **Korant, B. D.** 1975. Proteases and biological control, p. 621–644. *In* E. Reich, D. B. Rifkin, and E. Shaw (ed.), Proteases and biological control: Cold Spring Harbor Conferences on Cell Proliferation, vol. 2. Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y.
23. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
24. **Lobel, L. I., and S. P. Goff.** 1984. Construction of mutants of Moloney murine leukemia virus by suppressor-linker insertional mutagenesis: positions of viable insertion mutations. Proc. Natl. Acad. Sci. U.S.A. **81**:4149–4153.
25. **Lu, A. H., M. M. Soong, and P. K. Y. Wong.** 1979. Maturation of Moloney murine leukemia virus. Virology **93**:269–274.
26. **Mandel, M., and A. Higa.** 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. **53**:159–162.
27. **Maxam, A., and W. Gilbert.** 1979. Sequencing end-labelled DNA with base specific chemical cleavages. Methods Enzymol. **65**:499–599.
28. **Maxwell, S., and R. B. Arlinghaus.** 1981. In vitro proteolytic cleavage of Gazdar murine sarcoma virus $p65^{gag}$. J. Virol. **39**:963–967.
29. **Miller, A. D., and I. M. Verma.** 1984. Two base changes restore infectivity to a noninfectious molecular clone of Moloney murine leukemia virus (pMLV-1). J. Virol. **49**:214–222.
30. **Moelling, K., A. Scott, K. E. J. Dittmar, and M. Owada.** 1980. Effect of p15-associated protease from an avian RNA tumor virus on avian virus-specific polyprotein precursors. J. Virol. **33**:680–688.
31. **Murphy, E. C., Jr., J. J. Kopchick, K. F. Watson, and R. B. Arlinghaus.** 1978. Cell-free synthesis of a precursor polyprotein containing both gag and pol gene products by Rauscher murine leukemia virus 35S RNA. Cell **13**:359–369.
32. **Oroszlan, S., L. E. Henderson, J. R. Stephenson, T. D. Copeland, C. W. Long, J. N. Ihle, and R. V. Gilden.** 1978. Amino- and carboxy-terminal amino acid sequences of proteins coded by *gag* gene of murine leukemia virus. Proc. Natl. Acad. Sci. U.S.A. **75**:1404–1408.
33. **Philipson, L., P. Anderson, U. Olshevsky, R. Weinberg, D. Baltimore, and R. Gesteland.** 1978. Translation of MuLV and MSV RNAs in nuclease-treated reticulocyte extracts: enhancement of the *gag-pol* polypeptide with yeast suppressor tRNA. Cell **13**:189–199.
34. **Pinter, A., and E. De Harven.** 1979. Protein composition of a defective murine sarcoma virus particle possessing the enveloped type-A morphology. Virology **99**:103–110.
35. **Reynolds, R. K., and J. R. Stephenson.** 1977. Intracistronic mapping of the murine type C viral *gag* gene by use of conditional lethal replication mutants. Virology **42**:1136–1139.
36. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labelling of DNA to high specific activity by nick translation. J. Mol. Biol. **113**:237–258.
37. **Rowe, W. P., W. E. Pugh, and J. Hartley.** 1970. Plaque assay techniques for murine leukemia viruses. Virology **42**:1136–1139.
38. **Schwartzberg, P., J. Colicelli, and S. P. Goff.** 1983. Deletion mutants of Moloney murine leukemia virus which lack glycosylated *gag* protein are replication competent. J. Virol. **46**:538–546.
39. **Schwartzberg, P., J. Colicelli, and S. P. Goff.** 1984. Construction and analysis of deletion mutations in the *pol* gene of Moloney murine leukemia virus: a new viral function required for productive infection. Cell **37**:1043–1052.
40. **Schwartzberg, P., J. Colicelli, M. L. Gordon, and S. P. Goff.** 1983. Mutations in the *gag* gene of Moloney murine leukemia virus: effects on production of virions and reverse transcriptase. J. Virol. **49**:918–924.
41. **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.
42. **Shinnick, T. M., R. A. Lerner, and J. G. Sutcliffe.** 1981. Nucleotide sequence of Moloney murine leukemia virus. Nature (London) **293**:543–548.
43. **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.
44. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
45. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
46. **Stephenson, J. R., and S. A. Aaronson.** 1973. Characterization of temperature-sensitive mutants of murine leukemia virus. Virology **54**:53–59.
47. **Stephenson, J. R., S. Tronick, and S. Aaronson.** 1975. MLV mutants with temperature-sensitive defects in precursor polypeptide cleavage. Cell **6**:543–548.
48. **Traktman, P., and D. Baltimore.** 1982. Protease bypass of temperature-sensitive murine leukemia virus maturation mutants. J. Virol. **44**:1039–1046.
49. **van de Ven, W. J. M., D. van Zaane, C. Onnekink, and H. P. J. Bloemers.** 1978. Impaired processing of precursor polypeptides of temperature-sensitive mutants of Rauscher murine leukemia virus. J. Virol. **25**:553–561.
50. **van Zaane, D., M. J. Dekker-Michielsen, and H. P. J. Bloemers.** 1976. Virus-specific precursor polypeptides in cells infected with Rauscher leukemia virus: synthesis, identification, and processing. Virology **75**:113–129.
51. **Vogelstein, B., and D. Gillespie.** 1979. Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. U.S.A. **76**:615–619.
52. **Vogt, V. M., A. Wight, and R. Eisenman.** 1979. In vitro cleavage of avian retrovirus gag proteins by viral protease P15. Virology **98**:154–167.
53. **von der Helm, K.** 1977. Cleavage of Rous sarcoma viral polypeptide precursor into internal structural proteins in vitro involves viral protein P15. Proc. Natl. Acad. Sci. U.S.A. **74**:911–915.
54. **Witte, O. N., and D. Baltimore.** 1978. Relationship of retrovirus polyprotein cleavage to virion maturation studied with temperature-sensitive murine leukemia virus mutants. J. Virol. **26**:750–761.
55. **Wong, P. K. Y., and J. A. McCarter.** 1974. Studies of two temperature-sensitive mutants of Moloney murine leukemia virus. Virology **58**:396–408.
56. **Wong, P. K. Y., L. J. Russ, and J. A. McCarter.** 1973. Rapid selective procedure for isolation of temperature-sensitive mutants of Moloney murine leukemia virus. Virology **51**:424–431.
57. **Yoshinaka, T., and R. B. Luftig.** 1977. Properties of a proteolytic factor of murine leukemia virus. Cell **12**:709–719.
58. **Yoshinaka, T., and R. B. Luftig.** 1977. Partial purification of a P70 proteolytic factor that is present in purified virions of Rauscher leukemia virus (RLV). Biochem. Biophys. Res. Commun. **76**:54–63.
59. **Yoshinaka, Y., and R. B. Luftig.** 1980. Physicochemical characterization and specificity of the murine leukemia virus gag proteolytic factor. J. Gen. Virol. **48**:329–340.
60. **Yoshinaka, Y., and R. B. Luftig.** 1981. A comparison of avian and murine retrovirus polyprotein cleavage activities. Virology **111**:239–250.
61. **Yoshinaka, Y., and R. B. Luftig.** 1982. P65 of Gazdar murine sarcoma viruses contains antigenic determinants from all four of the murine leukemia virus (MuLV) gag polypeptides (P15, P12, P30 and P10) and can be cleaved in vitro by the MuLV proteolytic activity. Virology **118**:380–388.