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Murine Leukemia Virus *pol* Gene Products: Analysis with Antisera Generated against Reverse Transcriptase and Endonuclease Fusion Proteins Expressed in *Escherichia coli*

STELLA C. HU,[1] DONALD L. COURT,[2] MARTIN ZWEIG,[3] AND JUDITH G. LEVIN[1]*

Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, Bethesda, Maryland 20892,[1] and Laboratory of Molecular Oncology, National Cancer Institute,[2] and Program Resources, Inc.,[3] Frederick Cancer Research Facility, Frederick, Maryland 21701

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The organization of the murine leukemia virus (MuLV) *pol* gene was investigated by expressing molecular clones containing AKR MuLV reverse transcriptase or endonuclease or both gene segments in *Escherichia coli* and generating specific antisera against the expressed bacterial proteins. Reaction of these antisera with detergent-disrupted virus precipitated an 80-kilodalton (kDa) protein, the MuLV reverse transcriptase, and a 46-kDa protein which we believe is the viral endonuclease. A third (50-kDa) protein, related to reverse transcriptase, was also precipitated. Bacterial extracts of clones expressing reverse transcriptase and endonuclease sequences competed with the viral 80- and 46-kDa proteins, respectively. These results demonstrate that the antisera are specific for viral reverse transcriptase and endonuclease. Immunoprecipitation of AKR MuLV with antisera prepared against a bacterial protein containing only endonuclease sequences led to the observation that reverse transcriptase and endonuclease can be associated as a complex involving a disulfide bond(s).

Studies on the molecular organization of the *gag-pol* region of the murine leukemia virus (MuLV) genome have indicated that the gene order in *pol* is 5′-protease-reverse transcriptase-endonuclease-3′ (2, 24, 43). The location of the protease coding region at the 5′ end of *pol* was originally inferred from work on the MuLV clone 23 frameshift *pol* mutant (10, 24, 28), which showed that there was additional coding potential in *pol* upstream of reverse transcriptase (24), and also from studies on in vitro-generated MuLV deletion mutants with a defect in processing of the *gag* and *gag-pol* precursor proteins (3, 16). However, sequence analysis of the isolated protease demonstrated that it is actually a *gag-pol* protein since it is synthesized by translational readthrough of the UAG termination codon separating *gag* and *pol*, and its four N-terminal amino acids are encoded by sequences at the extreme 3′ end of *gag* (43). The conclusion that the central portion of *pol* codes for reverse transcriptase is based on the precise localization of the molecular defect in the clone 23 mutant (24) and the determination of the amino- and carboxyl-terminal sequence of the enzyme (2). From the sequence data, it can be calculated that the molecular mass of reverse transcriptase is 75 kilodaltons (kDa), although in polyacrylamide gels it typically migrates with an apparent molecular mass of 80 kDa (30, 41). The remaining coding capacity in *pol* was assigned to endonuclease (2, 5, 19, 24, 36, 37), in part because of the analogy to the organization of the avian retrovirus *pol* gene (4).

The present study was undertaken in connection with our interest in correlating the genetic structure and functional activities of the MuLV *pol* gene and was directed toward identifying the protein specified by the endonuclease coding region. MuLV-associated endonuclease activity was first described several years ago by Nissen-Meyer and Nes (32) and also by Kopchick et al. (18). The enzyme was isolated from viral cores (32), was reported to have a molecular mass of approximately 40 kDa (18, 32), and was also shown to share methionine-containing tryptic peptides with $Pr200^{gag\text{-}pol}$ and a polymerase precursor molecule, $Pr135^{pol}$ (18). In more recent work, mutations constructed in sequences at the 3′ end of the *pol* gene have yielded MuLV particles which retain reverse transcriptase activity and the ability to synthesize full-length linear and circular viral DNA molecules but are defective in integration of viral DNA into the host chromosome (5, 36). Similar results have also been obtained by Panganiban and Temin (33) with spleen necrosis virus mutants. Since both the avian and murine virus endonuclease enzymes have been postulated on the basis of their in vitro activity to function as "integrase" proteins in viral replication (6, 13, 18, 23, 33, 40), these genetic results support the proposed map position for endonuclease.

In this study, we used a different approach to investigate the question of the endonuclease map position. We expressed portions of the AKR MuLV *pol* gene in *Escherichia coli*, made antibodies to the expressed fusion proteins, and tested the reactivity of the sera against detergent-disrupted virions. The results established that sequences at the 3′ end of the MuLV *pol* open reading frame encode a protein with a molecular mass of approximately 46 kDa. This value is in agreement with the size predicted for endonuclease based on the total coding potential in *pol* (8, 15, 37) and the molecular masses of reverse transcriptase (2) and the *pol*-encoded portion of protease (43). In addition, using an antiserum directed against endonuclease alone, we found that endonuclease and reverse transcriptase can be associated in a complex involving a disulfide linkage.

MATERIALS AND METHODS

Materials. ^{14}C-labeled molecular weight markers for protein gels and [^{35}S]methionine (approximately 1,100 Ci/mmol) were obtained from New England Nuclear Corp. Protein A–Sepharose CL-4B was purchased from Pharmacia, Inc., and aprotinin was obtained from Sigma Chemical Co. AKR

* Corresponding author.

