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The Human Foamy Virus *pol* Gene Is Expressed as a Pro-Pol Polyprotein and Not as a Gag-Pol Fusion Protein

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It has been reported recently that the human foamy virus (HFV) Pol polyprotein of 120 kDa is synthesized in the absence of the active HFV aspartic protease. To gain more information on how the 120-kDa Pro-Pol protein is synthesized, mutant HFV genomes were constructed and the resulting proviruses were analyzed with respect to HFV *pol* expression and infectivity. HFV proviruses that contain termination codons in the nucleocapsid domain of *gag* and thus lack a *gag-pol* overlap region assumed to be required for translational frameshifting, nevertheless expressed the 120-kDa Pro-Pol precursor, the 80-kDa reverse transcriptase/RNase H, and a 40-kDa integrase in amounts similar to those observed for wild-type genomes. Since a Gag-independent expression of authentic Pol proteins was detectable in cells transfected with eukaryotic HFV *pol* expression plasmids, the data indicate that the HFV Pol precursor of 120 kDa is expressed independently of Gag by a mechanism that does not rely on ribosomal frameshifting, since the postulated HFV Gag-Pol protein of 190 kDa was not detectable under the conditions used. Furthermore, replacement of the Met residue by Thr at position 9 in *pol* within the *gag-pol* overlap region resulted in strongly reduced HFV Pol polyprotein expression and infectivity of the resulting proviruses. This Met residue of *pol* conserved in foamy virus sequences is the likely candidate for translational initiation of the 120-kDa Pro-Pol polyprotein. *trans* complementation of the HFV mutant with the Met-to-Thr substitution in the *pol* gene by a eukaryotic plasmid that expressed the HFV Pro-Pol protein resulted in partial recovery of infectivity. When HFV *pol* was fused in frame to *gag*, an engineered 190-kDa Gag-Pol fusion protein was formed and the enzymatic activity of the HFV protease was partially retained. The results imply that HFV is the first retrovirus that expresses a Pol polyprotein without formation of a Gag-Pol fusion protein.

Retroviral *pol* gene products are translated as Gag-Pol precursor polyproteins from genomic RNAs (9). There is an intimate relation between the synthesis of Pol proteins as Gag-Pol fusion proteins and targeting of Pol into retrovirus particles. Gag and Gag-Pol fusion proteins are coassembled into forming capsids, thereby targeting the viral enzymes into virions (9). The Gag-Pol fusion proteins of well-studied retroviruses are translated by a −1 frameshifting event that takes place in the *gag-pol* overlap region or, for murine leukemia viruses, by stop codon suppression when both genes are in the same reading frame (9, 16). Translational frameshifting allows the regulation of the stoichiometry of the structural Gag proteins and the nonstructural, enzymatically active Pol proteins. This mechanism of fine control of translational efficiency also holds true for yeast retrotransposons, in the case of which the frameshifting events for individual Ty elements have been shown to be either +1 or −1 (11). In related viral systems in which Pol expression is also directed from genomic RNAs, e.g., hepatitis B virus, cauliflower mosaic virus, and murine leukemia viruses, various mechanisms for *pol* translation have been shown or put forward to reconcile the different requirements for appropriate expression of Pol (11, 36).

The human spumavirus or foamy virus (HFV) isolated from an East African nasopharynx carcinoma patient (1) is a member of the *Spumavirinae* group of complex retroviruses (10, 31). HFV is characterized by unique structural and functional features that set the *Spumavirinae* apart from most known retroviruses like oncoviruses and lentiviruses (for reviews, see references 23 and 31). HFV encodes special genes, the regulatory and accessory *bel* genes; among their products is the Bel1 transcriptional transactivator. The *bel* genes contribute to the large genomic size (12). The second distinguishing feature of HFV is the presence of a functionally active internal promoter, PII, that is also required for virus replication (25, 26). The third remarkable characteristic is the absence of the Cys motif in the HFV NC protein sequence, which instead has evolved to contain Gly-Arg-rich repeats which have been implicated in nuclear targeting of Gag proteins and assumed to be responsible for genomic RNA encapsidation (3, 30, 39).

The fourth unusual feature is the apparent absence of an HFV Gag-Pol fusion protein of 190 kDa that has so far escaped reproducible detection even upon transfection of HFV proviral genomes deficient in protease (PR) activity (19). PR is located in the amino terminus of *pol* (19). To express an HFV Gag-Pol fusion protein, a +1 frameshifting, and not the typical −1 frameshift, would be required for fusion of the *gag* and *pol* genes in frame (23, 31) (GenBank accession no. U21247).

To assess how the 120-kDa HFV Pro-Pol protein, the full-length expression product of the *pol* gene, is synthesized, we performed site-directed mutagenesis and immunoblot analysis to show that the synthesis of the HFV *pol* gene products is Gag independent. Translational initiation likely occurs at the first Met residue, i.e., at position 9 of the *pol* open reading frame (ORF), defining the coding capacity of the *pol* gene as 1,143 amino acid residues. The results imply that HFV utilizes mechanisms for synthesizing Pro-Pol and for targeting it into virions that are different from those of other known retroviruses.

