JOURNAL OF VIROLOGY, Feb. 1988, p. 528–533
0022-538X/88/020528-06$02.00/0

Vol. 62, No. 2

A C-Terminal Domain in the Avian Sarcoma-Leukosis Virus *pol* Gene Product Is Not Essential for Viral Replication

RICHARD A. KATZ† AND ANNA MARIE SKALKA†*

Department of Molecular Oncology, Roche Institute of Molecular Biology, Roche Research Center, Nutley, New Jersey 07110

Received 5 June 1987/Accepted 16 October 1987

The virion proteins encoded by the avian retroviral *pol* gene (reverse transcriptase and endonuclease) are formed by the proteolytic processing of a *gag-pol* fusion protein precursor. Recent studies have predicted that the avian sarcoma-leukosis virus *pol* precursor protein undergoes a previously undetected processing event resulting in the formation of common C termini for the endonuclease (pp32) and the β subunit of reverse transcriptase (F. Alexander, J. Leis, D. A. Soltis, R. M. Crowl, W. Danho, M. S. Poonian, Y.-C. E. Pan, and A. M. Skalka, J. Virol. 61:534–542, 1987; D. Grandgenett, T. Quinn, P. J. Hippenmeyer, and S. Oroszlan, J. Biol. Chem. 260:8243–8249, 1985). This processing event removes 37 amino acids, thus defining a new *pol* domain. In this report, we present evidence that this C-terminal domain is translated as part of the *gag-pol* precursor but is not required for replication of the virus in tissue culture cells.

The primary translation product of the retroviral *pol* gene is a large *gag-pol* fusion protein precursor (30). Biochemical (22) and genetic (4, 16) studies have demonstrated that the mature *pol* gene products found in virions (reverse transcriptase and endonuclease) are formed by proteolytic cleavage catalyzed by a virus-encoded protease. The avian sarcoma-leukosis virus (ASLV) primary *pol* translation product, $Pr180^{gag\text{-}pol}$ (24), is processed to form the α and β subunits (63 and 95 kilodaltons [kDa], respectively) of reverse transcriptase and the pp32 endonuclease (32 kDa) (Fig. 1). The endonuclease is thought to play a role in the integration of retroviral DNA into host cell DNA (2, 9). The α and pp32 domains comprise the amino two-thirds and the carboxyl one-third of β, respectively. Thus, a single precursor molecule can give rise to β or to α and pp32, depending on whether the processing site within the β domain is used. Domains which are structurally (and in some cases functionally) analogous to the α and pp32 domains are encoded in the *pol* genes of retroviruses isolated from a wide variety of species (12). However, the proteolytic processing patterns appear to differ. For example, in Moloney murine leukemia virus, a major large-virion *pol* protein analogous to the β subunit of ASLV has not been identified, indicating that processing to separate the putative endonuclease domain is complete (10, 27). In human immunodeficiency virus, two reverse transcriptase-related products (64 and 51 kDa) with common N termini have been identified in addition to a putative endonuclease fragment of 34 kDa (19). The functional significance of these varied *pol* processing patterns is unknown.

Recent structural and proteolytic processing studies of bacterially produced ASLV *pol* proteins revealed that the *pol* precursor undergoes a previously undetected C-terminal processing event (Fig. 1) (1). Alignment of the C-terminal protein sequence of pp32 from avian myeloblastosis virus with the nucleotide sequence of the closely related Prague C (PR-C) strain of avian sarcoma virus (ASV) indicates that a C-terminal *pol* domain of 37 amino acids (ca. 4.1 kDa) is removed (8). The cleavage is predicted to occur between *pol* amino acids 858 and 859 (alanine and glycine). Interestingly, this glycine codon also encodes the splice acceptor site for *env* mRNA (see Fig. 3A) (6). Therefore, the 4.1-kDa C-terminal *pol* domain is encoded entirely within the overlap with the *env* gene (+1 translational reading frame) (Fig. 1). The *env* sequences within the overlap region encode most of the 62-amino-acid leader peptide that is removed from the $Pr95^{env}$ glycoprotein precursor by cellular signal peptidase during the process of membrane insertion (31).

In this study, we have evaluated the function of this region of the *pol* gene by constructing and characterizing two viral mutants in which the *pol* translational reading frame is interrupted with termination codons at or near the C-terminal processing site. This strategy would determine whether the 4.1-kDa C-terminal domain performs as essential function as part of the *gag-pol* precursor or possibly as a free peptide. The results indicate that the 4.1-kDa C-terminal *pol* domain is normally synthesized as part of the $Pr180^{gag\text{-}pol}$ precursor as predicted but, surprisingly, is not essential for viral replication.