MuLV purified by two cycles of sucrose density gradient centrifugation (Electronucleonics; lot 5029-6-55) and antiserum directed against partially purified Rauscher MuLV reverse transcriptase (lot 76S-163) were provided by the National Cancer Institute. The slow BAL 31 enzyme was supplied by International Biotechnologies, Inc.

Mammalian cells and viruses. SC-1 (14) and 3T3FL (11) cell lines and AKR MuLV-infected SC-1 cells (25) were maintained in McCoy 5a medium containing 10% fetal bovine serum. The cells were labeled by incubating subconfluent monolayers on 100-mm (diameter) petri plates with Eagle medium containing 1/25 the normal level of methionine, 5% dialyzed fetal bovine serum, and 50 μCi of [^{35}S]methionine per ml. The culture fluids were collected after 18 to 24 h and clarified. A second harvest was obtained by incubating the cells for another 3 h with McCoy 5a medium containing 10% fetal bovine serum. The combined fluids were centrifuged at 4°C in a Beckman SW27 rotor at 26,000 rpm, and the pellets were suspended in immunoprecipitation buffer (20 mM Tris hydrochloride [pH 7.4], 50 mM NaCl, 0.5% [vol/vol] each sodium deoxycholate and Nonidet P-40) containing 0.1% sodium dodecyl sulfate (SDS).

Plasmids. A schematic diagram illustrating the structure of the expression vector pWS50 (W. P. Sisk, J. G. Chirikjian, J. Lautenberger, C. Jorcyk, T. S. Papas, M. Berman, R. Zagursky, and D. L. Court, Gene, in press) and the AKR MuLV fragments used to construct recombinant plasmid clones is shown in Fig. 1. pWS50 is a pBR322 derivative which has the λ p_L promoter on a fragment inserted at the *Cla*I site followed by 49 nucleotides encoding the first 13 amino acids of the λ *c*II protein. Translation is initiated from the *c*II sequence, joined out of frame to the *lacZ* coding region, which is missing its own promoter and translation initiation signals. The strategy used was to treat appropriate MuLV *pol* fragments at one or both ends with BAL 31 (27), incubate them with T4 DNA polymerase to repair single-stranded ends (27), and then insert individual fragments into the unique *Nru*I site of pWS50 by blunt-end ligation. In-frame fusions generate *lacZ*$^+$ clones which can be detected as blue colonies with the indicator X-Gal (Sigma). The clones were isolated in the *E. coli* host, DC519 (Sisk et al., in press), which is lysogenic for λ and has a *lac* deletion. The nucleotide sequence at the 5′ terminus of the inserts was determined on double-stranded plasmid DNA by the method of Zagursky et al. (44) by using a 17-base-pair primer containing sequences from the *c*II region of pWS50 (Sisk et al., in press). The nucleotide positions of the AKR MuLV sequence are numbered as described by Herr (15).

A large AKR MuLV segment (pHSB-5) was used as the source for the cloned fragments. This segment contains 82 base pairs of protease (2531 to 2612), all of the reverse transcriptase (2613 to 4625) and endonuclease (4626 to 5840) coding sequences, and 700 base pairs of the *env* region and was constructed by inserting two *pol* fragments derived from subclones of 623P (24, 26) into the plasmid pJL6 (21): a 1.2-kilobase (kb) *Hpa*I-*Sal*I fragment (2531 to 3720) and a 2.8-kb *Sal*I-*Bam*HI fragment (3721 to 6543). A subclone of pHSB-5 containing only the *Sal*I-*Bam*HI insert was designated pSB. The reverse transcriptase clones were constructed as follows. pHSB-5 was digested with *Cla*I (at the site 5′ to the AKR MuLV insert), treated for 20 min at 30°C with the slow BAL 31 enzyme, and then digested with *Xmn*I. The resulting fragments were inserted into pWS50, and one of the blue *lacZ*$^+$ clones characterized was termed pRT24. A *lacZ*$^-$ derivative of pRT24, designated pRT235, was obtained by making a partial *Bam*HI digest, filling in the

