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Genetic Evidence that the Avian Retrovirus DNA Endonuclease Domain of *pol* Is Necessary for Viral Integration

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We used in vitro mutagenesis in the 3′ region of the avian retrovirus polymerase (*pol*) gene to genetically define the role of the DNA endonuclease domain. In-frame insertional mutations, which were dispersed throughout the 5′ region of pp32, produced a series of five replication-deficient mutants. In contrast, a single point mutant (Ala→Pro) located 48 amino acids from the NH_2 terminus of pp32 exhibited a delayed replication phenotype. Molecular analysis of this mutant demonstrated that upon infection it was capable of synthesizing both linear and circular species of unintegrated viral DNA. The levels of unintegrated viral DNA present in cells infected with the mutant virus were several times greater than wild-type levels. Quantitation of the amount of integrated viral genomes demonstrated that the mutant virus integrated viral DNA one-fifth as efficiently as wild-type virus. This single point mutation in the NH_2 terminus of pp32 prevented efficient integration of viral DNA, with no apparent effect on viral DNA synthesis per se. Thus, the DNA endonuclease domain has been genetically defined as necessary for avian retrovirus integration.

The avian retrovirus pp32 DNA endonuclease is derived proteolytically from a multidomain translation product of the polymerase (*pol*) gene, designated the β polypeptide (M_r, 92,000) (23). The β polypeptide is processed to yield the α polypeptide (M_r, 63,000) and the pp32 protein (M_r, 32,000) (7, 14, 23). The αβ complex possesses several enzymatic activities which include RNA-directed DNA polymerase, DNA-directed DNA polymerase, ribonuclease H, and DNA endonuclease (6). The pp32 protein contains the same amino acid sequences as the COOH-terminal third of the β polypeptide (5, 6, 23). pp32 also possesses DNA endonuclease activity, although the cofactor (Mg^{2+}, Mn^{2+}) requirements are different from those of the ββ or αβ complexes (8, 18). Purified pp32 protein preferentially binds to specific regulatory regions of the Rous sarcoma virus (RSV) long terminal repeat DNA (20). In vitro, the Mg^{2+}-dependent endonuclease activity of pp32 generates nicks in one or the other viral DNA strands 2 nucleotides from the circle junction (9), whereas the Mn^{2+}-dependent endonuclease of αβ (4) or pp32 (9) generates nicks 3 nucleotides from the circle junction. The former results are consistent with models which propose a role for the pp32 DNA endonuclease in removal of two nucleotides from the long terminal repeat termini upon integration of the viral DNA in vivo. Point and deletion mutations generated in the pp32 coding region of infectious RSV Prague A (PrA) DNA suggest that the protein is involved in the establishment of infection and not in synthesis of viral DNA (12). The generation of replication-deficient viruses by introducing mutations in the 3′ regions of the Moloney murine leukemia virus (MuLV) and spleen necrosis virus *pol* genes has demonstrated that this domain is involved in the integration of viral DNA (3, 21, 26).

To ascertain the biological role(s) of the DNA endonuclease domain, in-frame linker insertion mutations and a point mutation were introduced into the 3′ region of the RSV PrA *pol* gene. The linker insertion mutations were dispersed throughout the highly conserved NH_2-terminal portion of the pp32 coding region (2) as well as the pp32 moiety located on the β polypeptide. The mutants were all replication-deficient as determined by transformation assay and release of virus particles containing active reverse transcriptase. Second, a single point mutation was introduced into the NH_2 terminus of the *pol* endonuclease domain. This mutant exhibited a decreased rate of virus replication with a concurrent decreased rate of cellular transformation. To further evaluate the molecular effects of the point mutant, DNA synthesis and integration of viral DNA were examined. The point mutant exhibited a significant reduction in viral integration while maintaining wild-type levels of reverse transcription, genetically demonstrating the importance of the endonuclease domain in viral DNA integration.

MATERIALS AND METHODS

Cells and virus. Chicken embryo fibroblast (CEF) cells (C/E, leukosis-free, chf^-, gs^-) were obtained in suspension from SPAFAS, Inc. Cells were grown at 37 or 41°C in a CO_2 incubator in medium 199 (GIBCO Laboratories) supplemented with 5% fetal calf serum–1% dimethyl sulfoxide–10% tryptose phosphate broth–penicillin (100 U/ml)–streptomycin (100 μg/ml)–sodium bicarbonate to pH 7.2. The PrA strain of RSV was used as wild type.