MATERIALS AND METHODS

Construction of mutant viral clones. An infectious DNA clone of the Schmidt-Ruppin B (SR-B) strain of ASV, pLD6 (15), was used as a substrate for mutagenesis. The oligonucleotide-directed mutagenesis method (5, 23) was used to introduce a termination codon after *pol* amino acid 866 (see Fig. 3A). The mutagenic oligonucleotide was 5'GACTGGA TACCCGGGTAAGACGAGCAA3'. A 259-base-pair *Kpn*I-*Xho*I fragment, which included the mutated C-terminal *pol* domain, was inserted into the wild-type clone (pLD6) to create pLD6T by using standard techniques (20). The presence of the mutation was confirmed by nucleotide sequencing (21). The derivation of pLD6IS-1 will be described more fully elsewhere (R. A. Katz, M. Kotler, and A. M. Skalka, submitted for publication). Briefly, a synthetic DNA fragment was introduced into pLD6, which created the reading frame for the mature C terminus of pp32 and preserved the *env* splice acceptor site. This insert affected replication in *cis*, but a stable derivative which contained a point mutation within the insert could be isolated. The derivative replicated

* Corresponding author.
† Present address: Institute for Cancer Research, Fox Chase Cancer Center, 7701 Burholme Ave., Philadelphia, PA 19111.

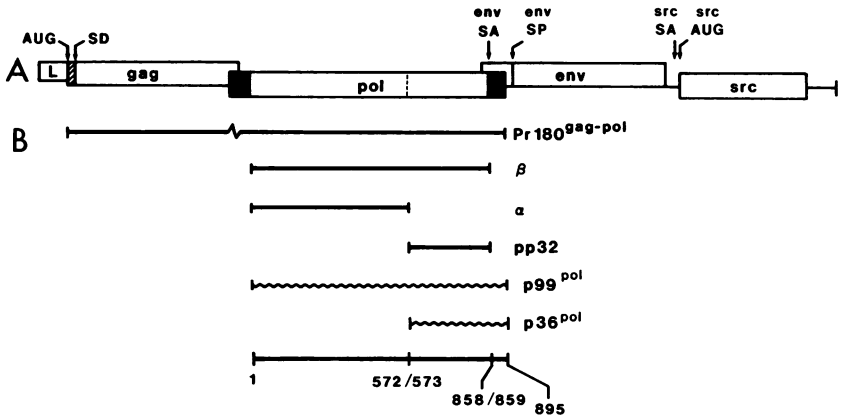

FIG. 1. Structure of the ASLV genome and *pol*-related products. (A) Map of the ASV genome depicting translational reading frames as blocks (not drawn to scale). The mRNA leader (L) is alternatively spliced to form *env* and *src* subgenomic mRNAs. SD, Splice donor site; SA, splice acceptor site; SP, *env* signal peptidase cleavage site. The positions of the translational initiation codons are indicated (AUG). Symbols: ▨, Region encoding the six-amino-acid peptide which is present in *gag* and *env* primary translation products (6); ■, translated *pol* regions which are proteolytically removed to form the major mature products (α, β, and pp32) found in the virion (not drawn to scale). (B) Major *pol* gene products, including the $Pr180^{gag\text{-}pol}$ precursor. The region in which ribosomal frame shifting occurs (11) to generate the *gag-pol* fusion protein is indicated by a break in the line. The bacterially produced *pol* proteins $p36^{pol}$ and $p99^{pol}$ (1) containing the 4.1-kDa C-terminal *pol* domain are shown as wavy lines. The amino acid positions of *pol* are indicated below; a threonine identified as the N-terminal amino acid of β is designated position 1 (3). Position 895 denotes the last amino acid encoded in the *pol* reading frame. Position 858 marks the C-terminal alanine residue of pp32 (see Fig. 3A) (8). The proteolytic cleavage between amino acids 572 and 573 which generates the N terminus of pp32 is indicated (8) although the C-terminal amino acid of α has not been identified.

like the wild-type virus. The *Kpn*I-*Xho*I cassette fragment from this virus was sequenced and cloned into pLD6; the resulting clone, pLD6IS-1, also gave rise to a virus that replicated like the wild type after transfection. The sequence of the altered region in pLD6IS-1 is shown in lower-case letters in Fig. 3A.

Transfection of chicken embryo fibroblasts. Chicken embryo fibroblasts (CEFs) (gs^- chf^-) were obtained as a suspension from SPAFAS, Inc., Norwich, Conn. Line 0-embryonated eggs were kindly provided by E. J. Smith of the U.S. Department of Agriculture Regional Poultry Research Laboratory, East Lansing, Mich. Viral DNA was introduced into CEFs by the DEAE-dextran-mediated transfection method which was modified as described previously (14). pLD6 and derivatives were cleaved with *Sal*I to release the pBR322 vector before transfection. The appearance of virus in the culture media was monitored by a reverse transcriptase assay (7).