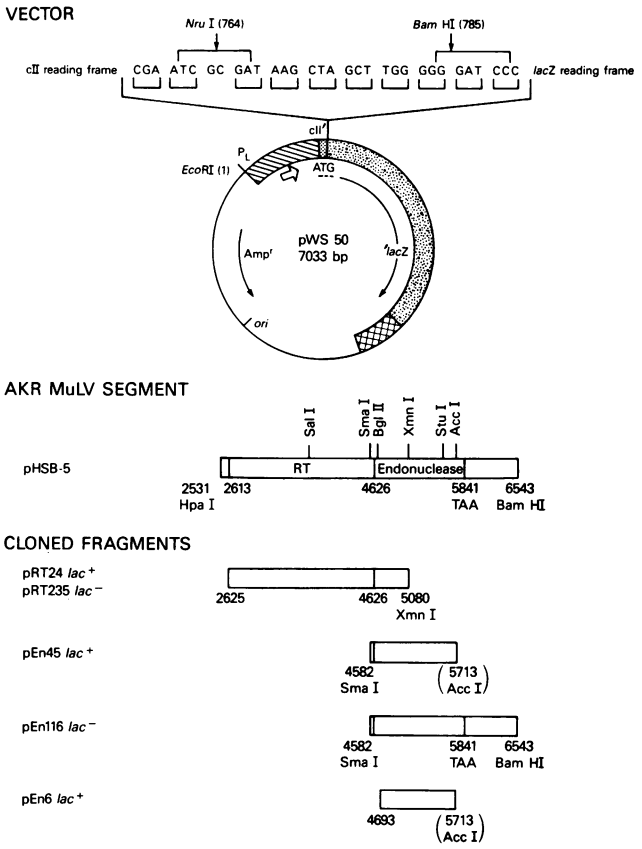

FIG. 1. Expression vector and plasmid clones with AKR MuLV *pol* inserts. A detailed description of the structure of the expression vector pWS50 (Sisk et al., in press) and construction of recombinant clones with AKR MuLV *pol* inserts is described in the text. The sequence illustrated above the diagram of pWS50 shows that the *c*II and *lacZ* reading frames are not in phase in pWS50. The unique *Nru*I cloning site is also indicated. Nucleotide positions of AKR MuLV fragments are numbered as described by Herr (15). Restriction sites used for cloning are indicated above the AKR MuLV segment. The 3′ termini of the pEn45 and pEn6 inserts were not determined exactly and are therefore shown in parentheses. RT, Reverse transcriptase.

*Bam*HI sites with the Klenow DNA polymerase, and screening for white colonies which retained the two *Bam*HI sites in reverse transcriptase but had lost the *Bam*HI site in the vector (Fig. 1), thereby creating a frameshift between reverse transcriptase and *lacZ*. The endonuclease clones pEn45 and pEn116 were constructed as follows. The 1.1-kb *Sma*I-*Acc*I (4582 to 5713) fragment of pSB was isolated, treated with the slow BAL 31 enzyme for 3 min, and then inserted into pWS50. Several *lacZ*$^+$ clones were isolated, and the one termed pEn45 was chosen for future experimentation. The 5′ terminus of the insert retained the nucleotides derived from the *Sma*I site; the exact 3′ terminus was not determined. A *lacZ*$^-$ derivative of pEn45, designated pEn116, was isolated by digesting pEn45 with *Stu*I and *Bam*HI, isolating the resulting large fragment, and then inserting the AKR MuLV 0.98-kb *Stu*I-*Bam*HI (5563 to 6543) fragment from pSB. To construct the pEn6 clone, the 1.0-kb *Bgl*II-*Acc*I (4677 to 5713) fragment of pSB was isolated, treated with the slow BAL 31 enzyme for 10 min, and then inserted into pWS50. pEn6 was one of several *lacZ*$^+$ clones

isolated by this procedure. The 5′ terminus of the insert begins at nucleotide position 4693; the precise 3′ terminus was not determined.

Expression of plasmid clones. Plasmids were transferred from *E. coli* DC519 to *E. coli* DC520 (Sisk et al., in press), which has a *lac* deletion and a prophage containing a temperature-sensitive repressor of the λ p_L promoter. Cells were grown at 32°C in L broth containing 50 μg of ampicillin per ml until the optical density at 600 nm was 0.2. To express genes under the control of the p_L promoter, the cultures were then shifted to 42°C and incubated for another hour.

Preparation of antisera. The pRT24, pEn45, and pEn6 fusion proteins were prepared from 500-ml cultures after induction for 1 h at 42°C. The proteins were separated on preparative SDS-polyacrylamide gels, and after staining with Coomassie blue, the appropriate gel bands were excised and minced in phosphate-buffered saline. Antisera were obtained by emulsifying the gel fragments with an equal volume of Freund adjuvant and injecting the suspension into rabbits intradermally three times at 10-day intervals. The sera were screened by immunoprecipitation (see below) or by Western blot analysis (38).

Immunoprecipitation. For immune precipitation, 5×10^4 to 10×10^4 cpm of each sample was mixed with 5 or 10 μl of antiserum in a final volume of 0.6 ml and incubated for 30 min at room temperature. A slurry of protein A–Sepharose CL-4B (40 μl) containing 1% aprotinin was then added, and incubation was continued for another 30 min. The immune complexes were centrifuged at 4°C in an Eppendorf microcentrifuge. Each pellet was washed five times with 0.5 ml of immunoprecipitation buffer and finally suspended in 50 μl of sample buffer (20).

Immune blocking experiments. DC520 cells transformed by the indicated clones were induced for 1 h at 42°C as described above and then sedimented. The bacterial cell pellets were suspended in 10 mM Tris hydrochloride (pH 7.4)–1% SDS–0.35 M 2-mercaptoethanol, boiled for 5 min, and centrifuged in an Eppendorf microcentrifuge. The supernatant fluid was saved. These extracts (30 μg of protein) were incubated with limiting amounts of the indicated antiserum (0.5 μl of anti-pRT24 and 2.5 μl of anti-pEn45) for 1 h at room temperature; ^{35}S-labeled AKR MuLV, which had been heated at 100°C for 5 min in the presence of 1% SDS–0.35 M 2-mercaptoethanol, was then added and incubation was continued for another 60 min. The rest of the immunoprecipitation procedure, beginning with addition of protein A–Sepharose CL-4B, was carried out as described above.