Cloned DNAs. Plasmid pJD100 contains a full-length nonpermutated copy of PrA DNA joined at the *Hin*dIII site of pBR322 (gift of J. T. Parsons).

Bacterial cultures. *Escherichia coli* HB101 cells were transformed to ampicillin resistance by plasmid constructs, using the $CaCl_2$ method of Mandel and Higa (19). Individual colonies were screened by a rapid alkaline lysis procedure (1).

Cell transfections and infections. Viral DNAs in pJD100 vectors were transfected intact at either 50 or 500 ng. DNAs were mixed with polybrene and applied to CEF cells as described by Kawai and Nishizawa (15). Virus production was detected by reverse transcriptase assay. Titers of virus stocks were determined by RNA slot-blot analysis and reverse transcriptase assay (11). Wild-type and mutant virus contained equivalent quantities of reverse transcriptase activity relative to viral RNA, as determined by RNA slot-blot analysis.

Viral protein analysis. Virus-infected CEF cells were la-

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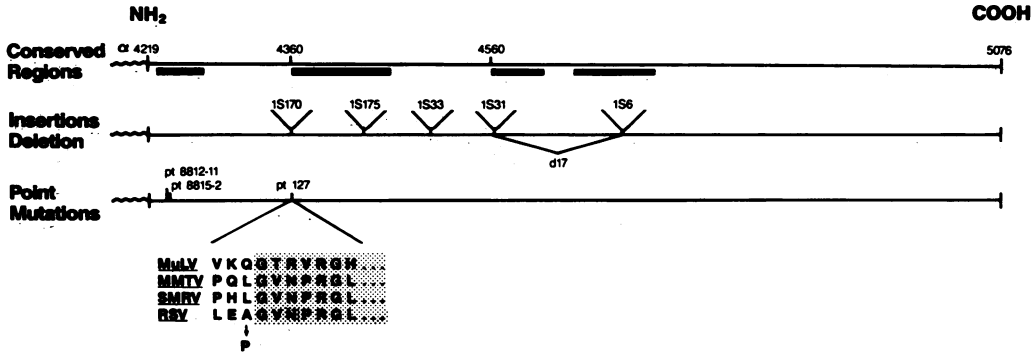

FIG. 1. Summary of conserved amino acids and site-specific mutations in the endonuclease region of *pol*. Conserved regions of amino acid sequences among MuLV, mouse mammary tumor virus (MMTV), squirrel monkey retrovirus (SMRV), and RSV (2), respectively, are designated by thick dark lines located immediately below the map of the pp32 coding domain. Insertion mutations (IS) contain four in-frame amino acid inserts at the designated locations. The sole deletion mutant (*d*17), contains a 126-base-pair in-frame deletion. Point mutants *pt*8812-11 and *pt*8815-2 were previously described (12). Point mutant *pt*127 contains a Pro substituted for an Ala at nucleotide number 4360. All DNA sequence numbers are according to Schwartz et al. (25). *pt*127 is located 1 amino acid 5′ to the second region of amino acid homology (2).

beled with [^{35}S]methionine (ICN Radiochemicals). Proteins from detergent-lysed virions were immunoprecipitated with rabbit antiserum against the αβ DNA polymerase or pp32 of avian myeloblastosis virus, as previously described (23).

Cell fractionation. Viral DNA was isolated from mass virus-infected cultures at 24-, 48-, and 72-h intervals. Two 150-cm^2 flasks of cells were harvested at each time point. CEF cells were lysed and fractionated into cytoplasmic and nuclear fractions. The unintegrated viral DNA was isolated by the method of Hirt (13), and the precipitated genomic DNA was analyzed for integrated viral sequences (10). Details of the procedure are described elsewhere (12).