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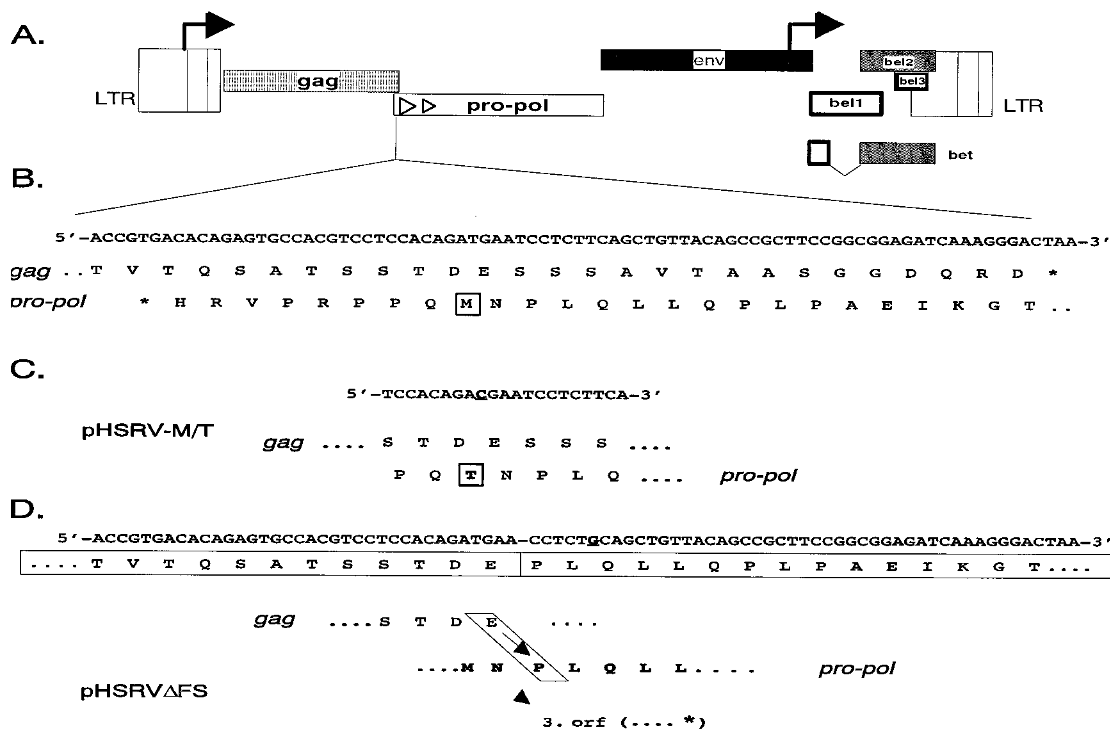

FIG. 1. Schematic presentation of the HFV provirus with emphasis on the *gag-pol* overlap region. (A) HFV DNA genomic organization. HFV genes are represented by boxes; both promoters are shown by bent arrows. Open triangles symbolize the first and the second Met residues of *pol*. (B) Nucleotide sequence of the *gag-pol* overlap region with the deduced sequences of the Gag and Pro-Pol proteins. The conserved Met codon shown to be used for Pro-Pol synthesis is boxed. (C) Nucleotide and deduced amino acid sequences of the deletion-substitution mutant pHSRV-M/T with the mutant Thr residue boxed. (D) Nucleotide and deduced amino acid sequences of the deletion mutant pHSRVΔFS. The shift from *gag* to *pol* is marked by a short arrow; that from *pol* to the third reading frame (3. orf) is marked by an arrowhead. The in-frame fusion of the Gag and Pro-Pol proteins is presented below the nucleotide sequence. Mutated bases in panels C and D are underlined and in boldface; the position of the deleted base in panel D is represented by a dash. Asterisks represent stop codons.

MATERIALS AND METHODS

Plasmids. The infectious HFV DNA clone pHSRV13 served as source of viral DNA; nucleotide numbering starts at the first base of the 5′ long terminal repeat of the HFV provirus (27). Plasmid pCMVβgal directs the expression of β-galactosidase from the cytomegalovirus immediate early promoter (25).

Construction of recombinant clones. Molecular cloning was performed according to standard techniques (36). Mutants of the proviral clone pHSRV13 were constructed by PCR-directed mutagenesis using plasmid pHSRV13 as the template as described previously (26).

Mutant pHSRVgagstop (see Fig. 2D) containing two stop codons in the HFV *gag* gene was constructed by PCR with sense primer gagsss (5′-AGATAATTTAAAT***T***AA***T***GAGGATATAATCTTCGACCC-3′; the *Swa*I restriction site is underlined) (in all sequences mutated bases are marked by bold italic letters) and antisense primer 3486a (5′-TTTGATCTTCTGGAAGAGC-3′). The reaction product was digested with *Swa*I and *Dra*III and inserted into pHSRV13 via a three-fragment ligation.

Eukaryotic *pol* expression plasmids were constructed by inserting the blunt-ended HFV *Avr*II-to-*Mro*I (see Fig. 4) DNA fragment from plasmids pHSRV13 and pHSRV-D/A into the blunt-ended *Hin*dIII- and *Sma*I-digested plasmid pBC12CMV (24).

To change the Met residue at position 9 of the *pol* ORF to Thr (M/T), products of PCR with primers 2936s (5′-GGTTCTCAAACTCCTAGG-3′) plus M/Ta (5′-TCCACAGA***C***GAATCCTCTTCAGCTGT-3′) and those of PCR with primers M/Ts (5′-GAGGATTC***G***TCTGTGGAGGACGTGGC-3′) plus 3486a using wild-type wt pHSRV13 DNA as the template were combined and reamplified with primers 2936s and 3486a (Fig. 1C). The reaction product was cleaved with *Dra*III and *Sap*I, and the DNA fragment containing the mutation was cloned into pHSRV13 in a three-fragment ligation. The resulting clones were designated pHSRV-M/T. In analogy, the *gag* and *pol* genes were fused in frame by deleting 1 bp in the *gag-pol* overlap region by PCR. The first two reactions were done with primers 2936s plus FRSHa (5′-AACAGCTG***C***AGAGG/TTCATCTGTGGAGGACGT-3′; the slash marks the position of the deleted nucleotide) and primers FRSHs (5′-TGAA/CCTCT***G***CAGCTGTTACAGCCGCTT-3′) plus 3486a (Fig. 1D). The reaction products were combined, reamplified, and similarly cloned into pHSRV13, yielding plasmid pHSRVΔFS. Control clones carrying in addition the inactivated PR of plasmid pHSRV-D/A with substitution of the Asp residue in the catalytical center of the HFV PR by Ala (D/A) were constructed in parallel. They were designated pHSRV-M/T-D/A and pHSRV ΔFS-D/A, respectively.