Analysis of intracellular viral proteins. At 9 days after transfection with wild-type or mutant viral DNA clones, infected-cell monolayers were lysed directly with protein gel loading buffer (18). Samples were applied to sodium dodecyl sulfate–7.5% polyacrylamide gels (18), and proteins were transferred to nitrocellulose paper by using a semidry electroblot apparatus (Sartorius Filters, Inc., Hayward, Calif.) (17) under the conditions described by the supplier (Vangard International, Neptune, N.J.). To detect *pol* proteins, rabbit antiserum directed against a synthetic *pol* peptide (corresponding to amino acids 840 to 854 in the pp32 domain) (see Fig. 6) was used as previously described (1). The *gag*-containing *pol* precursors were detected with rabbit antiserum directed against purified viral $p27^{gag}$ protein which was kindly provided by V. Vogt. The bound antibody molecules were detected by sequentially reacting the paper with goat anti-rabbit and rabbit anti-goat immunoglobulin G (Cooper Biomedical, West Chester, Pa.) followed by incubation with ^{125}I-labeled proteins A or G (Amersham Corp., Arlington Heights, Ill.) as described previously (1).

RESULTS

Nucleotide sequence analysis of the SR-B ASV region encoding the C-terminal *pol* peptide. Figure 1 shows the major ASLV *pol*-related products found in virions and infected cells. When the predicted translational reading frames of two of these products (β and pp32) were expressed in *Escherichia coli*, these products migrated more slowly than the mature virion proteins did, due to additional C-terminal amino acids that could be removed in vitro by the virus-encoded protease p15 (1, 29) (Fig. 1). We determined the nucleotide sequence of this C-terminal *pol* domain in an infectious SR-B ASV clone, pLD6 (15), which was used as a substrate for the mutagenesis experiments described below. For sequencing, we chose a 259-base-pair *Kpn*I-*Xho*I fragment which included the 3′ end of the *pol* gene (Fig. 2). This fragment served as a cassette which could be mutated and then inserted into our *E. coli*-based expression clone (1) or into an infectious viral clone. This allowed us to test the effect of mutations on the structure of the translation products or on viral growth, respectively. We note that there are eight nucleotide differences within this cassette, compared with the published sequence of the PR-C strain (25). Six of the eight nucleotide differences occur between nucleotides 5077 and 5187, the positions which delineate the borders of the 37-amino-acid (4.1-kDa) C-terminal *pol* domain coding region. Five of the six changes affect the predicted *pol* amino acid sequence (Fig. 2; see Discussion).

Mutations which prevent synthesis of most or all of the C-terminal *pol* domain. Two viral mutants were analyzed. In the first mutant, pLD6T, a termination codon was introduced downstream from the proteolytic processing site which separates the C-terminal 4.1-kDa domain from the pp32 and β domains of *pol*. The codon for a glycine residue (Fig. 3A, amino acid position 867) located nine amino acids downstream of the *pol* C-terminal cleavage site was changed to an ochre termination codon (GGA to TAA). This position corresponds to a glutamic acid residue (GAA codon) in the

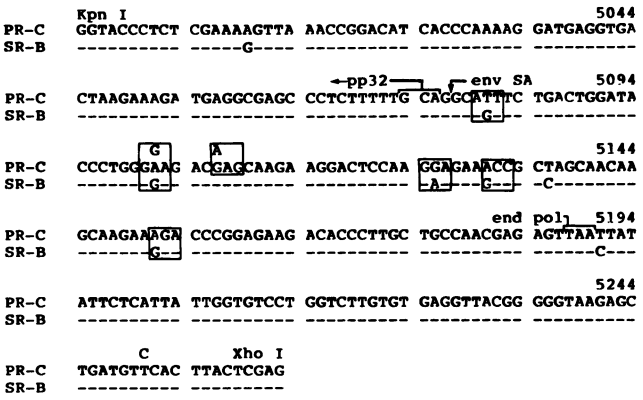

FIG. 2. Nucleotide sequence of the SR-B *Kpn*I-*Xho*I fragment that contains the coding region for the C-terminal *pol* peptide. The numbering of nucleotides corresponds to that of the PR-C sequence (25). Codons that are boxed contain nucleotide differences which are predicted to result in the amino acid changes shown in Fig. 6. PR-C, Sequence of pATV-8 (13, 25); SR-B, sequence of pLD6 (15); ----, identical nucleotides. The *env* splice acceptor site is indicated (SA). Nucleotides above the Pr-C sequence line (positions 5102, 5107, and 5251) denote alternative nucleotides identified in different Pr-C isolates (25).

PR-C strain at nucleotide positions 5101 to 5103 (Fig. 2 and 3A; see Fig. 6). The mutation which creates the termination codon in *pol* alters one codon in the *env* translational reading frame (+1 frame); a glutamic acid is changed to a lysine. This alteration in the *env* leader peptide is not expected to affect *env* function since either a glutamic acid or a lysine residue is found at this position in different isolates of PR-C (25). The codon for the tryptophan residue 866 (TGG) was altered (TGG to GGG) to create a *Sma*I restriction site (5′CC CGGG3′). This resulted in a tryptophan-to-glycine substitution of what would be the C-terminal amino acid (position 866) of the truncated Pr180$^{gag\text{-}pol}$ precursor. The second mutant, pLD6IS-1, was obtained by extended tissue culture passage of a virus that included an insertion in *pol* upstream of the 4.1-kDa domain. Details of the original construction will be provided elsewhere (Katz et al., submitted). The sequence of pLD6IS-1 in the region of the mutation is given in Fig. 3A. The net effect of the mutation is to separate the end of the pp32 and β reading frames from the *env* sequences and to precisely eliminate the reading frame for the C-terminal 4.1-kDa domain of *pol*.