Southern blot analysis. Hirt supernatant DNA was digested with a fourfold excess of the restriction enzyme *Nci*I (New England BioLabs, Inc.). A fourfold excess of the restriction enzyme *Bgl*II (Bethesda Research Laboratories, Inc.) was also added to 10 μg of genomic DNA. All samples were digested overnight at 37°C and were treated with 30 μg of proteinase K in the presence of 1% sodium dodecyl sulfate and 20 mM EDTA. DNA samples were routinely electrophoresed in 1% agarose gels at 40 V for the desired time in a Tris-borate-EDTA buffer, except for the *Nci*I digest which was electrophoresed in a 2% agarose gel (NuSieve; FMC BioProducts). The DNA was electrophoretically transferred from the agarose gels to charged nylon membranes in Tris-acetate-EDTA buffer (Bio-Rad Laboratories). Transfer reactions were performed at 80 V (0.83 A) and 4°C for the desired time. Membranes were baked at 80°C for 2 h. Prehybridization, hybridization, and membrane washing were performed in accordance with the instructions from Bio-Rad. DNA fragments used as probes were electroeluted from 1% agarose gels and purified over NACS columns (Bethesda Research Laboratories). The purified DNA fragments were nick translated (Bethesda Research Laboratories) by using [^{32}P]dCTP (ICN) as label. The labeled probe was separated from unincorporated label on a NACS column. Membranes were hybridized with 10^7 cpm of probe.

In vitro mutagenesis. Linker insertion mutagenesis was performed by the method of Lathe et al. (17). The synthetic DNA linker 5′ CCCGAATTCGGG was synthesized by Collaborative Research, Inc., with an *Eco*RI restriction site (underlined sequence). Oligonucleotide mutagenesis was performed by the method of Kunkel (16). The synthetic oligonucleotide 5′ TACTCCGGGTTCCAACG was synthesized by P-L Biochemicals, Inc., and contained an *Nci*I restriction site. The mutation was confirmed by DNA sequencing as described by Sanger et al. (22).

RESULTS

Experimental design. In-frame linker mutagenesis as well as oligonucleotide mutagenesis was used in the 3′ region of the *pol* gene to develop viable virus mutants for studying the role of the endonuclease domain in viral replication. Site-specific mutagenesis was used to alter a cloned copy of the *pol* gene, which was then assembled into the virus DNA genome. CEF cells were transfected with the mutant DNAs to determine whether the virus mutants were viable. Production of replication-competent PrA virus was assayed by cellular transformation and release of virus containing active reverse transcriptase into the medium. Virus stocks isolated from the transfection procedures were used to infect CEF cells to study the mutants at the molecular level.

Linker mutations. Figure 1 illustrates the location of the linker insertions. Twelve-base-pair linkers, containing an *Eco*RI site, were inserted at six *Hae*III sites located throughout the 5′ region of the pp32 DNA endonuclease or in the pp32 moiety of the β polypeptide. The linker mutants contained a 4-amino-acid insert. Five plasmids containing an additional *Eco*RI site at different locations were isolated along with one plasmid which contained an in-frame deletion. These mutant *pol* genes were substituted back into the viral vector pJD100. CEF cells were transfected with 50 and 500 ng of mutant DNA or wild-type DNA by using the Polybrene-dimethyl sulfoxide transfection procedure of Kawai and Nishizawa (15). Cells were routinely passaged for 14 days, during which time they were analyzed for cell transformation and virus release. Neither the linker insertion mutants nor the deletion mutant gave rise to detectable virus, and they were not studied further. However, these results demonstrate the sensitivity of the NH_2-terminal half of the endonuclease domain to perturbation and confirm the necessity of the region for viral replication (11, 12). Most of the insertion mutations were located in the highly conserved regions of the endonuclease domain (2) (Fig. 1), which may account for the high sensitivity of this region to mutation. In addition, it is also possible that these mutations affect other *pol* gene-encoded activities.

Oligonucleotide mutagenesis. To increase the possibility of isolating a replication-competent virus mutant, oligonucleotide mutagenesis was used to make a single point mutation. The point mutation (*pt*127) was located 5′ to a major conserved region of amino acids within the endonuclease domain (Fig. 1) (2). This location was selected to increase the likelihood of isolating a viable virus mutant by avoiding the most highly conserved amino acids. Previously, point mutants (Fig. 1, *pt*8812-11, *pt*8815-2) were constructed at the *Bgl*II site located at the NH_2 terminus of pp32 (11). These mutants were replication competent and displayed a delayed growth phenotype but appeared to exhibit wild-type levels of integration (12). Therefore, the point mutation in this study was located between the *Bgl*II site and the second region of highly conserved amino acids. The target for mutagenesis was an Ala residue at nucleotide number 4360 (25), which was replaced with Pro. The mutation was confirmed by DNA sequence analysis (data not shown). The mutagenized *pol* fragment was substituted into the viral vector pJD100 to produce the mutant vector designated pJD127. CEF cells were transfected with 50 or 500 ng of pJD127, using the Polybrene-dimethyl sulfoxide procedure of Kawai and Nishizawa (15). The CEF cells were continuously passaged for 14 days at 41°C, during which time they were monitored for virus release and cell transformation. The quantitation of released virus by measuring reverse transcriptase from wild-type PrA and mutant *pt*127 transfections is displayed in Fig. 2. Virus was detected in the supernatant of wild-type virus DNA-transfected cells considerably sooner than from mutant-virus DNA-transfected cells. Once initiated, virus release was similar for PrA and *pt*127. Transfections of CEF cells by PrA and *pt*127 were also carried out at 35 and 37°C. The relative amount of virus released at all temperatures was similar for the mutant and wild-type virus grown at 41°C, indicating that the mutant phenotype was not temperature sensitive.