In analogy, a splice acceptor consensus sequence located directly upstream of the first AUG codon of *pol* was changed from 5′-TCCTCCACAG**ATG**-3′ (splice acceptor consensus sequence underlined; ATG initiator in boldface) to 5′-***AGTAGT***ACTGATG-3′ by using primers 2936s plus dSAa (5′-CATC***A***GT***ACTACT***CGTGGCACTCTGTGTCAC-3′) and primers dSAs (5′-CACG***AGTAGT***AC***T***GATGAATCCTCTCCAGC-3′) plus 3486a. The mutants were characterized by restriction enzyme analysis and DNA sequencing, demonstrating that the PCR-amplified DNAs contained the original pHSRV13 sequence except for the mutations introduced.

Cells. COS7 and BHK cells were grown and virus infections were done as described previously (27). The HFV indicator cell line FAB was cultured as described previously (42). Cell-free HFV particles were harvested and HFV titrations on FAB cells were performed as described previously (26). The titer was expressed as the number of blue cells per milliliter of HFV inoculum.

DNA transfection and expression assays. Transfections by electroporation of 10 μg of DNA and reporter gene assays were performed and quantitated as described previously (25, 27). Transfection efficiencies were about 20 to 30% of the transfected cells as measured by β-galactosidase expression from cotransfected pCMVβgal DNA.

Immunological techniques. The preparation of cell-associated antigen, Western blotting (immunoblotting), and the sera used were described previously (4, 18, 19, 24, 27, 28, 35). Specifically bound antibodies were reacted with protein A-peroxidase (Sigma, Deisendorf, Germany) and detected with diaminobenzidine as chromogen (37) or with a commercially available enhanced chemiluminescence (ECL) kit (Gibco BRL, Karlsruhe, Germany) and exposed to X-ray films (Kodak).

Preparation of HFV-specific antisera. An HFV NC-specific polyclonal rabbit antiserum was induced against the predicted HFV NC part of the *gag* gene also containing some carboxy-terminal CA residues by expressing HFV DNA sequences from genomic position 2381 to 3128 corresponding to codons 388 to 636 of the *gag* gene in the pET16b expression vector as described previously (28). Similarly, HFV sequences from genomic position 3117 to 3590 corresponding to codons 9 to 167 of the HFV *pol* covering the PR region and extending into the reverse transcriptase (RT) domain were expressed in pET16b, and the resulting recombinant antigen was used to induce the *pol*-specific antiserum pol 9-167.