In both cases, the *Kpn*I-*Xho*I fragments that contained the mutated 4.1-kDa regions were assembled into an otherwise wild-type infectious ASV genome (pLD6), so that the only differences were in the region of interest. To determine if the introduced termination codons functioned, we also inserted the mutated *Kpn*I-*Xho*I fragments into our *E. coli pol*-endonuclease expression vector pFA3-RT36 (1). This vector normally directs the expression of a 36-kDa product that contains the 4.1-kDa C-terminal domain (Fig. 1). Insertion of the mutated *pol* C-terminal coding regions corresponding to pLD6T and pLD6IS-1 resulted in the synthesis of the expected truncated proteins in *E. coli* (Fig. 3B). The endonuclease product containing the C terminus encoded in the pLD6T fragment migrated slightly slower than the equivalent protein containing the pp32 C terminus did (pLD6IS-1) (Fig. 3B, lanes 2 and 3), as expected from the eight-amino-acid difference in their length. Thus, the introduced termination codons were functional.

Effect of truncation or elimination of the 4.1-kDa C-terminal *pol* segment on viral growth. The two viral mutants, pLD6IS-1 and pLD6T, were tested for infectivity by trans-

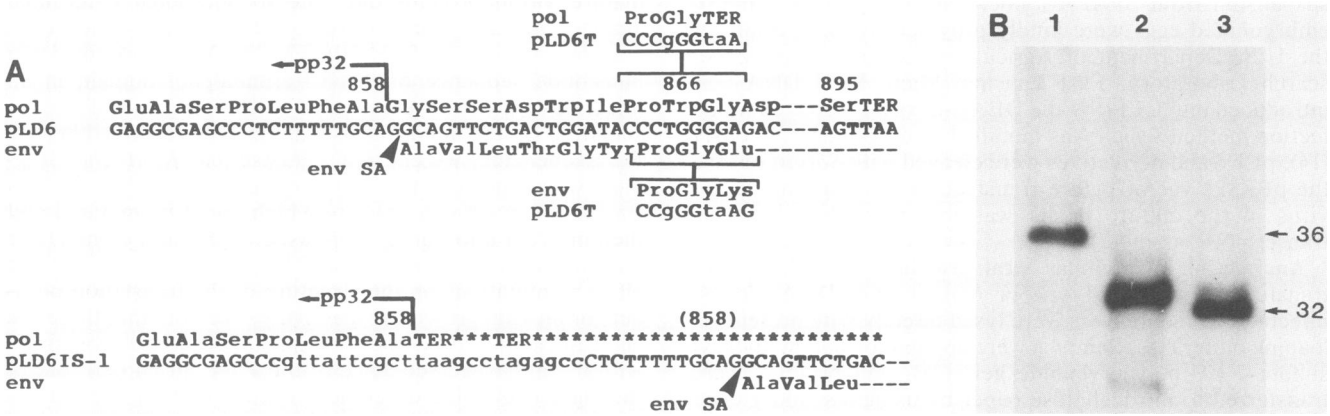

FIG. 3. Nucleotide sequence of mutations in the C-terminal *pol* region and analysis of corresponding bacterially produced endonuclease products. (A) Nucleotide sequences of pLD6 (wild type) and pLD6T and pLD6IS-1 mutants. In the pLD6 (wild-type) sequence, a termination codon (TER) was introduced after *pol* amino acid 866 by using oligonucleotide-directed mutagenesis to generate pLD6T (see Materials and Methods). Nucleotide changes are indicated by lower-case letters. The resulting alterations in the predicted amino acid sequences of *pol* and *env* are shown. In the sequence of the pLD6IS-1 mutant, an insert (lower-case letters) generated a reading frame for the C terminus of pp32 while preserving the *env* splice acceptor site (SA) motif. The pLD6IS-1 sequence shown is that of a mutant provirus containing the insert, which was molecularly cloned after multiple cycles of infection of CEFs, demonstrating that the complete *pol* reading frame was not restored by reversion mutations. *, Untranslated regions; ---, continuation of sequence; (858), GCA triplet that encoded the C-terminal amino acid of pp32 in the pLD6 (wild type) sequence. (B) Immunoblot analysis of *pol* endonuclease products synthesized in *E. coli*. An *E. coli* expression clone (pFA3-RT36) (1) which directs the synthesis of p36^{pol} (Fig. 1) was altered by replacing the *Kpn*I-*Xho*I cassette (Fig. 2) with mutant cassettes to generate *pol* reading frames that terminate after positions 866 (pLD6T) or 858 (pLD6IS-1). The products were identified by using goat antisera directed against avian myeloblastosis virus reverse transcriptase (1). Lysates of induced bacteria were fractionated on a sodium dodecyl sulfate–10% polyacrylamide gel as described previously (1). Lanes: 1, lysate from pFA3-RT36 (producing p36^{pol}); 2, pFA3-RT36 containing the pLD6T *Kpn*I-*Xho*I cassette; 3, pFA3-RT36 containing the *Kpn*I-*Xho*I cassette encoding the pp32 C terminus corresponding to pLD6IS-1. The positions of the 36- and 32-kDa products are indicated.