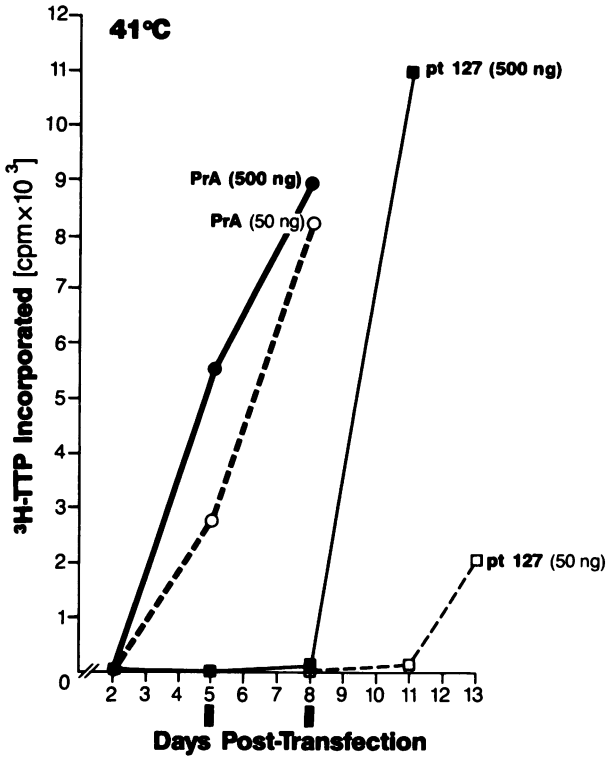

FIG. 2. Release of virus containing reverse transcriptase activity from transfected CEF cultures. Subconfluent 60-mm-diameter dishes of CEF cells were treated with 50 or 500 ng of PrA or *pt*127 DNA at 41°C. The cells were split into 75-cm^2 flasks on day 2 posttransfection. Cells in the 75-cm^2 flasks were split 1:3 on days 5 and 8 (▮). The amount of medium per culture was held constant between each experiment. Before each passage, 30 μl of supernatant from each infected culture was assayed for release of virus containing reverse transcriptase (12). Assays were always done under conditions of linear incorporation.

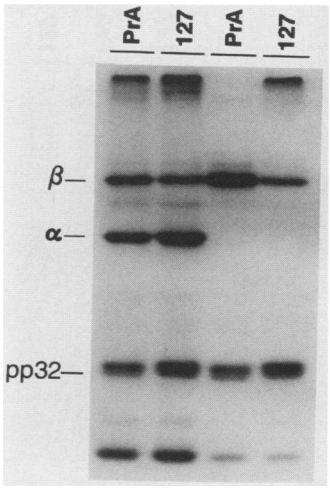

FIG. 3. Virion protein analysis. A constant number of trichloroacetic acid-precipitable counts of [^{35}S]Met-labeled virion protein was treated with 15 μl of either rabbit anti-αβ serum (lanes 1 and 2) or rabbit anti-pp32 serum (lanes 3 and 4). Equal counts of *pol* proteins were immunoprecipitated, and half of each sample was electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel as described in Materials and Methods.