RESULTS

Bacterial expression of plasmid clones containing AKR MuLV reverse transcriptase and endonuclease gene segments. Our studies on the genetic organization of the MuLV *pol* gene (24) were continued by expressing viral proteins in *E. coli*. Figure 1 illustrates the structure of the expression vector pWS50, which carries the bacteriophage λ p_L promoter and sequences encoding a portion of the λ *c*II protein joined out of frame to the *lacZ* coding region (Sisk et al., in press). Figure 1 also shows the nucleotide boundaries of AKR MuLV *pol* fragments cloned between *c*II and *lacZ*. Initial experiments were performed with clones containing both reverse transcriptase and putative endonuclease coding sequences. For example, clone pRT24 contains virtually the entire reverse transcriptase coding region (12 nucleotides encoding the four N-terminal amino acids are missing) plus

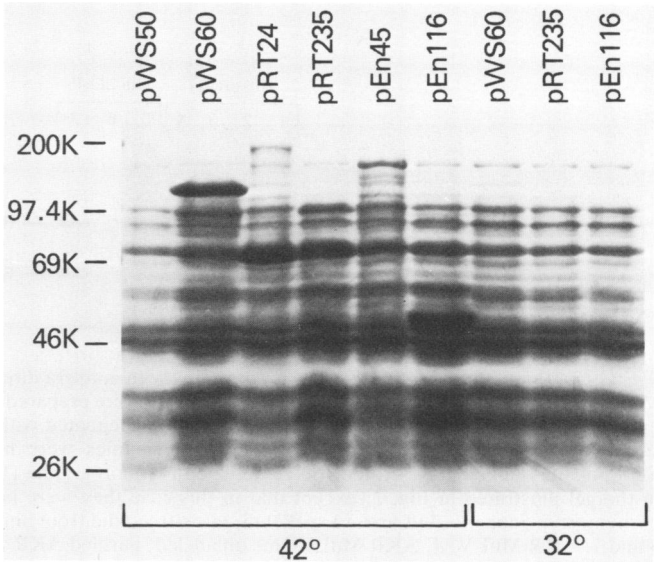

FIG. 2. Gel analysis of proteins expressed in *E. coli* DC520 cells. Plasmid clones were expressed in *E. coli* DC520 (Sisk et al., in press) as described in Materials and Methods. Expression was carried out for 1 h at 32 or 42°C, as indicated. The cells were then spun down, suspended in loading buffer (50 mM Tris hydrochloride [pH 6.8], 0.7 M 2-mercaptoethanol, 4% SDS, 18% glycerol, 0.0025% bromphenol blue), and heated at 100°C for 5 min. Samples (50 μl) were subjected to electrophoresis in a 10% SDS-polyacrylamide gel, and proteins were detected by Coomassie blue staining. The molecular weight markers shown on the left are myosin (200K), phosphorylase B (97.4K), bovine serum albumin (69K), ovalbumin (46K), and α-chymotrypsinogen (26K). The plasmid used is indicated above each lane.

456 nucleotides encoding the 5′ end of endonuclease. Clone pEn45 contains 45 nucleotides encoding the 15 C-terminal amino acids of reverse transcriptase and the adjoining approximately 1,088 base pairs (about 90% of the endonuclease coding region). Derivatives of these two clones having inserts no longer fused to β-galactosidase were also constructed. Thus, the derivative of pRT24, designated clone pRT235, has a frameshift between the 3′ end of the *pol* insert and the *lacZ* coding region. pEn116, derived from pEn45, contains all of the putative endonuclease coding region, including the natural UAA termination codon at the end of the MuLV *pol* open reading frame.

Each of these clones was expressed in *E. coli* DC520 (Sisk et al., in press), a strain possessing a *lac* deletion, as well as a prophage with a temperature-sensitive λ repressor. Induction was carried out at 42°C, the temperature at which the repressor is inactivated and the λ p_L promoter is functional. The clone pWS60 (Sisk et al., in press), which contains the λ *c*II sequence joined directly in frame to the *lacZ* coding region, was included as a positive control. Figure 2 shows the analysis of proteins present in whole-cell extracts on a Coomassie blue-stained polyacrylamide gel. As expected, the same protein pattern was observed for all clones tested at 32°C. In contrast, after incubation at 42°C, pWS60 expressed a large amount of β-galactosidase, and the *pol*-containing clones exhibited new bands not seen at 32°C. Moreover, these new bands were missing from extracts of pWS50 and pWS60 at 42°C. Thus, pRT24 expressed a unique 190-kDa protein. In addition, an approximately 72-kDa protein, which appeared in the gel to be superimposed on an endogenous bacterial protein band, was also present in the extract.

