Analysis of Retroviral *pol* Gene Products with Antisera Raised against Fusion Proteins Produced in *Escherichia coli*

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Portions of the *pol* gene of Moloney murine leukemia virus (MuLV) were expressed as fusion proteins in *Escherichia coli*, and the purified proteins were used to elicit antibodies in rabbits. The sera were used to examine the mature *pol* gene products contained in virion particles and identified the reverse transcriptase and a second protein, P46^{pol}, encoded by the 3' portion of the gene. The P46 protein was not phosphorylated and was present at the same molar abundance as the reverse transcriptase. The sera were also used to detect the Pr200^{gag-pol} intracellular precursor protein and to analyze its processing to the mature forms. The proteins formed by several Moloney MuLV mutants were analyzed. Further tests revealed cross-reactivity with Friend MuLV and feline leukemia virus proteins, but not with avian retrovirus proteins.

The *pol* gene of mammalian retroviruses encodes a large precursor protein (1, 24, 29, 30, 52) that is divided into three functional domains: a protease required for multiple cleavages of virion proteins (6, 59), the reverse transcriptase (RT; 5, 14, 53, 54), and a proviral integration function (8, 48). Genetic analyses have shown that mutations in each of these three domains result in distinctive phenotypes. Mutations in the protease domain block cleavage of both the *gag* and *gag-pol* precursors, permitting virus assembly but abolishing infectivity (6). Mutations in the RT domain block DNA synthesis in the early stages of infection (34, 48). Finally, mutations in the third domain permit synthesis of all the DNA forms but block the integration of the viral DNA into the cell genome (8, 48). Exploring the functions of each of these portions of the gene is important for understanding the replication of these viruses.

Direct biochemical analysis of the *pol* gene products isolated from murine retroviral particles has shown that these three functional domains correspond to three separate polypeptides that are all cleaved from a single large precursor protein (1, 5, 12, 29, 30, 52). The protease activity resides in a 14-kilodalton (kDa) protein encoded by the 5' portion of the *pol* gene (59, 60); the RT is a 77-kDa monomeric protein encoded by the center of the gene (5, 14, 53, 54); and the third enzyme is an endonuclease of about 40 kDa encoded by the 3' part of the gene (28, 40). The cleavage sites in the precursor protein that determine the termini of the mature enzymes have been localized (5). Sequence comparisons of various retroviral genomes have shown that the *pol* genes of other murine viruses, such as Rauscher murine leukemia virus (R-MuLV) and Friend MuLV (F-MuLV), and the feline leukemia viruses (FeLVs), are similarly organized.

The identification and characterization of the major virion proteins, the *gag* and *env* gene products, have been enormously facilitated by the preparation of polyclonal antisera (7, 12, 24, 58). In some cases such sera have been raised in animals by immunization with purified virion preparations, with or without disruption of the virions; such sera allow detection of all the major proteins of the virus and have been used to examine the synthesis and processing of these gene products (24, 58). In other cases sera specific for one or a few

of the viral proteins, such as P30^{gag} and gP70^{env}, have been prepared by immunization with individual proteins purified from the virion preparations (e.g., see reference 58). Sera specific for the *pol* gene products, however, are difficult to prepare because the antigens are present in small quantities in the virions and are thus more difficult to purify. Several sera reactive with various RTs, however, have been prepared (19, 24, 31, 39, 56, 57). Sera specific for the R-MuLV RT are available but show considerable reactivity with *gag* proteins (see references 24 and 58 and below) and are not helpful in detection of the other *pol* proteins. To characterize further the *pol* gene products encoded by the murine retroviruses and by mutants derived from them, high-titer antisera specific for these proteins would be useful reagents. We describe here the preparation of such sera by immunization with fusion proteins synthesized in bacteria. These sera allow the detection of both the RT and the C-terminal endonuclease. We also describe the characterization of these sera and their use in the analysis of the processing of *pol* proteins of wild-type Moloney MuLV (M-MuLV) and of mutants of the virus.

MATERIALS AND METHODS

Cells and viruses. NIH/3T3 cells were grown in Dulbecco modified Eagle medium (DMEM; GIBCO Laboratories) containing 10% newborn calf serum (M. A. Bioproducts). The clone 4 cell line (47) was the source of wild-type M-MuLV. NIH/3T3 cells infected with F-MuLV (13) were obtained from F. Lilly (Albert Einstein College of Medicine, Bronx, N.Y.). Feline embryo fibroblasts, both uninfected and infected with FeLV (25) subgroup A, were obtained from P. Besmer (Sloan-Kettering Institute for Cancer Research, New York, N.Y.) and maintained in DMEM supplemented with 15% fetal calf serum. Chicken embryo fibroblasts and chicken cell lines producing four different avian retroviruses were obtained from H. Hanafusa (The Rockefeller University, New York, N.Y.) and grown in DMEM supplemented with 5% newborn calf serum and 10% tryptone phosphate broth. The four avian viruses consisted of two nontransforming viruses, Rous-associated virus 1 (44) and Fujinami-associated virus, a subgroup C virus (23), and two transforming viruses, the Bryan high-titer strain of Rous

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sarcoma virus (RSV) (4) and the Schmidt-Ruppin strain of RSV (46).

Enzymatic manipulations of DNA. Plasmid pT11, carrying a full-length infectious proviral DNA copy of the M-MuLV genome (35), was the source of *pol* sequences in all constructions. The *trpE* fusion vector pATH3 was the generous gift of T. J. Koerner and A. Tzagaloff (Columbia University). Restriction enzymes (New England BioLabs, Inc.) were used in accordance with the recommendations of the supplier. T4 DNA ligase was the generous gift of J. Van Oostrum. All cloning manipulations were done by standard procedures (37). DNA fragments were prepared by electrophoresis in agarose gels and eluted by the glass powder method (55). *Escherichia coli* HB101 (3) was transformed to ampicillin resistance by standard procedures (36). Plasmids in small-scale cultures were analyzed by electrophoresis after rapid lysis (2). Large-scale plasmid preparations were made by equilibrium banding in CsCl-ethidium bromide (26).