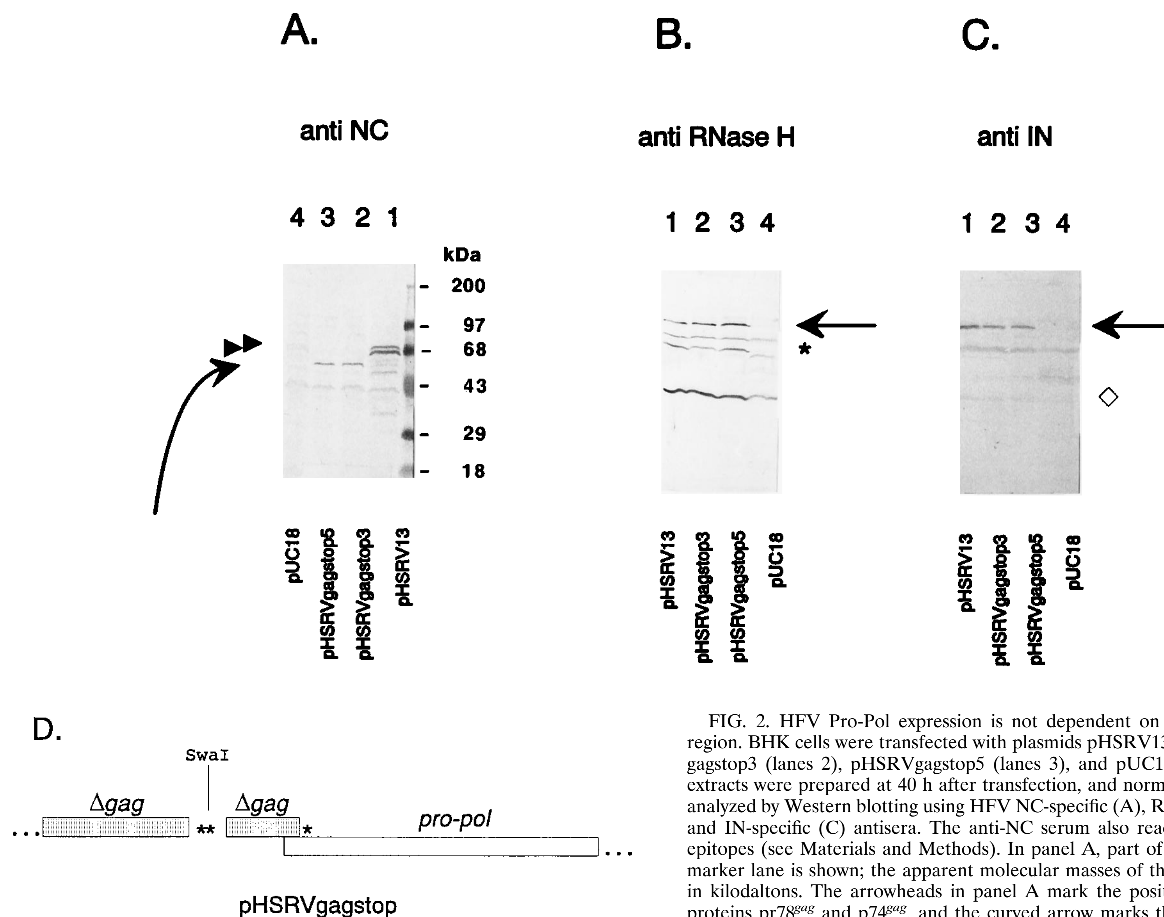

FIG. 2. HFV Pro-Pol expression is not dependent on the *gag-pol* overlap region. BHK cells were transfected with plasmids pHSRV13 (lanes 1), pHSRV-gagstop3 (lanes 2), pHSRVgagstop5 (lanes 3), and pUC18 (lanes 4). Protein extracts were prepared at 40 h after transfection, and normalized aliquots were analyzed by Western blotting using HFV NC-specific (A), RNase H-specific (B), and IN-specific (C) antisera. The anti-NC serum also reacts with CA-specific epitopes (see Materials and Methods). In panel A, part of the molecular mass marker lane is shown; the apparent molecular masses of the proteins are given in kilodaltons. The arrowheads in panel A mark the positions of the wt Gag proteins pr78*gag* and p74*gag*, and the curved arrow marks that of the truncated form of about 60 kDa expressed from pHSRVgagstop. In panels B and C, the position of the 120-kDa Pro-Pol polyprotein is indicated by the arrow; the 80-kDa RT/RNase H is marked by an asterisk in panel B, and the 40-kDa IN protein which becomes visible upon overexposure is marked by a diamond in panel C. Panel D schematically shows the location of the stop codons introduced into *gag* for the construction of plasmid pHSRVgagstop, which does not contain the HFV *gag-pol* overlap region. Asterisks represent stop codons.

RESULTS

HFV Pol expression is not affected by termination codons in *gag*. Two consecutive stop codons were introduced into the *gag* gene at the *Swa*I site substituting for Gag residues 534 (Gln) and 535 (Gly). The first stop codon is 114 codons upstream of the end of *gag* and 88 codons upstream of the overlap region of *gag* and *pol* (Fig. 2D; asterisks within *gag* mark the two stop codons introduced). This double mutation should lead to a truncated Gag protein, and it is expected to prevent *pol* expression by ribosomal frameshifting in the *gag-pol* overlap region. The mutation was introduced into the infectious HFV provirus pHSRV13, and the phenotype of the mutant was analyzed upon transfection into COS7 and BHK cells in parallel to the wild-type (wt) genome.

HFV antigen expression was analyzed by Western blotting using *gag* (Fig. 2A)- and *pol* (Fig. 2B and C)-specific antisera. wt plasmid pHSRV13 expressed the Gag precursor protein pr78*gag* and the HFV PR proteolytic cleavage product p74*gag* (pair of arrowheads in panel A), the CA of 33 kDa, and a CA-NC processing intermediate of 50 kDa consistent with the calculated values for NC and CA (2, 4, 19). In contrast, plasmids pHSRVgagstop3 and -5 (two independent recombinant clones carrying the truncated *gag* gene) expressed only a single *gag*-specific precursor of about 60 kDa (curved arrow in panel A) consistent with the calculated size of the resulting truncated Gag protein and the location of the engineered stop codons. In contrast, Pro-Pol expression was virtually unchanged in comparison with transfections with wt pHSRV13 DNA when analyzed with RNase H- and IN-specific antisera (Fig. 2B and C). The typical patterns of the unprocessed 120-kDa Pro-Pol precursor (arrows in panels B and C), the 80-kDa RT/RNase H (asterisk in panel B), and the 40-kDa IN (diamond in panel C) were detectable in extracts from wt pHSRV13- and pHSRV-gagstop-transfected cells. This result suggests that HFV *pol* is not expressed as a Gag-Pol fusion protein, since the *gag* gene of pHSRVgagstop does not overlap the amino terminus of *pol*. The result supports the conclusion that a Pro-Pol polyprotein of 120 kDa and not a 190-kDa Gag-Pol precursor protein is produced in HFV-infected cells and that the HFV PR cleavage site used to form p74*gag* is located close to the carboxy terminus of Gag (19).

When supernatants of pHSRVgagstop-transfected BHK cells were titrated on FAB cells, plasmid pHSRVgagstop was not infectious. Since Pol expression was not changed, the carboxy-terminal 114 amino acid residues of Gag containing two of the three Gly-Arg-rich sequences of HFV NC are required for infectivity. The results show that the full-length 120-kDa Pro-Pol protein is synthesized independently of Gag expression, since a Met initiation codon to express the carboxy terminus of Gag is not available in the HFV *gag* sequence in plasmid pHSRVgagstop. The Pro-Pol protein expressed in pHSRVgagstop-transfected cells exhibited specific protease