fection of CEFs. Transfection with either mutant clone produced progeny virus which was able to efficiently infect cells in the culture, as indicated by the presence of high levels of virion-associated reverse transcriptase activity in the culture media at 7 days posttransfection. The kinetics of virus appearance of the mutants was indistinguishable from that of the wild-type parent virus pLD6. Data for pLD6T are shown in Fig. 4. In these tests, CEFs prepared from SPAFAS or line 0 embryos were used. Line 0 fibroblasts lack endogenous viral genomes that are highly related to exogenous retroviruses and, therefore, presumably cannot supply homologous sequences that could recombine with the mutant to form a replication-competent retrovirus (26). The kinetics of the appearance of progeny virus was the same in both transfected CEF cultures. Since both mutant and wild-type viral clones contained the *src* gene, culture supernatants could also be assayed for the presence of focus-forming virus. At 8 days posttransfection, both wild-type and mutant culture supernatants contained approximately 6×10^5 FFU/ml.

Analysis of intracellular mutant *pol* translation products. To verify that the 4.1-kDa *pol* domain was translated in the wild-type virus but not in the mutant clones, we analyzed the proteins produced after transfection and subsequent virus spread. CEFs were transfected with the wild-type parent and the pLD6T and pLD6IS-1 mutants. After 9 days, when the cultures were morphologically transformed by viral *src* expression, total cell lysates were prepared and analyzed by immunoblotting (Fig. 5). With peptide-specific rabbit antiserum directed against the *pol* pp32 domain (1), the expected specific bands of approximately 180 and 95 kDa ($Pr180^{gag\text{-}pol}$ and β, respectively) were detected (Fig. 5A). $Pr180^{gag\text{-}pol}$ precursors synthesized from pLD6T and pLD6IS-1 had increased mobilities compared with that of the wild-type protein. The *gag-pol* precursors of pLD6T and pLD6IS-1 are predicted to be 29 and 37 amino acids shorter, respectively, than that of the wild type. This represents a rather small change in size from the ca. 1,600 amino acids present in the *gag-pol* precursor, and it is possible that the differences in the gel migration rates reflect conformational or modification differences between wild-type and mutant proteins as well as size differences.

The processed β subunits (95 kDa) from the mutant and wild-type infected cultures comigrated (Fig. 5A), as did the pp32 endonuclease products (not shown). Therefore, as predicted, the structures of the mature *pol* products (β and pp32) are not affected by mutations in the C-terminal 4.1-kDa reading frame. The results of a parallel immunoblot in which rabbit antiserum directed against the $p27^{gag}$ protein was used are shown (Fig. 5B). This antiserum detected the $Pr180^{gag\text{-}pol}$ and $Pr76^{gag}$ precursors. As with the results of the *pol* antiserum analysis, the $Pr180^{gag\text{-}pol}$ precursor in the mutant-infected cells had increased mobility compared with that of the wild type, while the $Pr76^{gag}$ precursors from all three sources comigrated. These results confirm that the bands of ca. 180 kDa detected by antiserum directed against pp32 (Fig. 5A) correspond to $Pr180^{gag\text{-}pol}$. As expected, the mutations did not affect the mobility of the *gag* precursor, $Pr76^{gag}$, which corresponds to the N-terminal one-half of the $Pr180^{gag\text{-}pol}$ and is translated independently (11). The results shown in Fig. 5 localize the regions which account for the mobility differences of the $Pr180^{gag\text{-}pol}$ products of pLD6T and pLD6IS-1 to the C-terminal 4.1-kDa domain. From these results, we conclude that the mutant viral clones direct the synthesis of truncated *gag-pol* precursors which lack all or part of the C-terminal 4.1-kDa *pol* domain.

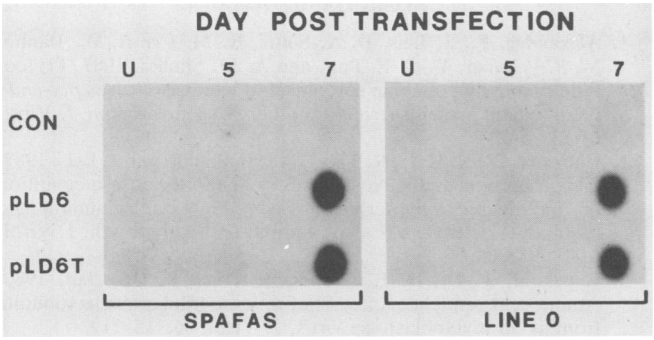

FIG. 4. Reverse transcriptase assay for detecting viral particles in the media of transfected cultures. Cultures of CEFs (SPAFAS or Line 0) were transfected with pLD6 and pLD6T as described in Materials and Methods. Control cultures (CON) were mock transfected. The culture supernatants were assayed for virion-associated reverse transcriptase activity at 5 and 7 days posttransfection by incorporation of [α-^{32}P]dGTP by using a poly(C) · oligo (dG) template primer. The reaction mixtures were applied to DEAE paper which was washed and exposed to X-ray film (7). Media from untransfected cultures were assayed as a negative control (U).