Preparation of bacterial cell extracts. Bacterial cultures were grown, starved for tryptophan, and harvested as described previously (27, 50). Total protein extracts and extracts enriched for insoluble proteins were prepared as before (51). Proteins were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (32) and visualized by staining with Coomassie blue.

Preparation of rabbit antisera against bacterial fusion proteins. Antisera were prepared by standard protocols (e.g., see reference 42). Two groups of New Zealand White female rabbits were subjected to immunization regimens with two different antigens. One group of two animals was injected biweekly with either 5 or 50 μ g of a homogeneous preparation of the RT fusion protein encoded by plasmid pB6B15.23 (43), first in complete Freund adjuvant (GIBCO) and for subsequent boosts, in incomplete adjuvant. The second group, consisting of four animals, was injected with approximately 3 to 15 μ g of the fusion protein encoded by plasmid pSC1. To prepare the antigen, the insoluble fraction of a lysate of bacteria carrying pSC1 was isolated and subjected to SDS-polyacrylamide gel electrophoresis. The position of the 116-kDa protein was visualized after staining with potassium-SDS precipitates (22), and the protein was recovered from the gel slice. Approximately 2 μ g of the protein was obtained per ml of culture. The animals were bled 10 to 14 days after each immunizing injection.

Other antisera. A variety of antisera were obtained for analysis of retroviral gene products. The sera included goat anti-M-MuLV serum (NCI 77S-591), goat anti-R-MuLV P30^{gag} serum (NCI 77S-102), anti-*pol* sera (NCI 77S-424 and 74S-454), goat anti-Gross-MuLV P12^{gag} serum (NCI 75S-711), rabbit anti-*trpE* serum obtained from O. N. Witte (University of California, Los Angeles), anti-FeLV serum from P. Besmer and rat anti-RSV serum from H. Hanafusa. Other sera included normal goat serum (National Cancer Institute [NCI] and GIBCO) and rabbit preimmune serum.

Analysis of virion proteins. Animal cells at approximately 80% confluence in 10-cm dishes were starved for 30 min in 5 ml of DMEM lacking methionine. Approximately 150 μ Ci of [³⁵S]methionine (600 Ci/mmol; Amersham Corp.) was added, and the cells were grown for 17 h. Virus particles released into the medium during the labeling period were collected and freed of cell debris by centrifugation (SS34 rotor at 2,000 rpm for 10 min). The virus was passed through Swinex Acrodisc filters (pore size, 0.45 μ m; Gelman Sciences, Inc.) and layered over 5 ml of a solution of 25% (wt/vol) sucrose in TNE buffer (50 mM Tris hydrochloride [pH 8.3], 100 mM NaCl, 1 mM EDTA). The virus was pelleted by centrifuga-

tion (SW41 rotor at 25,000 rpm for 4 h) and suspended in electrophoresis sample buffer (62.5 mM Tris hydrochloride [pH 6.8], 1% [wt/vol] SDS, 6.25 mM EDTA, 120 mM beta-mercaptoethanol, 10% [wt/vol] glycerol, 0.005% bromophenol blue). A portion of the sample was analyzed directly by SDS gel electrophoresis (32), and the remainder was immunoprecipitated as follows. The sample was diluted by the addition of 10 volumes of phospholysis buffer (PLB; 1% [vol/vol] Triton X-100, 0.5% [wt/vol] sodium Desoxycholate, 0.1% [wt/vol] SDS, 0.1 M NaCl, 10 mM sodium phosphate [pH 7.5]), and in some cases nonspecific proteins were preadsorbed by incubation of the samples for 2 h at 0°C with normal goat serum (NCI and GIBCO) and then for 1 h at 0°C with fixed, washed *Staphylococcus aureus* (Pansorbin; Calbiochem-Behring). The samples (200 to 250 μ l) were clarified, the labeled proteins were bound by incubation overnight with 4 μ l of selected antisera, and the immune complexes were collected by incubation with *S. aureus* for 1 h followed by centrifugation. The labeled proteins were eluted from the pellets by boiling in 2 $\times$ electrophoresis sample buffer and analyzed by electrophoresis on 5 to 15% gradient gels (32) followed by fluorography. Virus released by avian cells was analyzed similarly, but the growth medium during the labeling period was supplemented with 2% dialyzed calf serum and contained no tryptone phosphate broth.

Phosphate labeling of virion proteins. NIH/3T3 cells at confluence in 10-cm dishes were starved for 20 min in 3 ml of phosphate-free medium supplemented with 10% DMEM and 10% dialyzed calf serum. One millicurie of ³²P_i was added per dish, and the cells were labeled for 4 h. As controls, duplicate cultures were labeled with [³⁵S]methionine (150 μ Ci; Amersham) in methionine-free DMEM for 4 h. Virions were harvested and purified as described above, and the virion proteins were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography.

Analysis of intracellular viral proteins and virion proteins by pulse-chase experiments. Cell cultures (four duplicate 10-cm dishes) were starved for 15 min in 3 ml of methionine-free DMEM. [³⁵S]methionine (200 μ Ci) was added for 15 min (pulse), the medium was removed, and the cells were washed with buffered saline. The cells in one dish were lysed immediately (0 min of chase) with 3 ml of ice-cold PLB (see above), and the lysate was transferred to prechilled tubes in ice. DMEM (3 ml) was added to the remaining three dishes, and the cells were incubated at 37°C for a 15-min, 45-min, or 4-h chase period. At the end of the chase periods the medium over the cells was harvested for analysis of virion proteins, and the cells were lysed in 3 ml of cold PLB for analysis of intracellular proteins. Virion particles were purified, and the virion proteins were immunoprecipitated and analyzed by electrophoresis as described above. Cell lysates were clarified by centrifugation in a Ti50 rotor at 35,000 rpm for 6 h and analyzed by immunoprecipitation, electrophoresis, and fluorography.