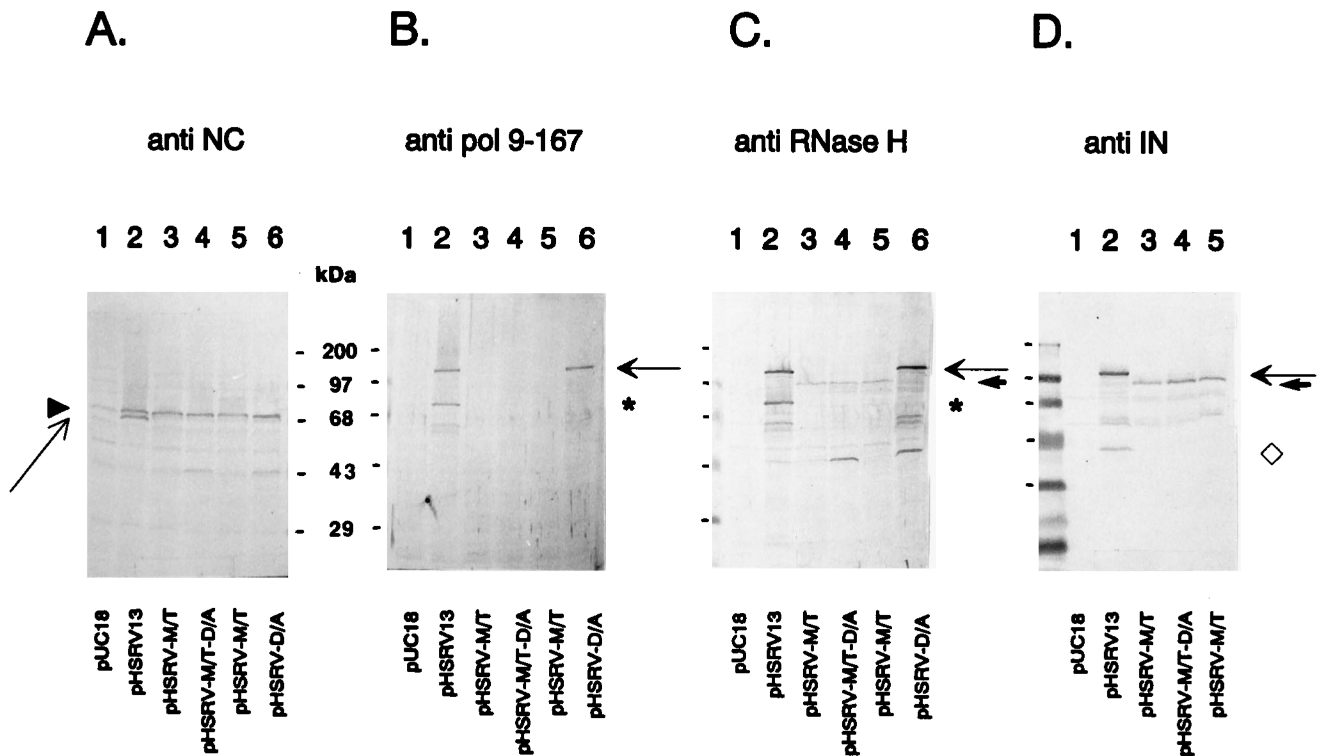

FIG. 3. Substitution of the first Met of *pol* by Thr abrogates Pro-Pol expression. Cellular extracts harvested from BHK cells at 40 h after transfection with plasmids pUC18 (lanes 1), pHSRV13 (lanes 2), pHSRV-M/T (lanes 3 and 5, representing two independent recombinants), pHSRV-M/T-D/A (lanes 4), and pHSRV-D/A (lanes 6) were used for Western blot analysis with antisera against HFV NC (A), pol 9-167 (B), RNase H (C), and IN (D). In panel A, the arrowhead points to the position of pr78^{gag} and the long arrow marks the position of p74^{gag}. In panels B to D, the long arrow points to the position of the 120-kDa Pro-Pol polyprotein; the 80-kDa RT/RNase H protein is marked by an asterisk in panels B and C; and the 40-kDa IN protein in panel D is indicated by a diamond. The position of the truncated Pol protein of 95 kDa is indicated by a small arrow in panels C and D. For some blots, half of the protein marker lane with the apparent molecular masses is given.

activity, since the 80-kDa RNase H and 40-kDa IN domains of the Pro-Pol polyprotein were detectable.

We conclude that the first Met codon of *pol*, which is located at amino acid position 9 in the *pol* ORF, is the likely candidate for the initiation of HFV Pro-Pol expression (Fig. 1A and B). Correspondingly located Met codons are also present in the *pol* genes of the known simian foamy viruses (23).

Substitution of the first Met in HFV *pol* by Thr abrogates efficient Pol expression. The Met codon at position 9 of the *pol* ORF in plasmid pHSRV13 was changed to ACG (M/T) by site-specific mutagenesis without affecting the encoded protein sequence of the overlapping *gag* gene (Fig. 1B and C). In parallel, the control clone pHSRV-M/T-D/A, which additionally contained a mutation in which an Ala replaced the Asp residue in the PR catalytic center, inactivating the HFV PR, was constructed.

The constructs were transfected into BHK cells in parallel with wt pHSRV13 DNA, the PR-inactive mutant pHSRV-D/A, and pUC18 DNA (Fig. 3). Protein blots performed with antisera against *pro-pol* residues 9 to 167 (panel B), RNase H (panel C), and IN (panel D) demonstrated that the expression of the full-length 120-kDa Pro-Pol polyprotein (arrow) was almost absent in pHSRV-M/T (panels B to D, lanes 3 and 5)- or pHSRV-M/T-D/A (lanes 4)-transfected cells compared with pHSRV13 (lanes 2)- or pHSRV-D/A (lanes 6)-transfected cells. Minute amounts of the 120-kDa Pro-Pol protein were nevertheless detectable by means of the ECL detection system (data not shown). In transfections with the M/T substitution mutants, a protein band of about 95 kDa (thick arrow in panels C and D; see also Fig. 5A, B, and E) that was absent in pHSRV13-transfected cells was detectable with antisera against RNase H and IN. This band likely represents a Pol polyprotein initiated at the second Met residue of the *pol* gene 198 codons downstream from the mutated Met residue (Fig. 1A, second Met represented by the smaller triangle in *pol*). In line with this assumption, antiserum pol 9-167 specific for epitopes upstream of codon 167 of the *pol* ORF did not detect the 95-kDa protein (Fig. 3B). Protein bands corresponding to the PR-mediated mature products 80-kDa RT/RNase H (asterisk in panels B and C) and 40-kDa IN (diamond in panel D) were not detectable in extracts from pHSRV-MT- or pHSRV-M/T-D/A-transfected cells, whereas they were present in wt pHSRV13-transfected cells.

Immunoblot analyses performed with *gag*-specific antisera directed against NC (panel A) or CA or MA domains (data not shown) showed comparable amounts of pr78^{gag} in wt and M/T or M/T-D/A mutant provirus transfections (arrowhead in panel A). The p74^{gag} intermediate (arrow in panel A) was virtually absent in the M/T mutant-transfected cells but was efficiently expressed in wt-transfected cells (19). This result shows that the PRs of mutants pHSRV-D/A, pHSRV-M/T-D/A, and pHSRV-M/T were either inactivated by mutagenesis of the active center, only minimally expressed, or both.