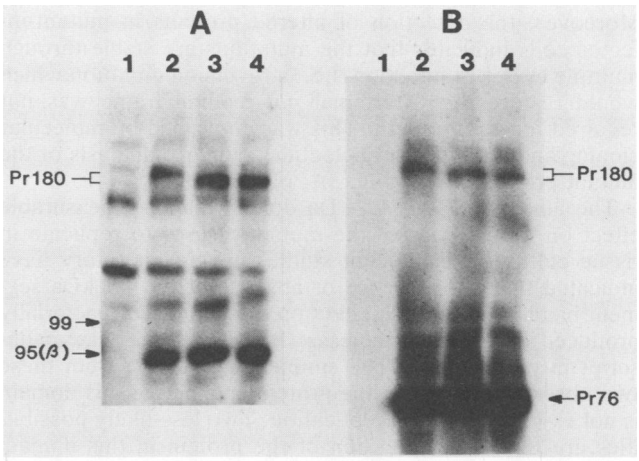

FIG. 5. Immunoblot analysis of intracellular mutant and wild-type viral proteins. CEFs were transfected with wild-type (pLD6) and mutant viral clones (pLD6T and pLD6IS-1). Virus was detected in the culture supernatant ca. 7 days posttransfection. After 10 days, lysates of infected CEFs were prepared and fractionated on a sodium dodecyl sulfate–7.5% polyacrylamide gel (18). Viral proteins were detected by using rabbit antisera directed against a peptide corresponding to *pol* amino acids 840 to 854 (see Fig. 6) in the pp32 domain of *pol* (1) (A) or against purified *gag* protein p27 (B) as described in Materials and Methods. Lysates were prepared from uninfected cells (lanes 1), wild-type (pLD6) infected cells (lanes 2), pLD6T-infected cells (lanes 3), and pLD6IS-1-infected cells (lanes 4). The positions of appropriate marker proteins, $p99^{pol}$ (99) and β (95[β]), are indicated.

DISCUSSION

In this report, we evaluated the function of the C-terminal 4.1-kDa domain in the *pol* gene of ASLV by constructing viral mutants containing translation termination codons which should abolish its expression. We showed that the termination codons function in bacterial cells (Fig. 3B). The termination codons also function in infected CEFs, as judged by the more-rapid migration of the $Pr180^{gag\text{-}pol}$ precursors in mutant- compared with wild-type-infected cells (Fig. 5).

Moreover, the detection of altered proteins in mutant-infected cells indicates that the mutations are stable through multiple cycles of infection, i.e., a reversion mutation which would restore the C-terminal *pol* reading frame was not required for viral growth. This was confirmed by molecular cloning and subsequent nucleotide sequence analysis of the mutant proviral DNA (Fig. 3A, pLD6IS-1).

The absence of the 4.1-kDa domain had no measurable effect on the abilities of the mutant viruses to replicate in tissue culture cells. Recent studies in our laboratory have indicated that the presence or absence of the 4.1-kDa segment has no measurable effect on the activity of bacterially produced ASLV endonuclease (R. W. Terry et al., manuscript in preparation). The simplest conclusion from these two observations is that the protein encoded in this domain is not essential for viral replication. Two less-likely possibilities are that the expression of the protein in this domain provides a selective advantage to the virus in some cells or most cells express a protein that can complement the viral defect. Neither of these can be tested, since the hypothetical cells or putative functions have not been identified.

A structural comparison with another avian retrovirus provided independent evidence that this region of *pol* is not required to establish an integrated provirus. An examination of the *pol* gene coding capacity of an *env*-deleted virus, avian erythroblastosis virus (AEV-H), indicated that this *pol* domain is absent (33). In this virus, the 5′ site of recombination with the cellular oncogene (c-*erbB*) occurs near the *env* splice acceptor site. This results in a change in the *pol* reading frame downstream of *pol* amino acid position 859 (Fig. 6). Six new *pol* amino acids are encoded before the first termination codon is encountered. Thus, AEV-H contains a *pol* mutation, similar to those described in this work, in which the 4.1-kDa coding region is lost. Introduction of helper-free AEV-H particles into cells by polyethylene glycol-mediated fusion results in transformation (32). Thus, neither reverse transcription nor viral DNA integration appears to be abolished by this *pol* mutation. However, the relative efficiencies of these reactions in AEV-H-infected cells have not been determined. Consistent with the findings described above, we have noted that the deduced amino acid sequences of the PR-C and SR-B strains vary considerably in the 4.1-kDa C-terminal *pol* domain; 5 of 37 amino acids differ (Fig. 6). Several of these are not conservative changes and, thus, seem likely to produce significant alterations in protein structure. These changes are not obviously deleterious. This observation suggests that conservation of the C-terminal domain of the *pol* protein is not highly selected in the evolution of these viruses.