Treatment of viral proteins with endo H. Labeled proteins were immunoprecipitated and eluted from the antibodies in electrophoresis sample buffer, as described above. The samples were then diluted fourfold with buffer (50 mM sodium acetate [pH 6.0]) to give a final SDS concentration of 1%. Endoglycosidase H (endo H; 30 ng; obtained from P. Robbins, Massachusetts Institute of Technology, Cambridge) was added, and the reaction mixture was incubated at 37°C for 1 h. A one-third volume of 4 $\times$ electrophoresis sample buffer was added, and the products were analyzed on 5 to 15% polyacrylamide gradient gels.

FIG. 1. Structure of gene fusions and proteins encoded by various plasmids. All of the constructs were carried on a small circular plasmid vector. The genes are positioned so that the viral sequences are aligned with the corresponding sequences on the M-MuLV genome (bottom line). The *SalI* and *ClaI* sites in the polylinker of vector plasmid pATH3 and in the M-MuLV genome, used in the construction of the pSC1 plasmid, are indicated. The inducible promoter from the *trp* operon is indicated by the solid box labeled p. The *trpE*, RT, and endonuclease domains present on each plasmid are indicated by open boxes. The sizes of the protein products (solid arrows) are indicated. kb, Kilobases.

RESULTS

Construction of a gene fusion expressing the 3' half of the *pol* gene. To generate large quantities of the M-MuLV *pol* gene products for use as immunogens, we expressed portions of the gene in the form of fusion proteins in *E. coli*. A 3.9-kilobase DNA fragment extending from the unique *SalI* site in the center of the *pol* gene to the *ClaI* site on the 3' side of the gene was isolated from an infectious proviral DNA clone of the M-MuLV genome (35). The fragment was inserted into the polylinker site of the pATH3 vector (T. J. Koerner, unpublished data), appending the *pol* coding sequences to a portion of the *trpE* gene contained on the plasmid. The resulting plasmid, pSC1, was designed to express a large fusion protein of 116 kDa containing the C-terminal half of the RT domain and all of the downstream polypeptides (Fig. 1).

Bacterial cells containing this plasmid were starved for tryptophan to induce expression of the *trp* operon, and lysates were prepared. Portions of the extracts were treated to isolate the proteins that were insoluble in the presence of the detergent Nonidet P-40 and high salt. Analysis of these proteins by SDS-polyacrylamide gel electrophoresis, followed by staining with Coomassie blue, revealed that an abundant 116-kDa protein was specifically synthesized in cells bearing the pSC1 plasmid (Fig. 2, lanes 3 and 4). Additional smaller proteins were also detected. Analogous extracts from bacteria carrying the control plasmid pATH3 did not express the large protein but instead contained a major 37-kDa protein, which is the size of the truncated *trpE* protein encoded by this plasmid. The insoluble fraction of the lysates was enriched for these abundant proteins.

A second plasmid was constructed by the removal of the bulk of the *trpE* and RT regions from plasmid pSC1 (details to be described elsewhere). It was predicted that this plas-

mid, pSCB150.32, would encode a fusion protein of 50 kDa, consisting of 18 amino acids of the *trpE* protein, 16 amino acids of the RT, and 408 amino acids from the C terminus of the *pol* protein. Examinations of the proteins in bacteria carrying this new plasmid indeed revealed the presence of a novel protein migrating at approximately 50 kDa (Fig. 2, lanes 5 and 6).

Preparation and characterization of antisera specific for the *pol* gene products. To produce polyclonal antibodies against the *pol* polypeptides, large quantities of the pSC1 fusion protein were isolated by preparative gel electrophoresis of the insoluble fraction of the lysates from bacteria carrying pSC1. The protein was used to immunize rabbits by standard protocols, and sera were collected at various intervals (anti-pSC1 sera). The highly purified RT enzyme isolated from bacteria containing the pB6B15.23 plasmid (43) was used to immunize a second set of rabbits by the same regimen (anti-pB6B15.23 RT sera). Two sera specific for the R-MuLV RT and a serum specific for the *trpE* protein were obtained for comparison.

The pSC1 protein contains sequences of three different gene products and might elicit an immune response against only one or two of these regions. To determine whether the antisera were reactive against all of the domains present on the immunogen, the sera were tested for their ability to immunoprecipitate ³⁵S-labeled proteins from various bacterial extracts (Fig. 3). The sera were first tested on extracts of control cultures containing only the pATH3 vector plasmid (panel A). The sera raised against the pSC1 fusion protein, but not those raised against the pB6B15.23 enzyme, specifically recognized the truncated *trpE* fragment encoded by pATH3. Next, the sera were tested on extracts from bacteria expressing the pB6B15.23 RT (panel B). Both the anti-pSC1 and anti-pB6B15.23 RT sera reacted strongly with the 71-kDa enzyme expressed in these cells, showing that they

FIG. 2. SDS-polyacrylamide gel analysis of proteins extracted from bacterial cultures. Total cell protein lysates (lanes 1, 3, and 5) and fractions enriched for insoluble proteins (lanes 2, 4, and 6) were analyzed on a 10% SDS-polyacrylamide gel, and the proteins were visualized by staining with Coomassie blue. Lanes 1 and 2, Cells containing the pATH3 control plasmid; the truncated *trpE* protein migrated at approximately 37 kDa. Lanes 3 and 4, Cells containing pSC1. The full-length 116-kDa fusion protein is indicated by the arrow; several smaller proteins specific to these cells were also formed. Lanes 5 and 6, Cells containing pSCB150.32, a derivative of pSC1 retaining only the 3' portions of the *pol* gene. The stable 50-kDa protein is indicated by the asterisk. The migration positions of molecular size standards are indicated.

recognized determinants on the RT portion of the *pol* gene products (lanes 3 to 7). Thus, the portion of the RT present in the pSC1 protein was sufficient to elicit formation of antibodies reactive with the active enzyme. Finally, the sera were tested against extracts of cells carrying pSCB150.32 and therefore expressing only the C-terminal *pol* region (panel C). Neither the anti-R-MuLV RT sera nor the anti-pB6B15.23 RT sera recognized the 50-kDa protein expressed by these cells. The anti-pSC1 sera, however, strongly reacted with this protein (lanes 6 and 7). Thus, the C-terminal portion of the pSC1 protein was also immunogenic and elicited the formation of antibodies specific for C-terminal *pol* proteins. This specificity allowed the detection of proteins that could not be seen with sera prepared against either the R-MuLV or pB6B15.23 RTs.