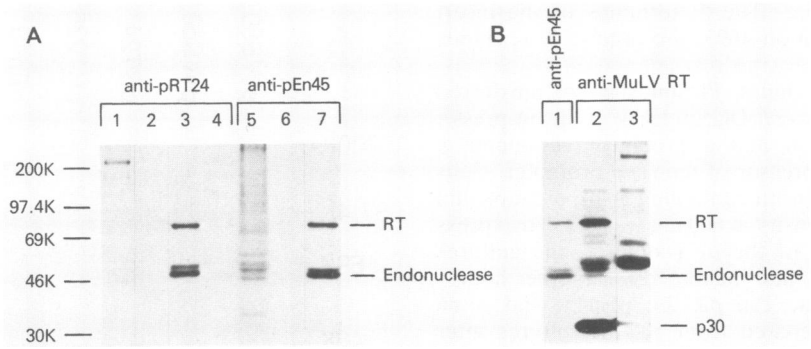

FIG. 3. Immunoprecipitation of MuLV proteins with antisera directed against the pRT24 and pEn45 fusion proteins and partially purified MuLV reverse transcriptase (RT). ^{35}S-labeled samples were prepared and immunoprecipitated as described in Materials and Methods. Where unlabeled virus was added (lane 4), the antiserum was incubated with 90 μg of disrupted AKR MuLV for 30 min prior to addition of labeled sample. After suspension in sample buffer (20), samples were heated at 100°C for 3 min and analyzed by electrophoresis in 10% SDS-polyacrylamide gels. Proteins were detected by fluorography (1). The marker proteins shown on the left were the same as those used in the gel illustrated in Fig. 2, except that in this case they were labeled with ^{14}C, and carbonic anhydrase (30K) was present instead of α-chymotrypsinogen. (A) Lanes: 1, pelleted, virus-free fluid from uninfected SC-1 cells (14); 2, AKR MuLV reacted with preimmune serum; 3 and 7, AKR MuLV; 4, AKR MuLV plus unlabeled, purified AKR MuLV; 5, pelleted, virus-free fluid from uninfected 3T3FL cells (11); 6, AKR MuLV reacted with preimmune serum. (B) Lanes: 1 and 2, AKR MuLV; 3, pelleted, virus-free fluid from uninfected SC-1 cells. The antisera used are indicated on the figure, except for lanes 2 and 6, in A, in which the corresponding preimmune sera were used, as indicated above. The anti-MuLV reverse transcriptase used was obtained from the National Cancer Institute (lot 76S-163). The background bands in lanes 1 and 5 were not competed by unlabeled, purified AKR MuLV.

Extracts of the *lacZ*$^-$ clone, pRT235, contained a 93-kDa product, which migrated slightly ahead of an endogenous 97-kDa band, as well as the 72-kDa protein found in pRT24 extracts. The 72-kDa protein is derived from the N-terminal portion of the larger protein products since it could be detected by Western blot analysis with an antibody directed against the *c*II leader peptide (data not shown). It is also of interest that extracts of pRT235 and another *lacZ*$^-$ derivative of pRT24 (pRT250), which contains a termination codon near the C terminus of reverse transcriptase, express high levels of reverse transcriptase activity. This activity has been extensively purified and has been shown to be inhibited by the immunoglobulin G fraction of anti-MuLV reverse transcriptase (unpublished observations). pEn45 and pEn116 expressed 150- and 49-kDa proteins, respectively. In every case, the molecular mass of the primary protein product was consistent with that predicted by the sequence (8, 15) of the MuLV *pol* insert.

Immunoprecipitation of viral proteins with antisera directed against bacterial fusion proteins containing reverse transcriptase and endonuclease sequences. The ability to obtain high-level expression with this system made it possible to prepare specific antibodies against MuLV *pol* proteins. Large cultures of pRT24 and pEn45 were induced at 42°C, and bands corresponding to the *lacZ* fusion proteins were excised from preparative gels and injected into rabbits. The resulting antisera were tested by immunoprecipitation of ^{35}S-labeled virus followed by polyacrylamide gel electrophoresis (Fig. 3). Antibodies directed against the pRT24 fusion protein reacted with wild-type AKR MuLV to yield three bands (Fig. 3A, lane 3): a prominent band at approximately 80 kDa, which is the expected size of the viral reverse transcriptase (30, 41); a less prominent band at approximately 50 kDa; and another prominent band at approximately 46 kDa. Addition of unlabeled, purified virus to the immunoprecipitation reaction (lane 4) reduced the intensity of these bands to the background level, indicating that they represent virus-related proteins.

Antisera made against the pEn45 fusion protein, which contains amino acid sequences expressed from the 3′ end of *pol*, reacted strongly with the AKR MuLV 46-kDa protein (Fig. 3A, lane 7, and 3B, lane 1). The prominence of the 46-kDa band led us to believe that it represents the viral endonuclease. More evidence for this conclusion is provided below. In addition to the 46-kDa band, the 50- and 80-kDa bands seen with the anti-pRT24 serum (lane 3) were also detected. The finding that the viral reverse transcriptase could be immunoprecipitated with the anti-pEn45 serum was unexpected, suggesting that the 15 C-terminal amino acids of the enzyme may be sufficient to elicit an immune response. The 50-kDa band is discussed below.

Control experiments revealed that these three bands present in AKR virions were missing from culture fluids harvested from uninfected cells (Fig. 3A, lanes 1 and 5) and also from AKR MuLV samples reacted with the corresponding preimmune sera (lanes 2 and 6). Additional results obtained with antisera to partially purified MuLV reverse transcriptase are shown for comparison (Fig. 3B). This antiserum, which contains antibodies directed against the viral polymerase, as well as antibodies to p30 determinants, immunoprecipitated both the AKR 80-kDa and p30 proteins (lane 2). Interestingly, a distinct AKR band of approximately 46 kDa (lane 2) comigrating with the endonuclease band in lane 1 was also immunoprecipitated by this serum but, in agreement with the data shown in panel A, was missing from control fluids (lane 3).