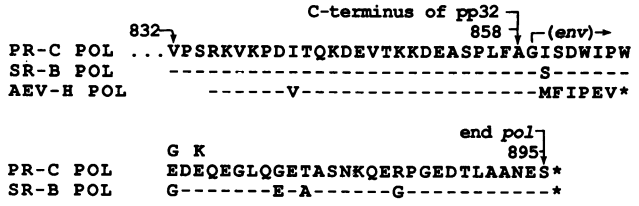

FIG. 6. Deduced amino acid sequences of C termini of avian retroviral *pol* products. The PR-C (25) and SR-B sequences (Fig. 2) are included for comparison. The deduced sequence of AEV-H is from published data (33). ----, Sequence identical to that of PR-C; *, termination codons. Amino acids above PR-C sequence line indicate variable residues (25). Amino acid positions are numbered as described in the legend to Fig. 1. The C terminus of pp32 is indicated. The codon for glycine at position 859 encodes the *env* splice acceptor site; the overlap with the *env* gene begins at that position.

Four of the nucleotide differences between the PR-C and SR-B strains of ASV also affect the amino acid sequence of the overlapping *env* reading frame (not shown). The overlapping region includes a major portion of the 62-amino-acid *env* leader peptide which is removed from the $Pr95^{env}$ precursor to form the N terminus of the gp85 envelope surface glycoprotein. The long leader peptide has no known function, and the amino acid differences between the two strains suggest that differences in protein structure can also be tolerated in this region. Interestingly, although this *pol-env* gene overlap feature is found in a number of retroviruses from a variety of species (28), the functionally required *env* hydrophobic signal peptide and the signal peptidase cleavage sites (31) are always found downstream of the overlap region (Fig. 1). Although this overlapping structure exists in the Moloney murine leukemia virus genome, it is not known whether C-terminal *pol* processing analogous to that of ASLV occurs (10).

The results presented here indicate that the amino acids encoded within the C-terminal *pol* domain are not required for viral replication. Nucleotide sequence analysis indicates that the amino acids encoded in this region and the overlapping *env* regions vary between viral strains. If the *pol* (or possibly *env*) protein sequences encoded in the overlapping translational reading frames are not important for replication, one may ask why the region has been retained in the retroviral genome. One possibility is that the nucleotide sequence itself is important, perhaps because it encodes *cis*-acting elements which affect the translation or processing of viral mRNAs. Such putative *cis*-acting elements might be unaffected by the mutations described here. This possibility is being explored by an analysis of additional mutations in the *pol-env* region of the ASLV genome.

ACKNOWLEDGMENTS

We are grateful to D. Soltis and F. Alexander for helpful suggestions on oligonucleotide mutagenesis strategies. We also thank E. J. Smith and V. Vogt for generous gifts of materials and D. Stacey, B. Cullen, and D. Cobrinik for critical review of the manuscript.

LITERATURE CITED

1. **Alexander, F., J. Leis, D. A. Soltis, R. M. Crowl, W. Danho, M. S. Poonian, Y.-C. E. Pan, and A. M. Skalka.** 1987. Proteolytic processing of avian sarcoma and leukosis viruses *pol-endo* recombinant proteins reveals another *pol* gene domain. J. Virol. **61**:534–542.
2. **Cobrinik, D., R. Katz, R. Terry, A. M. Skalka, and J. Leis.** 1987. Avian sarcoma and leukosis virus *pol*-endonuclease recognition of the tandem long terminal repeat junction: minimum site required for cleavage is also required for viral growth. J. Virol. **61**:1999–2008.
3. **Copeland, T. D., D. P. Grandgenett, and S. Oroszlan.** 1980. Amino acid sequence analysis of reverse transcriptase subunits from avian myeloblastosis virus. J. Virol. **36**:115–119.
4. **Crawford, S., and S. P. Goff.** 1985. 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. J. Virol. **53**:899–907.
5. **DeChiara, T. M., F. Erlitz, and S. J. Tarnowski.** 1986. Procedures for *in vitro* DNA mutagenesis of human leukocyte interferon sequences. Methods Enzymol. **119**:403–415.
6. **Ficht, T. A., L.-J. Chang, and C. M. Stoltzfus.** 1984. Avian sarcoma virus *gag* and *env* gene structural protein precursors contain a common amino-terminal sequence. Proc. Natl. Acad.