Titrations of cell culture supernatants from pHSRV13-, pHSRV-M/T-, and pHSRV-M/T-D/A-transfected BHK cells on FAB cells showed that pHSRV-M/T DNA gave only 1 to 10 blue FAB cells per ml whereas the transfection of pHSRV13 DNA induced about 10^4 blue FAB cells per ml. The low-level infectivity of plasmid pHSRV-M/T may be due to low-level *pol* expression. Plasmid pHSRV-M/T-D/A was not infectious.

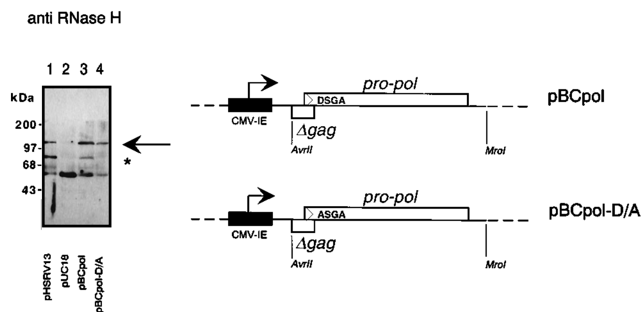

FIG. 4. Uncoupling of the expression of authentic HFV Pro-Pol proteins from Gag. Protein extracts from COS7 cells were prepared at 40 h after transfection with plasmids pUC18 (lane 2), pBCpol (lane 3), and pBCpol-D/A (lane 4) and analyzed by Western blotting using an HFV antiserum against RNase H. Extracts from pHSRV13-transfected BHK cells were analyzed in parallel (lane 1). Immunoblots were developed with the ECL system. The position of the 120-kDa Pro-Pol polyprotein is marked by an arrow, and the position of the 80-kDa RT/RNase H is indicated by an asterisk. The positions of the size markers with their apparent molecular masses (in kilodaltons) are given. The structures of the HFV Pro-Pol expression plasmids are schematically shown on the right. The HFV *Avr*II-to-*Mro*I DNA fragment containing the complete HFV *pro-pol* and part of the *gag* gene is under the control of the cytomegalovirus immediate-early (CMV-IE) promoter. The wt and mutant amino acid sequences of the catalytic center of HFV PR are shown; the triangle indicates the location of the first Met codon of the *pol* ORF.

The experiments indicate that the HFV Pro-Pol protein is encoded by codons 9 to 1151 of the *pol* ORF.

Uncoupling of authentic Pol protein expression from Gag synthesis. To confirm that carboxy-terminal regions of HFV Gag are not required for the synthesis of authentic and functionally active Pro-Pol proteins, the full-length *pro-pol* genes from wt pHSRV13 and PR mutant pHSRV-D/A were expressed under the control of the cytomegalovirus immediate early promoter in COS7 cells. Plasmid clone pBCpol harbors the complete Pro-Pol sequence containing an intact PR and, in addition, 71 *gag* codons (Fig. 4; for details, see Materials and Methods). This part of the *gag* ORF does not contain a Met codon for translational initiation. In plasmid clone pBCpol-D/A, an Ala replaced the Asp residue in the PR catalytic center.

When transfected into COS7 cells, plasmids pBCpol and pBCpol-D/A (Fig. 4, lanes 3 and 4, respectively) directed the expression of a 120-kDa Pro-Pol polyprotein (straight arrow) indistinguishable from that expressed from pHSRV13-transfected BHK cells (lane 1). As expected, mature cleavage products corresponding to the 80-kDa RT/RNase H (asterisk) were detectable in extracts from wt pBCpol-transfected cells but not upon transfection of PR mutant pBCpol-D/A DNA.

Coexpression of HFV Pro-Pol complements the defect of pHSRV-M/T mutants in *trans*. To determine whether the reduced infectivity and the absence of $p74^{gag}$ intermediate in plasmid pHSRV-M/T can be attributed to a lack of Pro-Pol expression, cotransfections of plasmids pHSRV13 and pHSRV-M/T with pUC18, pBCpol, and pBCpol-D/A were performed. Different ratios of proviral DNA and Pro-Pol expression vectors were used. Cell culture supernatants taken at 40 h after transfection of BHK or COS7 cells were assayed for HFV infectivity by using FAB cells. Representative titers of supernatants from BHK cells transfected with 1 μg of proviral DNA and 9 μg each of pUC18, pBCpol, and pBCpol-D/A are shown in Table 1. The titer of mutant pHSRV-M/T was always below 10 blue cells per ml, whereas coexpression of wt Pro-Pol proteins from plasmid pBCpol resulted in an approximately 100-fold increase in titer. As expected, the cotransfection of pBCpol-D/A DNA did not increase the viral titer. Coexpression of either Pro-Pol expression plasmid and wt pHSRV13 DNA did not significantly alter the titer.

TABLE 1. *trans* complementation of infectivity of mutant provirus pHSRV-M/T by coexpression of eukaryotic HFV Pro-Pol expression plasmids

Provirus	Titer (no. of blue cells/ml of inoculum) after cotransfection with[a]:		
	pUC18	pBCpol	pBCpol-D/A
pHSRV13	4×10^3	6.2×10^3	5.4×10^3
pHSRV-M/T	4.5	6×10^2	1.2

[a] BHK cell culture supernatants harvested at 40 h after cotransfection of the wt and mutant HFV proviruses with the Pro-Pol expression plasmids were titrated for HFV infectivity on FAB cells.

When cell extracts from *trans*-complemented BHK cells were analyzed by immunoblotting (data not shown), coexpression of pHSRV-M/T DNA with pBCpol DNA resulted in a faint band of $p74^{gag}$ that was absent upon cotransfection of pUC18 or pBCpol-D/A DNA. This result confirms that plasmid pBCpol directs the expression of biologically active PR.