Further evidence of antibody specificity was obtained by testing the abilities of various bacterial extracts to block immunoprecipitation of AKR reverse transcriptase and endonuclease by antisera to the *pol* fusion proteins. Preabsorption of anti-pRT24 with extracts of clone pRT24 (Fig. 4A, lane 2) blocked the reactivity of this antiserum. The analogous experiment with anti-pEn45 and extracts of clone pEn45 (Fig. 4B, lane 3) gave the same type of result. Similar results were also obtained if the *lacZ*$^-$ clones pRT235 and pEn116 were used (data not shown). However, when anti-pRT24 was preabsorbed with an extract of clone pEn45 (Fig. 4A, lane 3), the viral 80-kDa band was partially eliminated, whereas only trace amounts of the 46-kDa band could be seen. This observation indicates that the sequence at the 3′

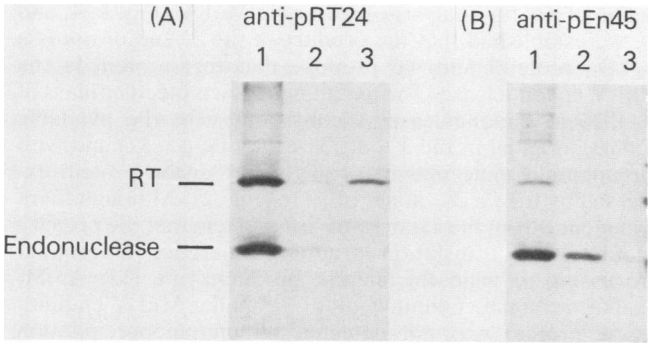

FIG. 4. Ability of bacterial extracts to block immunoprecipitation of MuLV reverse transcriptase (RT) and endonuclease. Antibodies to the pRT24 (A) and pEn45 (B) fusion proteins were incubated with bacterial blocking extracts prior to reaction with ^{35}S-labeled AKR MuLV, as described in Materials and Methods. The addition of blocking extracts was as follows (lanes): 1, no addition; 2, pRT24 extract; 3, pEn45 extract.

end of *pol*, the putative endonuclease coding region (2, 5, 19, 24, 36, 37), specifies a 46-kDa protein. Similarly, preabsorption of anti-pEn45 with extracts of clone pRT24 led to a partial reduction in the intensity of the 46-kDa band and complete elimination of the 80-kDa reverse transcriptase band. This finding shows that the *pol* insert in clone pRT24 contains sequences primarily encoding the reverse transcriptase protein and only a portion of the 46-kDa protein.

Immune reaction of MuLV proteins after exposure of detergent-disrupted virions to dissociating and nondissociating conditions. The preceding results describe antisera generated against the pRT24 and pEn45 bacterial fusion proteins; in each case, these antisera react with reverse transcriptase and endonuclease determinants. To facilitate further characterization of the MuLV endonuclease, we also prepared an antiserum directed against endonuclease alone. The *lacZ*$'$ clone, pEn6 (Fig. 1), was isolated for this purpose. This clone contains most of the endonuclease sequence, but is missing 68 nucleotides at the 5$'$ terminus and, like pEn45, about 10% of the 3$'$-terminal sequence. Immunoprecipitation of MuLV with antisera directed against the pEn6 fusion protein unexpectedly resulted in the appearance of three major bands in the gel: the 46-kDa endonuclease band as well as the reverse transcriptase and 50-kDa bands (Fig. 5A, lane 1). Since the anti-pEn6 serum should react only with endonuclease and not with any other MuLV protein, this finding suggested the possibility that endonuclease is bound to reverse transcriptase (and the 50-kDa protein) in a complex, which is not dissociated in the usual immunoprecipitation buffer (20 mM Tris hydrochloride [pH 7.4], 50 mM NaCl, 0.5% each Nonidet P-40 and deoxycholate) containing 0.1% SDS.

To investigate this possibility, detergent-disrupted MuLV was heated at 100°C for 5 min in the presence of added 1% SDS and 0.35 M 2-mercaptoethanol, diluted at least 10-fold in immunoprecipitation buffer, and reacted with the antiserum. Gel analysis of the immune complex (Fig. 5A, lane 2) showed a prominent 46-kDa band and only a faint trace of the 80-kDa reverse transcriptase band. No bands were detected after reaction of treated virus with preimmune serum (lane 3). These results are consistent with the idea that virus particles contain a reverse transcriptase-endonuclease complex which is almost completely dissociated by treatment with SDS and a sulfhydryl reducing agent. Under dissociating conditions, the anti-pEn6 serum exhibits monospecific anti-endonuclease activity.