In summary, the anti-pSC1 sera showed reactivity against all three portions of the fusion protein: the *trpE* protein of pATH3, the RT of pB6B15.23, and the C-terminal portion encoded by pSCB150.32. The sera raised against the purified pB6B15.23 RT reacted only with the RT domain and did not react with the *trpE* protein or with the C-terminal protein.

Analysis of virion particle proteins by immunoprecipitation with antisera raised against *pol* gene products: detection of a novel C-terminal *pol* protein. To identify the *pol* gene prod-

ucts present in M-MuLV virion particles, producer cells were labeled with [35 S]methionine overnight, and the released virions were purified. The virions were disrupted with detergent, and the proteins were immunoprecipitated with specific sera and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 4).

The rabbit antiserum raised against the purified pB6B15.23 enzyme reacted specifically with the P80^{pol} protein (Fig. 4, lane 15). The rabbit serum raised against the pSC1 fusion protein recognized two major proteins (lanes 16 and 17): the P80^{pol} protein and a novel, smaller protein which migrated at approximately 46 kDa. This novel protein was present at an approximately equimolar level with the P80 RT. It was not recognized by the anti-pB6B15.23 RT serum but only by those sera capable of recognizing the C-terminal portion of the *pol* gene product; this suggests that the 46-kDa protein is a virion-associated protein cleaved from the C terminus of the major *pol* precursor protein. This protein has approximately the same size and origin as the endonuclease previously identified in R-MuLV and F-MuLV virions (28, 30, 40).

The anti-pB6B15.23 RT serum and the anti-pSC1 sera identified one additional protein, a minor species which migrated at 51 kDa (Fig. 4, lanes 15', 16', and 17') and was present at a lower abundance than the P80 and P46 proteins. This minor species was detected with both sera and therefore contains at least some determinants from the RT domain. The properties and function of this minor protein were not investigated further.

Previously available sera identified the expected virion proteins. Goat anti-M-MuLV serum reacted with the two major viral proteins, gPr80^{env} and P30^{gag} (Fig. 4, lane 11). Goat anti-R-MuLV P30 serum was highly specific for the P30^{gag} protein (lane 12). The goat anti-R-MuLV RT sera reacted with P80^{pol}, the mature RT, but also showed significant reactivity against P30^{gag} (lanes 13 and 14).

P46 protein is not phosphorylated. In avian retroviruses, the major *pol* gene product, the beta subunit of the RT, is cleaved to form the smaller alpha subunit and a C-terminal protein designated pp32 (9, 15–21, 33, 38). The latter protein is phosphorylated and is encoded in the avian viruses by the same portion of the *pol* gene as that which encodes the 46-kDa protein in the murine viruses. To determine whether the P46 protein of M-MuLV is similarly phosphorylated, producer cells were labeled with 32 PO₄ for 4 h, the virus was collected from the medium and purified, and the virion proteins were displayed by electrophoresis on SDS-polyacrylamide gels (Fig. 5). Examination of the total virion proteins revealed only a single major phosphoprotein of approximately 12 kDa (lane 3). Several other proteins were detected with smaller amounts of label, but these proteins did not comigrate with any viral proteins and were presumably nonspecific proteins which copurified with the virions. The major phosphoprotein was shown by serology to be pp12^{gag}, a virion protein known to be phosphorylated (41, 58); the phosphate-labeled protein could be immunoprecipitated by goat antiserum specific for Gross MuLV P12 (lane 7). Analysis of the proteins labeled in parallel with [35 S]methionine showed that all of the expected virion proteins were present, including the major *gag*, *pol*, and *env* gene products (lane 4). Immunoprecipitation of the virion proteins with anti-pSC1 serum clearly revealed the P46 and P80 *pol* proteins from the 35 S-labeled virus (lane 12), but these proteins could not be detected with the 32 PO₄ label (lane 11) even after very long autoradiographic exposures. Thus, we could not demonstrate any phosphorylation of the

FIG. 3. Specificity of antisera raised against fusion proteins. Bacterial cultures were labeled with [35 S]methionine, extracts were prepared, and equal amounts of the labeled proteins were immunoprecipitated and analyzed by gel electrophoresis, as described in Materials and Methods. Extracts were made from cells bearing the following plasmids: (A) pATH3 control plasmid, expressing the *trpE* domain; (B) pB6B15.23, expressing the RT domain; (C) pSCB150.32, expressing the endonuclease domain. Immunoprecipitations were performed with the indicated antisera. Lanes: 1, normal goat serum; 2, rabbit preimmune serum; 3, goat anti-R-MuLV RT serum (NCI 74S-454); 4, goat anti-R-MuLV RT serum (NCI 77S-424); 5, rabbit anti-pB6B15.23 RT serum; 6 and 7, rabbit anti-pSC1 sera 1 and 3; 8, rabbit anti-*trpE* serum. The migration positions of molecular weight standards (10^3) are indicated on the right. bpb, Bromophenol blue.

P46^{pol} protein under conditions that readily allowed the detection of phosphate in pp12^{gag}. We estimate that we could have detected such phosphorylation if P46 contained phosphate at 1/20 the level of pp12^{gag}, even taking into account the low abundance of P46 in the virions.

Processing of precursor proteins: analysis of intracellular viral proteins and virion proteins by using antisera specific for the *pol* gene products. A series of pulse-chase labeling experiments were performed to analyze the proteolytic processing of the *gag* and *gag-pol* precursor proteins of wild-type virus. Cells infected with virus and uninfected NIH/3T3 cells were pulse-labeled for 15 min with [35 S]methionine, the medium was removed, and the label was chased for 0 min, 15 min, 45 min, or 4 h. At the end of each chase period both the intracellular proteins and the extracellular virion proteins were isolated and analyzed. The intracellular proteins were prepared by lysis of the cells in PLB; the virions were purified from the medium by sucrose density gradients and similarly lysed in electrophoresis sample buffer and PLB. To minimize processing of the proteins after harvesting, these steps were performed rapidly and in the cold. Both protein fractions were subjected to immunoprecipitation followed by electrophoresis on SDS-polyacrylamide gels. The processing of the *gag* proteins was analyzed with goat anti-R-MuLV P30 serum, and the processing of the *pol* proteins was analyzed with rabbit anti-pB6B15.23 RT and anti-pSC1 sera.