Virus protein analysis. To ensure that the point mutation did not affect the processing of β, α, or pp32, [^{35}S]Met-labeled virus proteins were examined. Labeled virions were lysed, and the *pol* proteins were immunoprecipitated and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as shown in Fig. 3. Antiserum to the αβ DNA polymerase (first two lanes) demonstrated that the relative levels of α, β, and pp32 were similar for PrA and *pt*127. Antiserum to pp32 (last two lanes) immunoprecipitated β and the characteristic pp32 doublet, which represents phosphorylated and nonphosphorylated forms of the protein (24). The mutant virions appear to contain all the *pol*-encoded proteins and at wild-type levels. All of the other [^{35}S]Met-labeled virus proteins also appeared normal (data not shown).

Molecular analysis. Since *pt*127 contained a normal complement of viral *pol* proteins and exhibited wild-type reverse transcriptase activity, further analysis at the molecular level was necessary to determine the nature of the delayed growth phenotype. CEF cells were infected with an equal amount of PrA and *pt*127. Virus stocks were quantitated by reverse transcriptase activity and slot-blot analysis of viral RNA. At 24, 48, and 72 h postinfection, cells were harvested and fractionated into a Hirt fraction (13) and a genomic fraction. The Hirt fraction contains mostly unintegrated viral DNA, whereas the genomic fraction contains mostly integrated viral genomes.

It is important to evaluate the stability of the mutation. The presence of the mutation generates a new *Nci*I site. Figure 4 shows the result of *Nci*I digestion of PrA and *pt*127

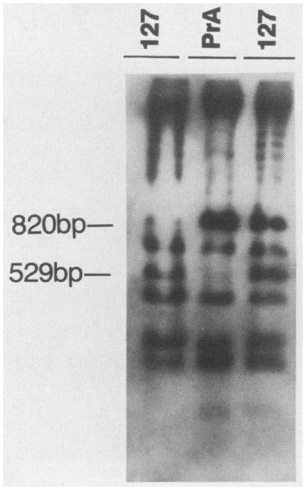

FIG. 4. *Nci*I digest of unintegrated viral DNA. CEF cells were infected with PrA or *pt*127 (127) virus stocks. Lane 1 contained *Nci*I-digested unintegrated viral DNA isolated from infected cultures (two 150-cm^2 flasks) which were passaged for a total of 4 weeks. Lane 2 contained *Nci*I-digested unintegrated wild-type viral DNA. Lane 3 contained a partial *Nci*I digest of unintegrated viral DNA isolated from infected cultures (two 150-cm^2 flasks) which were passaged for 2 weeks. Unintegrated viral DNA isolated from each culture was digested with *Nci*I, electrophoresed on a 2% NuSieve agarose gel, electroblotted to a nylon membrane, and probed with a 2.2-kilobase *Hpa*I-*Kpn*I viral *pol* probe (nucleotide numbers 2731 to 4995) (25).

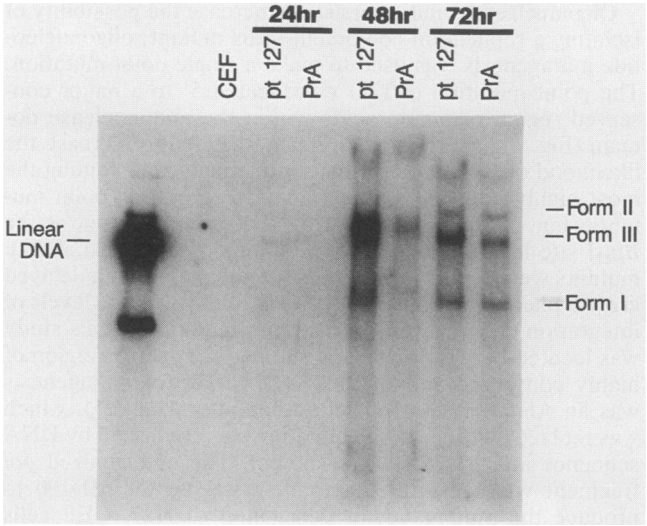

FIG. 5. Unintegrated virus-specific DNA. CEF cells were infected with equal reverse transcriptase units of PrA or *pt*127. CEF cells from two 150-cm^2 flasks were harvested for each infection at 24, 48, and 72 h postinfection. The cytoplasmic and nuclear Hirt supernatant fractions of each infection were electrophoresed on a 1% agarose gel, transferred to a nylon membrane, and probed with nick-translated viral DNA.

unintegrated viral DNAs which were isolated from virus-infected CEF cells passaged for either 2 or 4 weeks. The presence of the point mutation was confirmed by the digestion of the 820-base-pair PrA *pol* fragment. Whereas the PrA viral DNA exhibited and expected restriction pattern, the *pt*127 viral DNA contained a new *Nci*I site confirming the stability of the mutation.