As expected, when MuLV was reacted with anti-pEn45 serum after exposure to either nondissociating (Fig. 5A, lane 4) or dissociating (lane 5) conditions, both the endonuclease and reverse transcriptase bands were observed. However, the intensity of the reverse transcriptase band was reduced about fivefold by the dissociating treatment (compare lanes 4 and 5). A weak immune reaction is consistent with the rather small representation of reverse transcriptase sequences in the pEn45 fusion protein and with the results presented in Fig. 4. The 50-kDa band was not detected at all under dissociating conditions (lane 5). Lane 6 shows that the reverse transcriptase, endonuclease, and 50-kDa bands were each present in the gel when MuLV was exposed to dissociating conditions prior to reaction with anti-pRT24; as

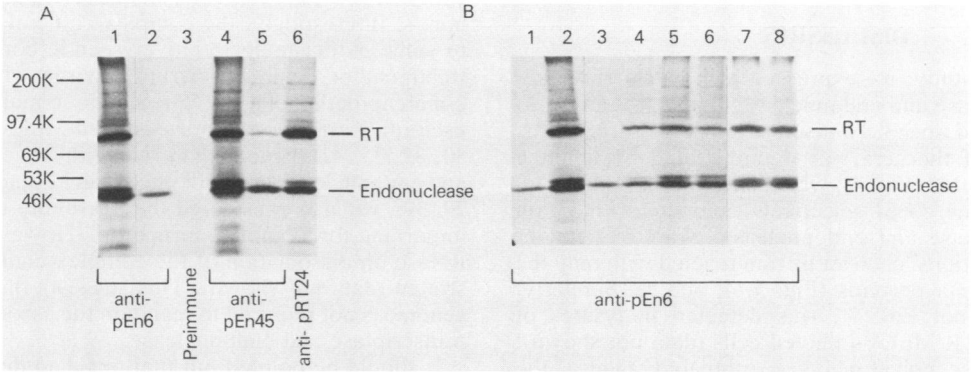

FIG. 5. Dissociation of MuLV reverse transcriptase (RT)-endonuclease complex. (A) ^{35}S-labeled AKR MuLV was disrupted in immunoprecipitation buffer–0.1% SDS with no further treatment (lanes 1 and 4) or, in addition, heated at 100°C for 5 min in the presence of 1% SDS–0.35 M 2-mercaptoethanol (lanes 2, 3, 5, and 6). The virus was then diluted at least 10-fold in immunoprecipitation buffer and incubated with the antiserum indicated below each lane. The preimmune serum was taken from the rabbit, which was subsequently injected with the pEn6 fusion protein. (B) Antiserum directed against the pEn6 fusion protein was reacted with ^{35}S-labeled AKR MuLV, which had been treated as follows (lanes): 1, heated at 100°C for 5 min in the presence of 1% SDS–0.35 M 2-mercaptoethanol; 2, no treatment; 3, 0.35 M 2-mercaptoethanol–1% SDS, no heat; 4, 0.035 M 2-mercaptoethanol–1% SDS, no heat; 5, 2 M NaCl–0.35 M 2-mercaptoethanol; 6, 2 M NaCl–0.035 M 2-mercaptoethanol; 7, heated at 100°C for 5 min in the presence of 1% SDS; 8, 0.35 M 2-mercaptoethanol, no heat. Immunoprecipitation was performed as described in Materials and Methods. The molecular weight markers were the same as those in Fig. 3, with the addition of the 53K human γ-globulin protein.

expected, the reverse transcriptase band was the most prominent. The finding that the 50-kDa protein was immunoprecipitated with anti-pRT24 and not by either anti-pEn45 or anti-pEn6 after exposure to dissociating conditions suggests that this protein is related to reverse transcriptase and may represent a degradation or specific cleavage product of the enzyme. Not surprisingly, in the reaction with antibodies to the partially purified viral reverse transcriptase, dissociating conditions prevented the appearance of the endonuclease band in Fig. 3; however, the reverse transcriptase and p30 bands were still readily detected in the gel (data not shown).

To further explore conditions which lead to dissociation of the reverse transcriptase-endonuclease complex, AKR MuLV was subjected to various treatments and then immunoprecipitated with antiserum to the pEn6 fusion protein (Fig. 5B). Densitometry scans were performed to verify the visual observations. Results obtained under the previously demonstrated dissociating (lane 1) and nondissociating (lane 2) conditions are shown for comparison. Lane 3 shows that treatment with SDS–0.35 M 2-mercaptoethanol at room temperature led to a 10- to 15-fold reduction in the intensity of the reverse transcriptase and 50-kDa bands, an effect almost as great as that seen at 100°C (lane 1). Thus, high temperature is not needed to disrupt the complex. Lowering the concentration of 2-mercaptoethanol to 0.035 M (lane 4) gave results more closely resembling those obtained under nondissociating conditions, although some dissociation did occur (compare with lane 2). When the virus was heated at 100°C with 1% SDS alone (lane 7) or treated with 2-mercaptoethanol at room temperature in the absence of SDS (lane 8), the complex remained intact. In addition, 6 M urea, RNase (50 μg/ml), or 2 M NaCl did not dissociate the complex (data not shown). Treatment with 2 M NaCl in the presence of 0.35 M (lane 5) or 0.035 M (lane 6) 2-mercaptoethanol had a small but noticeable effect on dissociation of the complex. The results of this series of experiments strongly suggest that disulfide bonds are involved in the observed association of MuLV reverse transcriptase and endonuclease. Addition of SDS and, to a lesser extent, high salt can potentiate the activity of the sulfhydryl reducing agent in vitro.