The anti-P30 serum, but not the *pol*-specific sera, detected the two *gag* precursor proteins, Pr65^{gag} and the glycosylated gPr80^{gag} (Fig. 6A). These proteins could be readily detected after the 15-min labeling with no chase period. The *gag* protein precursors gradually disappeared during the chase; as previously reported (11), the glycosylated protein was

very labile and could barely be detected after 45 min of chase. The amount of the major *gag* precursor, Pr65^{gag}, decreased as the mature P30^{gag} protein appeared, showing a half time of roughly 1 h for the process (panel A). Some processed P30 could be detected after a chase period as short as 15 min. At this time labeled virus also could be recovered in the medium, and the amount of label in the purified virions increased steadily thereafter (panel D). After a 4-h chase the amount of P30 in the intracellular fraction decreased slightly, suggesting that much of the *gag* protein had been released from the cell in the form of virion particles. Indeed, at late times most of the counts were recovered in the extracellular fraction. No residual precursor proteins could be seen in the virions under any conditions; all of the virion-associated label was in the form of mature P30 even at the earliest time points. These results suggest that cleavage normally precedes or very rapidly follows virion release.

The *gag*- and *pol*-specific sera all immunoprecipitated a doublet of proteins of very high molecular weight (Fig. 6, panels A, B, and C): Pr200^{gag-pol} and the glycosylated Pr200^{gag-pol} (45). Like the *gag* precursors, these proteins could be detected after the 15-min labeling and gradually disappeared during the chase; they also showed a half-life of roughly 1 h for the process. The ratio between the two *gag-pol* precursor proteins did not change dramatically during the chase, suggesting that the glycosylated species was not grossly more labile than the nonglycosylated form. The precursors were no longer detectable after 4 h of chase. A very large number of smaller proteins were also detected by the *pol*-specific sera, ranging in size from 80 to 200 kDa (panels B and C). The label in these bands first increased and then disappeared during the chase period, suggesting that they represented intermediates in the processing to mature

FIG. 4. Immunoprecipitation of proteins in virion particles from uninfected NIH/3T3 cells (lanes 1 to 9) and clone 4 cells releasing wild-type M-MuLV (lanes 10 to 18). Lanes: 1 and 10, total virion proteins without immunoprecipitation; 2 and 11, goat anti-M-MuLV serum (NCI 77S-591); 3 and 12, goat anti-R-MuLV P30^{gag} serum (NCI 77S-102); 4 and 13, goat anti-R-MuLV RT serum (NCI 74S-454); 5 and 14, goat anti-R-MuLV RT serum (NCI 77S-424); 6 and 15, rabbit anti-pB6B15.23 RT serum; 7 and 16, rabbit anti-pSC1 serum 1; 8 and 17, rabbit anti-pSC1 serum 3; 9 and 18, rabbit preimmune serum; 15' to 18', longer exposure of lanes 15 to 18 to show the minor *pol*-related protein band migrating at 51 kDa. The migration positions of molecular weight standards (10^3) are indicated.

proteins. These proteins were undetectable after 4 h of chase. The anti-pB6B15.23 RT serum first allowed the detection of mature P80^{pol} intracellularly after 15 min of chase, and the level increased after the 45-min chase (panel B). The anti-pSC1 serum showed the same proteins intracellularly but also revealed the appearance of the P46 protein (panel C). After 45 min of chase, the anti-pB6B15.23 RT serum detected the mature P80^{pol} protein in the extracellular virions (panel E); the anti-pSC1 serum detected this protein, as well as the P46 protein (panel F). The P80 and P46 proteins appeared with identical kinetics, and the ratio between them remained constant during the chase. No precursor forms of these proteins were detected in the virion particles, suggesting that processing was completed before or very soon after the release of the virions from the cells.

Analysis of mutant viral precursor proteins and treatment of labeled intracellular proteins with endo H. Several mutants of M-MuLV have been constructed in this laboratory that carry deletions and insertions in the *pol* gene (6, 48; N. Tanese and S. P. Goff, manuscript in preparation). We used the anti-pSC1 serum to examine the *gag-pol* precursor proteins synthesized by several of these mutants. Cells were labeled for 15 min and then chased for 15 min, and the intracellular proteins were extracted, immunoprecipitated, and analyzed as before (Fig. 7A). Cells infected with the wild-type virus showed the same doublet of *gag-pol* precursors as was seen before (lane 2). Mutant *dl2901* (6) contains a 126-base-pair deletion in the 5' part of the *pol* gene; the deletion preserves the reading frame and allows formation of

active RT. Clone 4H12 cells expressing this mutant also showed a doublet of *gag-pol* precursors, and as expected from the genome structure, the proteins were slightly shorter than those of the wild-type virus (lane 3). Mutant *dl5401* contains a 91-base-pair deletion in the 3' part of the *pol* gene that does not affect RT activity but does abolish the ability of the viral DNA to establish itself in the cell as an integrated provirus (48). Clone F7 cells containing this mutant genome expressed a very short pair of *gag-pol* proteins (lane 4); the lower, more abundant of the proteins migrated at 165 kDa, exactly as predicted by the position and size of the deletion in this virus.