Sci. USA **81**:362–366.
7. **Goff, S., P. Traktman, and D. Baltimore.** 1981. Isolation and properties of Moloney murine leukemia virus mutants: use of a rapid assay for release of virion reverse transcriptase. J. Virol. **38**:239–248.
8. **Grandgenett, D., T. Quinn, P. J. Hippenmeyer, and S. Oroszlan.** 1985. Structural characterization of the avian retrovirus reverse transcriptase and endonuclease domains. J. Biol. Chem. **260**: 8243–8249.
9. **Hippenmeyer, P. J., and D. P. Grandgenett.** 1984. Requirement of the avian retrovirus pp32 DNA binding protein domain for replication. Virology **137**:358–370.
10. **Hu, S. C., D. L. Court, M. Zweig, and J. G. Levin.** 1986. Murine leukemia virus *pol* gene products: analysis with antisera generated against reverse transcriptase and endonuclease fusion proteins expressed in *Escherichia coli*. J. Virol. **60**:267–274.
11. **Jacks, T., and H. E. Varmus.** 1985. Expression of the Rous sarcoma virus *pol* gene by ribosomal frameshifting. Science **230**:1237–1242.
12. **Johnson, M. S., M. A. McClure, D.-F. Feng, J. Gray, and R. F. Doolittle.** 1986. Computer analysis of retroviral *pol* genes: assignment of enzymatic functions to specific sequences and homologies with nonviral enzymes. Proc. Natl. Acad. Sci. USA **83**:7648–7652.
13. **Katz, R. A., C. A. Omer, J. H. Weiss, S. A. Mitsialis, A. J. Faras, and R. V. Guntaka.** 1982. Restriction endonuclease and nucleotide sequence analyses of molecularly cloned unintegrated avian tumor virus DNA: structure of large terminal repeats in circle junctions. J. Virol. **42**:346–351.
14. **Katz, R. A., R. W. Terry, and A. M. Skalka.** 1986. A conserved *cis*-acting sequence in the 5′ leader of avian sarcoma virus RNA is required for packaging. J. Virol. **59**:163–167.
15. **Kopchick, J. J., and D. W. Stacey.** 1984. Differences in intracellular DNA ligation after microinjection and transfection. Mol. Cell. Biol. **4**:240–246.
16. **Kramer, R. A., M. D. Schaber, A. M. Skalka, K. Ganguly, F. Wong-Staal, and E. P. Reddy.** 1986. HTLV-III *gag* protein is processed in yeast cells by the virus *pol*-protease. Science **231**:1580–1584.
17. **Kyhse-Anderson, J.** 1984. Electroblotting of multiple gels: a simple apparatus without buffer tanks for rapid transfer of proteins from polyacrylamide to nitrocellulose. J. Biochem. Biophys. Methods **10**:203–209.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **277**:680–685.
19. **Lightfoote, M. M., J. E. Coligan, T. M. Folks, A. S. Fauci, M. A. Martin, and S. Venkatesan.** 1986. Structural characterization of reverse transcriptase and endonuclease polypeptides of the acquired immunodeficiency syndrome retrovirus. J. Virol. **60**: 771–775.
20. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavage. Methods Enzymol. **65**:499–560.
22. **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.
23. **Morinaga, Y., T. Franceschini, S. Inouye, and M. Inouye.** 1984. Improvement of oligonucleotide-directed site-specific mutagenesis using double-stranded plasmid DNA. Bio/Technology **2**: 636–639.
24. **Oppermann, H., J. M. Bishop, H. E. Varmus, and L. Levintow.** 1977. A joint product of the genes *gag* and *pol* of avian sarcoma virus: a possible precursor of reverse transcriptase. Cell **12**:993–1005.
25. **Schwartz, D. E., R. Tizard, and W. Gilbert.** 1983. Nucleotide sequence of Rous sarcoma virus. Cell **32**:853–869.
26. **Schwartzberg, P., J. Colicelli, and S. P. Goff.** 1985. Recombination between a defective retrovirus and homologous sequences in host DNA: reversion by patch repair. J. Virol. **53**:719–726.
27. **Tanese, N., M. J. Roth, and S. P. Goff.** 1986. Analysis of retroviral *pol* gene products with antisera raised against fusion proteins produced in *Escherichia coli*. J. Virol. **59**:328–340.
28. **Van Beveren, C., J. Coffin, and S. Hughes.** 1985. Nucleotide sequence complemented with functional and structural analysis, p. 567–1148. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), Molecular biology of tumor viruses: RNA tumor viruses, 2nd ed., vol. 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Von der Helm, K.** 1977. Cleavage of Rous sarcoma viral polyprotein precursor into internal structural protein in vitro involves viral protein p15. Proc. Natl. Acad. Sci. USA **74**:911–915.
30. **Weiss, R., N. Teich, H. Varmus, and J. Coffin (ed.).** 1984. Molecular biology of tumor viruses: RNA tumor viruses, 2nd ed., vol. 1. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Willis, J. W., J. M. Hardwick, K. Shaw, and E. Hunter.** 1983. Alterations in the transport and processing of Rous sarcoma virus envelope glycoproteins mutated in the signal and anchor regions. J. Cell. Biochem. **23**:81–94.
32. **Yamamoto, T., S. Kawai, T. Koyama, H. Hihara, T. Shimizu, and K. Toyoshima.** 1983. Newly generated avian erythroblastosis virus produces noninfectious particles lacking env-gene products. Virology **129**:31–39.
33. **Yamamoto, T., T. Nishida, N. Miyajima, S. Kawai, T. Ooi, and K. Toyoshima.** 1983. The *erb*B gene of avian erythroblastosis virus is a member of the *src* gene family. Cell **35**:71–78.