The infectivity of pHSRV-M/T DNA was only partially restored, and proteolytic cleavage did not reach wt levels, since the expression levels of both pBCpol plasmids were low (Fig. 4). The low-level expression may explain why plasmid pBCpol-D/A did not reduce proteolytic processing and infectivity of pHSRV13 in *trans*. The results show that the full-length 120-kDa Pro-Pol protein expressed from plasmid pBCpol is capable of providing the enzymatic activities encoded in the HFV *pol* gene in *trans*.

In-frame fusion of the HFV *gag* and *pol* genes results in a partially active PR. HFV *gag* was fused in frame to *pol* by deleting 1 bp in the *gag-pol* overlap region in clones pHSRVΔFS to imitate the situation in oncoviruses and lentiviruses (Fig. 1D). bp 3122 in the *gag-pol* overlap region was deleted, since this base pair was assumed to be located close to the assumed HFV +1 frameshifting site (23). The mutagenesis resulted in the in-frame fusion of the *gag* and *pro-pol* genes, giving rise to a genetically engineered 190-kDa Gag-Pol fusion protein (Fig. 1D). A 120-kDa Pro-Pol protein that starts at Met residue 9 of the *pol* ORF cannot be formed by any of the ΔFS clones, as this Met was fused to the third, neither *gag*- nor *pol*-specific reading frame (Fig. 1D).

BHK cells were transfected with mutant pHSRVΔFS, pHSRV-M/T, and pHSRV-D/A DNAs; double-mutant pHSRVΔFS-D/A DNA; and wt pHSRV13 DNA, and cellular extracts were used for immunoblot analysis. Immune reactions with HFV *pol*-specific antisera directed against RNase H (Fig. 5A) and IN domains (panels B and E) showed that a *pol*-specific protein of approximately 190 kDa was present in pHSRVΔFS-D/A-transfected cells (panels A and B, lanes 5, open arrow) but was not detectable, e.g., in pHSRV13 (lanes 2)-, or pHSRV-D/A (lanes 7)-transfected cells. The 190-kDa polyprotein was faintly detectable in extracts from pHSRVΔFS-transfected BHK cells (panel E, lanes 3 and 4 [two independent recombinants]) when the more sensitive ECL detection system was used, and again it was not detectable in pHSRV13-transfected cells (panel E, lane 2). The 190-kDa protein in pHSRVΔFS-D/A-transfected cells reacted also with HFV *gag*-specific MA and CA antisera (panels C and D, lanes 5, open arrow), confirming that it corresponded to the engineered HFV Gag-Pol fusion protein that was not detectable in wt-transfected cells (lanes 2). The 190-kDa protein was

faintly detectable in pHSRVΔFS-transfected cells when the ECL detection system was used with the CA antiserum (data not shown).

In addition, a *pol*-specific protein that comigrated with the 120-kDa Pro-Pol precursor from pHSRV13- and pHSRV-D/A-transfected cells (Fig. 5A and B, lanes 2 and 7; Fig. 5E, lane 2) was overexpressed in pHSRVΔFS-transfected cells (Fig. 5A, B, and E, lanes 3 and 4). The 120-kDa protein was absent in pHSRVΔFS-D/A-transfected cells (lanes 5), indicating that it is an HFV PR cleavage product of the engineered 190-kDa Gag-Pol fusion protein (see below). The mature 80-kDa RT/RNase H (asterisk in panel A) and the 40-kDa IN (diamond in panels B and E) were not observed in any mutant but were expressed by pHSRV13 DNA (lanes 2). We assume that the increased concentrations of the 95-kDa Pol protein expressed from plasmids pHSRVΔFS (lanes 3 and 4, thick arrow) were possibly formed by translational initiation of *pol* expression at the second Met residue in *pol* in analogy to plasmid pHSRV-M/T (Fig. 1A and 5A, B, and E).

A *gag*-specific protein comigrating with the PR cleavage product $p74^{gag}$ from wt-transfected cells (Fig. 5C and D, long arrow) was present in pHSRVΔFS-transfected cells but not in pHSRVΔFS-D/A-transfected cells. The $p74^{gag}$ and the 120-kDa Pro-Pol protein derived from plasmid pHSRVΔFS are most probably formed by the HFV PR-mediated proteolysis of the engineered 190-kDa fusion protein (Fig. 1D), since these specific cleavage products were absent when the HFV PR had been inactivated by mutagenesis (clone pHSRVΔFS-D/A). Accordingly, the full-length $pr78^{gag}$ (Fig. 5C and D, arrowhead) was not expressed from pHSRVΔFS or pHSRVΔFS-D/A (lanes 7) mutants, since the *gag* stop codon is absent because of the in-frame fusion of *gag* to *pol* (Fig. 1D). Control plasmids pHSRV-M/T (Fig. 5, lanes 6) and pHSRV-D/A (lanes 7) exclusively expressed $pr78^{gag}$.

The titration of cell culture supernatants derived from these transfections showed that the infectivity of pHSRVΔFS was more than 100-fold reduced in comparison with that of pHSRV13 (40 blue FAB cells per ml for pHSRVΔFS versus 7 $\times$ 10^3 blue FAB cells per ml for pHSRV13). None of the plasmids carrying the mutated PR directed the synthesis of infectious HFV particles.

The results indicate that the PR encoded by plasmid pHSRVΔFS is capable of cleaving the site that is used to