The sensitivity of the doublet of large *gag-pol* precursor proteins to endo H was tested. The viral proteins were immunoprecipitated as before and eluted from the *S. aureus* pellets, and a portion of the samples was diluted and treated with endo H. The digestion products were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 7B). Samples from wild-type infected cells contained the doublet of proteins near 200 kDa as before (lane 3). After endo H digestion, the upper band of the doublet migrated slightly more rapidly, whereas the mobility of the lower band was unchanged (lane 4). The shift was small but reproducible. These results suggest that the protein corresponding to the upper band was glycosylated. The migration of the majority of the smaller, processed proteins was not shifted, with the exception of one prominent protein migrating at approximately 105 kDa (lanes 3 and 4). Analysis of cells expressing *dl5401* virus showed the truncated doublet of *gag-pol* proteins as before

FIG. 5. SDS-polyacrylamide gel analysis of virion proteins metabolically labeled with $^{32}\text{PO}_4$ and $[^{35}\text{S}]$ methionine. Uninfected NIH/3T3 cells (controls; lanes C) and infected cells producing M-MuLV (lanes V) were labeled with $^{32}\text{PO}_4$ (lanes P) or $[^{35}\text{S}]$ methionine (lanes S) for 4 h, virion particles were purified, and the viral proteins were analyzed on a 5 to 15% gradient gel, as described in Materials and Methods. Lanes: 1 to 4, total proteins analyzed without immunoprecipitation; 5 to 8, protein immunoprecipitated with goat anti-Gross-MuLV P12^{gag} serum (NCI 75S-711); 9 to 12, proteins immunoprecipitated with rabbit anti-pSC1 serum. The migration positions of molecular size standards are indicated. Whereas $^{32}\text{PO}_4$ label was readily detected in P12^{gag} (lanes 3 and 7), no label was found in P46^{pol} (lanes 3 and 11).

(lane 5). Treatment of these proteins with endo H reduced the apparent size of the upper protein (lanes 5 and 6). Thus, the mutant virus synthesizes truncated versions of both the unglycosylated and glycosylated *gag-pol* precursors. The fact that both proteins are similarly affected by the deletion is further strong evidence that these proteins are products of the viral genome.

Analysis of mature virion proteins of M-MuLV mutants with rabbit anti-*pol* antisera. The rabbit anti-*pol* sera were helpful in determining the effect of mutations on the processing of the altered *pol* proteins. We examined two mutants to test for alterations in the mature *pol* proteins. Mutant *dl5401*, described above, contains a deletion in the 3' part of the *pol* gene, affecting the establishment of the provirus; mutant *in4900-16* contains a frameshift insertion in the 3' part of the gene that reduces the level of RT activity (Tanese and Goff, in preparation). Cells were grown in the presence of $[^{35}\text{S}]$ methionine, and the labeled virions were collected; the virions were disrupted, and the virion proteins were immunoprecipitated with various sera and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 8). All the producer cell lines released virus and processed the *gag* protein. Immunoprecipitation with anti-P30 serum revealed that wild-type virus and both mutants released virus particles with

P30^{gag} (lanes 3 and 4); as previously reported, *dl5401* particles showed only partial cleavage of the *gag* precursor to the mature products, so that Pr65^{gag} was also seen. Immunoprecipitation of wild-type virus with anti-pSC1 serum revealed both P80^{pol} and P46^{pol} as before (lane 6). The mutant *dl5401* contained normal levels of P80^{pol} but no detectable P46 (lane 7). Thus, the *pol* precursor of this mutant was processed to form an intact RT, with both cleavages flanking this domain occurring normally, but no stable C-terminal product was formed. The mutant *in4900-16* contained a truncated RT and no P46 (lane 8). The position of the frameshifting insertion in this mutant at nucleotide 4900 predicts termination to yield a mature protein 36 amino acids shorter than would be formed by cleavage of the wild-type precursor at the normal C terminus of the RT (5). Thus, the *pol* precursor of this mutant is apparently processed at the N-terminal sites, forming a nearly normal P80 protein (shorter by only 36 amino acids) and no mature C-terminal protein product. The altered RT presumably accounts for the lower enzyme activity and the inability of the mutant to form full-length viral DNA upon infection. The results further support the identity of the proteins detected by the antisera as virus-encoded proteins.

Reactivity of anti-*pol* sera with heterologous retroviral

FIG. 6. Pulse-chase studies of intracellular viral proteins and released virion proteins. Uninfected NIH/3T3 cells and infected cells producing M-MuLV were pulse-labeled with [35 S]methionine for 15 min, the label was removed, and the cultures were incubated with unlabeled methionine for various lengths of time. Equal portions of the proteins were immunoprecipitated and analyzed on 5 to 15% gradient gels. The upper three panels (A, B, and C) display intracellular proteins, and the lower three panels (D, E, and F) display virion proteins. (A) and (D) Proteins immunoprecipitated with goat anti-R-MuLV P30^{gag} serum; (B) and (E) proteins immunoprecipitated with rabbit anti-pB6B15.23 RT serum; (C) and (F) proteins immunoprecipitated with rabbit anti-pSC1 serum. Lanes: 1 to 4, uninfected NIH/3T3 cells; 5 to 8, clone 4 producer cells. The lanes in each panel show samples labeled by the indicated protocols. Lanes: 1 and 5, 15-min pulse, no chase; 2 and 6, 15-min chase; 3 and 7, 45-min chase; 4 and 8, 4-h chase. The migration positions of molecular size standards are indicated.

proteins. We determined whether the antisera raised against the M-MuLV *pol* proteins showed cross-reactivity with gene products of other retroviruses. Cells infected with several strains of avian sarcoma and leukosis viruses, with FeLV, or with F-MuLV were labeled with [35 S]methionine for 9 h, and virion particles were harvested from the medium and purified. The virions were disrupted with detergent, and the viral proteins were analyzed by SDS-polyacrylamide gel electro-

phoresis before and after immunoprecipitation with various antisera (Fig. 9). Antisera specific for the total virion proteins of each virus strain were used as controls.