To investigate the replication defect in the mutant *pt*127, we examined its ability to synthesize both linear and circular viral DNA. The Hirt supernatant DNA from PrA and *pt*127 was analyzed for the presence of unintegrated viral DNA at 24, 48, and 72 h postinfection. Figure 5 shows that the mutant *pt*127 synthesized both species of viral DNA. As soon as 24 h postinfection, *pt*127 exhibited increased levels of viral DNA compared with wild type, even though equivalent amounts of Hirt supernatant DNA were added in each lane. This pattern is also maintained at the two later time points. The mutant unintegrated viral DNA is present at two to three times the concentration of wild-type DNA as determined by densitometric analysis of these gels exposed at different times. This evidence suggests that the mutation does not affect the ability of the mutant to reverse transcribe the viral genome. The increased levels of mutant viral DNA present in the Hirt supernatant could be due to an "up" mutational effect to the αβ polymerase (which is not supported from the biochemical analysis) or reflects a defect in viral integration.

To examine the efficiency of viral integration, equal amounts of PrA- and *pt*127-infected CEF genomic DNAs were digested with *Bgl*II and visualized with a *src*-specific viral probe (Fig. 6). *Bgl*II digestion of viral DNA yields an internal 3.5-kilobase fragment containing pp32, the *env* region, and the 5′ half of v-*src*-coding domains. A probe consisting of the 5′ half of the v-*src* gene was used to detect the 3.5-kilobase viral fragment. By using this v-*src* probe, we can determine the efficiency of viral integration while avoiding the background signals from endogenous retroviral sequences. The v-*src* probe will also detect c-*src* present in the CEF cells (27). Since c-*src* levels within CEF cells are constant, the c-*src* signal can be used as an internal control to estimate integration efficiency. Figure 6 shows that mutant *pt*127 integrates significantly less efficiently than wild-type PrA at several time points after infection. Mutant pt127 integrates 17% as efficiently as wild-type as determined by densitometric scanning. These results suggest that the mutant *pt*127 contains a defect in integration. A defect in integration of viral DNA would be consistent with the increased levels of mutant viral DNA in the Hirt supernatant and with decreased levels of integrated mutant viral DNA.

DISCUSSION

To genetically demonstrate a role for the avian retrovirus DNA endonuclease domain in viral integration, site-specific mutations were constructed in the appropriate coding sequences. Of the mutants constructed, one point mutant (*pt*127) exhibited an altered replication phenotype, indicating a defect at some step in the initiation of infection. Mutant *pt*127 was replication competent, which permitted detailed molecular analysis that revealed a defect in the ability of the mutant virus to integrate viral DNA. Since the point mutation is located in the 3′ region of the *pol* gene, it was important to demonstrate that the mutation did not affect the processing of the *pol* gene products. Analysis of *pol* gene products isolated from *pt*127 virions indicated that the processing of the *pol* precursor was unaffected by the mutation. In vivo analysis of unintegrated mutant viral DNA demonstrated that the mutant virus could synthesize both linear and circular species of viral DNA. Although this does not rule out the possibility that the mutation has an effect on the viral DNA polymerase, it is apparent that sufficient quantities of unintegrated viral DNA were present for integration. The RNase H activity of the mutant polymerase was not