DISCUSSION

In the present study, we expressed high levels of MuLV reverse transcriptase and endonuclease fusion proteins in *E. coli* and generated specific antisera against these proteins. The specificity of the sera was demonstrated by immune blocking experiments (Fig. 4), which showed that the bacterial fusion proteins could selectively compete with virion reverse transcriptase and endonuclease. Moreover, under appropriate conditions, each antiserum reacted with only the expected MuLV *pol* proteins (Fig. 3, 4, and 5). Similarly, $Pr200^{gag\text{-}pol}$, but not $Pr65^{gag}$, was detected in lysates of pulse-labeled, AKR MuLV-infected cells (data not shown). In contrast, other polyclonal sera prepared against the partially purified reverse transcriptase enzyme were found to contain high levels of antibodies directed against p30 *gag* determinants (Fig. 3) (3, 10). It should also be noted that, prior to this report, an anti-MuLV endonuclease antiserum (Fig. 5) was not available.

Earlier studies from a number of different laboratories indicated that the 3′ end of the MuLV *pol* gene encodes endonuclease (2, 5, 19, 24, 36, 37). Using antisera made against bacterial proteins expressing sequences from this region of *pol* for analysis of wild-type MuLV (Fig. 3, 4, and 5), we established that the product of the 3′ end of *pol* is a 46-kDa protein, and we propose that this protein is the MuLV endonuclease. The assignment of a molecular mass of 46 kDa to endonuclease is consistent with the available coding potential in the 3′ portion of *pol* (8, 15, 37) and with the apparent molecular mass of the *c*II fusion protein expressed by the *lacZ*$^-$ clone, pEn116 (Fig. 2). Although there is evidence from the avian retrovirus system that the primary endonuclease translation product undergoes C-terminal processing to yield the mature pp32 enzyme (12; A. M. Skalka, personal communication), a smaller MuLV endonuclease protein was not detected by immunoprecipitation (Fig. 3, 4, and 5) or Western blot analysis (data not shown) of wild-type virus. In addition, preliminary studies on partially purified fractions of MuLV endonuclease indicate that the enzymatically active protein has a molecular mass of 46 to 47 kDa; this protein reacts with antisera directed against the pEn45 fusion protein (R. Furkes and W. K. Yang, personal communication). Thus, the data presented in this report complement the genetic results obtained with integration-defective MuLV mutants (5, 36) and suggest that the coordinates for endonuclease extend from sequences immediately 3′ of the reverse transcriptase coding region to the UAA termination codon at the 3′ terminus of the *pol* gene.

In addition to providing information on the product of the endonuclease coding region, the availability of an anti-endonuclease serum (anti-pEn6) led to an unanticipated finding, viz., that reverse transcriptase and endonuclease can be isolated as a complex involving one or more disulfide bonds. Thus, reaction of detergent-disrupted MuLV with this serum resulted in precipitation of endonuclease as well as reverse transcriptase and the reverse transcriptase-related 50-kDa protein; however, a single prominent band corresponding to endonuclease was observed if the virus was treated with SDS and 2-mercaptoethanol prior to immunoprecipitation (Fig. 5).

The conclusion that a disulfide linkage is a feature of the reverse transcriptase-endonuclease complex is based on the finding that treatment with SDS alone leaves the complex intact (Fig. 5). Treatment with 2-mercaptoethanol alone also fails to dissociate the complex. These results imply that the complex is held together by noncovalent as well as disulfide bonds. Interestingly, the complex could also be dissociated to some extent under relatively gentle conditions, e.g., by treatment of detergent-disrupted virus with high salt and 2-mercaptoethanol (Fig. 5). Similar conditions have been used for purification of unassociated MuLV *pol* proteins (9, 30, 32, 35, 41). Since avian retroviral reverse transcriptase and endonuclease are both nucleic acid-binding proteins (17, 29, 39), we also considered the possibility that viral RNA is important for complex formation. However, an effect of RNase on dissociation of the complex could not be demonstrated (data not shown). This suggests that the viral RNA genome is not required to maintain the association of reverse transcriptase and endonuclease.

It should be pointed out that our data do not exclude the possibility that some or even all of the reverse transcriptase, endonuclease, or both exist as uncoupled molecules in the virus particle and not as part of a covalently linked complex. Nevertheless, specific coprecipitation of endonuclease and reverse transcriptase by the monospecific anti-endonuclease serum indicates that these two proteins are located within close proximity in the virus core. Experiments are currently under way to define the nature of the association between reverse transcriptase and endonuclease.

The biological significance of the complex is not known at this time. It is interesting, however, that a portion of the major envelope proteins of MuLV, gp70, and Pr15E (formerly designated p15E), is found in virions as a complex linked by disulfide bonds (22, 31, 34, 42). If reverse transcriptase and endonuclease are linked within the virion and remain bonded to each other after the virus is uncoated, such a complex might be important in regulation and stabilization of the viral replicative and integrative functions. Thus, the existence of a complex might prevent the presence of free enzymes in the cytoplasm and nucleus, where they could interfere with normal cell activities. Participation in specific catalytic reactions could also be involved. For example, reverse transcriptase might play a role in directing the specificity of endonuclease-catalyzed cleavage at the long terminal repeat junction (6, 7, 12) of circular viral DNA. In addition, a complex would contribute to increased catalytic efficiency of the individual enzymes. Finally, since reverse transcriptase and endonuclease are cleaved from a common precursor (18, 19), having the mature proteins linked may permit more efficient packaging within the virus core.

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