Uninfected NIH/3T3 cells did not release any rapidly sedimenting label (Fig. 9, panel A), whereas cells infected with M-MuLV released particles containing the expected *gag*, *pol*, and *env* proteins (panel B). As in previous experiments, the anti-pB6B15.23 RT serum precipitated the P80^{pol}

FIG. 7. Analysis of glycosylation of viral *gag-pol* precursor proteins. (A) Intracellular proteins were labeled with [35 S]methionine for 15 min, the label was chased for 15 min, and the viral proteins were immunoprecipitated with rabbit anti-pSC1 serum. The products were subjected to electrophoresis on a 5 to 15% gradient SDS-polyacrylamide gel. Lanes: 1, uninfected NIH/3T3 cells; 2, clone 4 cells containing wild-type M-MuLV; 3, clone 4H12 cells containing *dl2901* virus, a mutant lacking the *pol* protease function (6); 4, clone F7 cells containing *dl5401* virus (48), a mutant with a deletion in the 3' part of the *pol* gene. (B) Analysis of *gag-pol* precursors with endo H. Lanes: 1 and 2, uninfected NIH/3T3 cells; 3 and 4, clone 4 cells releasing M-MuLV; 5 and 6, clone F7 cells containing the M-MuLV mutant *dl5401* (48). The first lane of each pair (lanes 1, 3, and 5) was untreated, and the second lane of each pair (lanes 2, 4, and 6) was treated with endo H. Virus-infected cells

FIG. 8. Analysis of virion proteins encoded by M-MuLV mutants with alterations in the *pol* gene. Producer cells were labeled with [35 S]methionine for 10 h, and virion particles were purified from the medium. The virions were disrupted, and the proteins were immunoprecipitated either with goat anti-R-MuLV P30 serum (lanes 1 to 4) or with rabbit anti-pSC1 serum (lanes 5 to 8). The cell lines tested were as follows. Lanes: 1 and 5, uninfected cells; 2 and 6, clone 4 cells releasing wild-type virus; 3 and 7, clone F7 cells releasing *dl5401* virus, a mutant bearing a deletion in the endonuclease portion of the *pol* gene; 4 and 8, clone H8G3 cells releasing *in4900-16* virus, a mutant bearing a frameshift mutation near the 3' end of the region encoding the RT. The migration positions of molecular size standards are indicated.

protein and the anti-pSC1 serum precipitated both the $P80^{pol}$ and $P46^{pol}$ proteins. Cells infected with F-MuLV released a similar pattern of proteins (panel C). The two anti-*pol* sera detected 80- and 45-kDa proteins; the sizes and relative abundance of the two F-MuLV proteins were very similar to those of the M-MuLV proteins. Thus, the anti-*pol* sera could be used to precipitate the virion proteins from the closely related F-MuLV.

Uninfected feline cells released some particulate label, but none reacted with anti-FeLV serum (Fig. 9, panel D). Cells infected with FeLV released several prominent new proteins, and serum raised against total FeLV virions allowed the identification of major *gag* proteins migrating at approximately 28 and 18 kDa. The anti-pB6B15.23 RT serum detected a single protein of about 75 kDa; the anti-pSC1 serum detected the same protein, as well as a protein of about 45 kDa. The proteins were visible among the total

each expressed a doublet of *gag-pol* proteins recognized by the antiserum; the two proteins encoded by *dl5401* were smaller, as predicted by the structure of the mutant genome. The positions of the smaller, nonglycosylated precursors are marked with arrowheads and labeled. The positions of the larger *gag-pol* proteins are indicated by the asterisks. The migration positions of molecular size standards are indicated.

FIG. 9. Characterization of antisera for cross-reactivity to protein products of heterologous murine, feline, and avian retroviruses. Cells infected with various retroviruses were labeled with [35 S]methionine for 9 h, and virion particles were purified from the supernatant medium and disrupted. The sera were tested for the ability to immunoprecipitate the labeled proteins. (A) Uninfected NIH/3T3 cells; (B) clone 4 cells producing wild-type M-MuLV; (C) cells producing F-MuLV; (D) uninfected feline embryo fibroblasts; (E) feline embryo fibroblasts infected with FeLV subgroup A; (F) uninfected chicken embryo fibroblasts; (G) chicken embryo fibroblasts producing Rous-associated virus 1; (H) chicken embryo fibroblasts producing Fujinami-associated virus, subgroup C; (I) chicken embryo fibroblasts producing the Schmidt-Ruppin strain of RSV; (J) chicken embryo fibroblasts producing the Bryan high-titer strain of RSV. The samples were treated with the indicated sera. Lanes 1, Total virion proteins without immunoprecipitation. Lanes 2, Sera prepared against whole virus; goat anti-M-MuLV (NCI 77S-591) for panels A to C; anti-FeLV for panels D and E; and rat anti-RSV for panels F to J. Lanes 3, rabbit preimmune serum. Lanes 4, Rabbit anti-pB6B15.23 RT serum. Lanes 5, Rabbit anti-pSC1 serum. The migration positions of molecular size standards are indicated.

virion proteins and were present at a similar abundance as the P80 and P46 proteins in the murine virions. These results show that the polyclonal sera do cross-react with FeLV proteins of size and abundance similar to those of the MuLVs.

A similar analysis was performed with chicken embryo fibroblasts infected with four different avian virus strains (Rous-associated virus 1, Fujinami-associated virus, subgroup C, Bryan-RSV with Rous-associated virus 1 helper virus, or Schmidt-Ruppin RSV). All of the infected cells released similar patterns of virion-associated proteins (Fig. 9, panels G to J), and serum from rats bearing RSV-induced tumors immunoprecipitated the major *gag* proteins of all four viruses. Neither the rabbit anti-pB6B15.23 RT nor the anti-pSC1 sera were able to immunoprecipitate any detectable proteins from these virions. Thus, any binding of the

antibodies to the avian viral proteins was too weak to be useful in the detection or recovery of the avian *pol* gene products.

DISCUSSION

The expression of portions of eucaryotic genes in bacterial cells is a powerful means of preparing antigenic proteins for use in immunization regimens. In our procedure, we used the *trp* operon promoter and a portion of the *trpE* gene of *E. coli* to generate fusion proteins containing portions of the M-MuLV *pol* gene products at their C termini (27, 50). The fusion proteins were readily identified; in one case the fusion protein displayed abundant RT activity, permitting its purification by conventional chromatography procedures (43), and in the case of pSC1, the fusion protein was large and

highly insoluble, permitting its isolation by selective extractions and SDS-polyacrylamide gel electrophoresis. The purified proteins served as potent immunogens in rabbits even when as little as 3 µg of protein per injection was used. The resulting antisera were highly specific for the *pol* proteins, showing no reactivity with other viral or eucaryotic cell proteins.