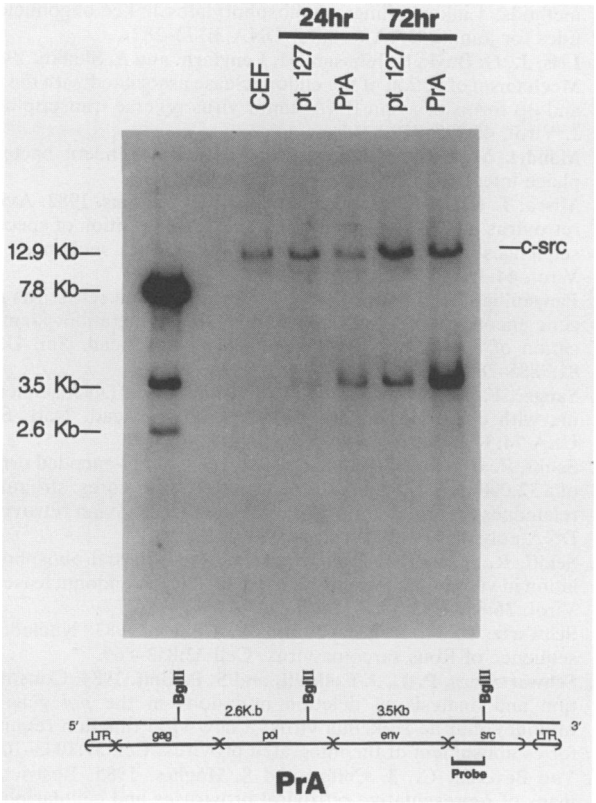

FIG. 6. *Bgl*II digest of genomic DNA from infected CEF cells. The same CEF cells as those used in the experiment shown in Fig. 5 were analyzed for integrated viral genomes. Equivalent amounts of genomic DNAs from each time point were digested with *Bgl*II, run on a 1% agarose gel, and probed with the 5′ half of v-*src*. The *Bgl*II digestion pattern and location of the *Xho*I-*Bgl*II v-*src* probe are illustrated in the diagram below the blot. The first lane of the genomic blot contains the *Bgl*II digest on mock-infected CEF cells. Kb, Kilobases.

examined directly, although we infer from the previous results that it is functional, since the mutant virus can reverse transcribe the RNA genome as efficiently as wild-type virus. Thus, mutant *pt*127 can carry out the early events of viral replication.

Quantitation of the amount of DNA synthesized by wild-type and mutant *pt*127 infections demonstrated that the mutant virus-infected cells contained more unintegrated viral DNA than wild-type virus-infected cells (Fig. 5). Conversely, Southern analysis of genomic DNA showed that mutant viral DNA was integrated 17% as efficiently as observed with wild-type. By using the *Bgl*II v-*src* probe, integrated viral DNA could be detected without interference from endogenous background retroviral sequences, enabling quantitation of the amounts of integrated viral DNA.

A standard concern with the analysis of integrated viral DNA is contamination by unintegrated viral DNA. This is not likely to be a major problem. The amount of c-*src* defines the concentration of genomic DNA per lane (Fig. 6). By direct comparison with c-*src* levels, the amount of integrated viral DNA (v-*src*) is always considerably less with mutant *pt*127 than with wild-type virus (Fig. 6), even though the mutant virus-infected cells contained more unintegrated viral DNA than wild-type infected cells (Fig. 5). This is opposite of what would be expected if there was significant contamination of unintegrated viral DNA with genomic DNA. Together, Southern analysis of unintegrated and integrated viral DNA demonstrated that mutant *pt*127 was partially blocked in integration. The increased levels of unintegrated viral DNA in the mutant Hirt supernatants were apparently caused by the decreased efficiency of viral DNA integration.

Due to the high rate of recombination during retrovirus replication, the stability of the point mutation (*pt*127) was examined. *Nci*I digestion of unintegrated mutant viral DNA isolated from infected CEF cells which had been passaged for an extended period demonstrated that the mutation was stable for these analyses. This result does not rule out the possibility of another mutational event occurring during extended passages. However, the mutant genotype remained consistent throughout these analyses.

The point mutation (*pt*127) and the first linker insertion mutation (IS170) are located at the same site in the endonuclease domain (see Fig. 1). The point mutation changed Ala to Pro at nucleotide number 4360 (25), whereas the linker inserted four amino acids, Arg-Ile-Arg-Ala, immediately downstream of this same Ala. The mutant viral genome which contained the insertion mutation IS170 did not produce replication-competent virus upon transfection. This mutation could have affected protein processing or any of the activities associated with the endonuclease domain.

The molecular data derived from the mutant *pt*127 indicates the mutation causes decreased efficiency in viral integration. These data are consistent with a published report from Donehower and Varmus (3) which showed that a single point mutation in the 3′ region of the MuLV *pol* gene reduced integration efficiency 10-fold. The MuLV mutation changed the second Arg residue to Cys (see stippled box of the second conserved region in Fig. 1). This alteration is located only 5 amino acids downstream from the *pt*127 mutation. These results confirm the functional similarity between the RSV and MuLV 3′ regions of their respective *pol* genes. Although we have confirmed the importance of the avian *pol* gene in viral integration genetically, the functional defect is unknown since this region encodes the endonuclease activities of both the reverse transcriptase (β chain) and pp32.

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