The sera allowed the detection of the RT and the protein encoded by the 3' portion of the *pol* gene in mature virions. These proteins, judging from their electrophoretic mobility, had molecular sizes of 80 and 46 kDa, respectively. They were present in virions at approximately equal molarities; there was no residual uncleaved precursor protein detected in mature virions. These results suggest that the primary translation product, Pr200^{*gag-pol*}, is completely cleaved into these products and that both are retained with equal efficiency in the virion particle. Analysis of the P46 protein did not reveal any phosphorylation. In addition to P80^{*pol*} and P46^{*pol*}, the sera identified a minor protein which was present in the virions at a lower molarity. The protein, which migrated at 51 kDa, was recognized by the anti-pB6B15.23 RT serum and must share at least some sequence with the P80^{*pol*} protein. We propose that it is formed by an alternate cleavage pathway from one of the two *gag-pol* precursors. The function of this protein, if any, is unknown. Analysis of its role awaits the formation of mutants specifically altered in its formation.

The pattern of processing of the *pol* precursor described above is quite distinct from that in the avian retroviruses, in which the *pol* gene serves to encode the large beta subunit of the RT and only a portion of this protein is cleaved to form the smaller alpha subunit and the pp32 endonuclease (14-21). The mature virions thus contain a mixture of uncleaved beta and cleaved alpha and pp32; the bulk of the RT activity resides in a heterodimer consisting of the alpha and beta subunits. The alpha subunit exhibits DNA polymerase activity (17), the pp32 protein contains a specific endonuclease activity (21), and the beta subunit contains both activities (15, 16, 18, 20, 33).

The new *pol*-specific sera, as well as *gag*-specific sera, identified two large *pol* precursors (approximately 200 kDa), the larger of which was glycosylated; these two proteins correspond to the two *gag* proteins made by M-MuLV, Pr65^{*gag*} and gPr80^{*gag*} (11, 24, 29, 30, 45). The two *pol* precursor proteins disappeared with similar kinetics during chase periods, exhibiting half-lives of roughly 1 h. Numerous smaller proteins, presumably intermediates in the processing of the large precursors, were also detected by the antisera, as was previously described (24). We cannot say whether the glycosylated *gag-pol* precursor contributes to most of these intermediates; very few of these proteins, however, were glycosylated. In any case, genetic evidence has shown that the glycosylated versions of the *gag* and *gag-pol* proteins are not required for viral replication (47).

We were unable to detect uncleaved *gag* and *gag-pol* precursor proteins in virions, even in those harvested very soon after labeling. This suggests that cleavage normally occurs before or very soon after release from the cell. The existence of cleaved virion proteins in the intracellular fraction is not in itself good evidence that cleavage occurs intracellularly; it is very likely that mature budded virions can remain attached, or reattach, to the cell surface and be retained in the intracellular fraction. The fact that the proteins of released virions are completely cleaved, however, does suggest that cleavage occurs concomitantly with budding or very soon thereafter in wild-type virus. We

cannot completely rule out the possibility that some processing occurred during the handling of the virions, but every effort was made to prevent it. Previous work has led to the suggestion that cleavage is incomplete at the time of release and continues for 1 to 2 h afterwards to form the completely processed proteins (58). These studies were made with the MuLV mutants *ts3* and *ts24*, however, and involved the arrest of assembly by a shift to high temperature, followed by a shift to low temperature to allow virion release. Under these unusual conditions it was clearly shown that precursor cleavage is incomplete at the time of virion release and that cleavage continues after release. We suggest that in wild-type virions forming continuously, cleavage normally precedes release. It should be emphasized that cleavage is known to be unnecessary for release; viral protease mutants, which never cleave the *gag* or *gag-pol* precursors, can assemble and release virions perfectly well (6). These results show that cleavage and release are not obligatorily coupled.

The *pol* proteins of several M-MuLV mutants were examined with the new sera. A mutant, *dl5401*, bearing a frameshift deletion near the 3' end of the gene, was shown to synthesize shortened versions of both *gag-pol* proteins; this confirms the genetic structure of the mutant and also confirms the identity of both of the wild-type 200-kDa proteins as viral gene products. It was also shown that these shortened precursor proteins were processed to form normal-size RT but that none of the 46-kDa protein was present. Since the mutant is defective in the establishment of the integrated provirus in the infected cell, that function can be firmly assigned to the P46^{*pol*} protein. Another mutant, *in4900-16*, showed formation of a truncated RT, as predicted from its genetic structure.

Our laboratory is continuing efforts to characterize the *pol* proteins, and we expect that the sera will be extremely helpful in these efforts. We have tested the ability of both the anti-pB6B15.23 RT serum and the anti-pSC1 serum to inhibit RT activity. Others have tested antisera directed against synthetic avian *pol* peptides and found that they did not inhibit RT activity (20). Preliminary results with our sera suggest that the anti-pB6B15.23 RT serum does inactivate DNA polymerase activity and that the anti-pSC1 serum does not; these analyses could lead to further definition of the functional domains of the RT. Similar experiments can be performed to analyze the functions of the P46 protein, which has been shown to contain endonuclease activity by several investigators (28, 40). The P46 protein may be functionally, as well as structurally, related to the avian pp32 endonuclease, a specific DNA-binding enzyme (21, 38) that can cleave DNA at the palindromic site at which circular viral DNAs integrate into host DNA (9, 10).

We were also able to test for the conservation of antigenic determinants on viruses related to M-MuLV by various evolutionary distances. The sera readily detected the closely related F-MuLV *pol* gene products, which were found to be similar in size to the M-MuLV proteins. The sera also reacted well with the evolutionarily more distant FeLV proteins. The FeLV proteins were similar in size but showed some differences; the RT was slightly smaller than the M-MuLV enzyme. The sera showed no detectable cross-reactivity with any of the four strains of avian retroviruses. Thus, the *pol* gene products of these viruses are so different that no determinants can be bound by the polyvalent sera tightly enough to allow immunoprecipitation in standard protocols. We cannot rule out the possibility that more sensitive assays such as Western blot transfers might detect weak cross-reactivity.

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