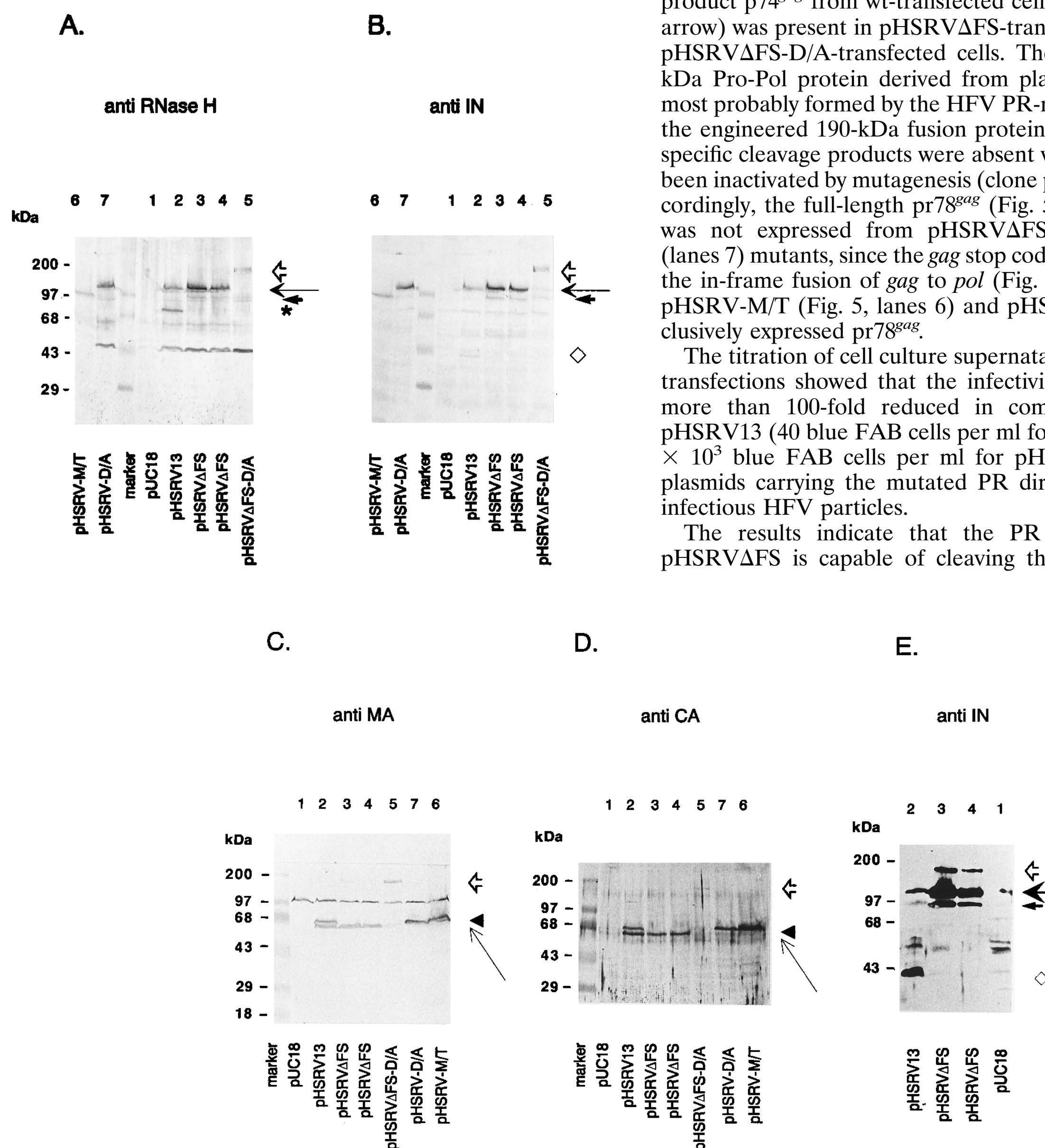

FIG. 5. In-frame fusion of HFV *gag* and *pol* results in a partially active HFV PR for the engineered 190-kDa Gag-Pol fusion protein. BHK cells were harvested at 40 h after transfection with pUC18 (lanes 1), pHSRV13 (lanes 2), pHSRVΔFS (lanes 3 and 4, representing two independent recombinants), pHSRVΔFS-D/A (lanes 5), pHSRV-M/T (lanes 6), and pHSRV-D/A (lanes 7) DNAs. Protein samples were analyzed by Western blotting using HFV RNase H-specific (A), IN-specific (B and E), MA-specific (C), and CA-specific (D) antisera. The open arrow in each of the panels indicates the position of the engineered 190-kDa Gag-Pol fusion protein. The long arrows in panels A, B, and E point to the 120-kDa Pro-Pol protein and the comigrating *pol*-specific protein expressed by pHSRVΔFS DNA; the short arrows indicate the position of the 95-kDa Pol protein. The asterisk in panel A marks the position of the 80-kDa RT/RNase H, and the diamonds in panels B and E mark that of the 40-kDa IN protein. The arrowheads in panels C and D point to $pr78^{gag}$, and the long arrows point upward to $p74^{gag}$. The positions of marker proteins and their apparent molecular masses are indicated. Immunoblots in panels A to D were developed by using diaminobenzidine as chromogen, whereas HFV-specific proteins in panel E were detected by ECL. Some of the blots were intentionally overexposed to detect the engineered 190-kDa Gag-Pol fusion protein expressed from plasmids pHSRVΔFS and pHSRVΔFS-D/A.

process pr78^{gag} to p74^{gag}. In contrast, additional sites in the Gag-Pol fusion protein were not cleaved by the PR expressed by pHSRVΔFS DNA.

DISCUSSION

A *pol* expression strategy that does not rely on the synthesis of a Gag-Pol polyprotein has not been reported for any other retrovirus. Substantial amounts of the HFV 120-kDa Pro-Pol protein are expressed by a mechanism that does not use ribosomal frameshifting in the overlap region of the *gag* and *pol* genes. Close inspection of the *gag-pol* overlap region of HFV revealed that there is no +1 frameshifting sequence as reported previously for the different classes of Ty retrotransposons (11) nor for frameshift of the recently identified human ornithine decarboxylase antizyme (29). Pseudoknot structures comparable in length and stability to those detected in other retroviruses (6) were not found in corresponding regions of HFV.

Ribosomal frameshifting and internal initiation of translation (as discussed below) at too short a distance have not been reported so far and seem to be incompatible. Competition for viral RNA binding sites between cellular factors necessary for ribosomal frameshifting and translational initiation might be the reason for this limitation (21, 33). When our data are taken together, it must be concluded that the +1 frameshifting deduced from the sequence of the HFV *gag-pol* overlap region and previously assumed to exist (23, 31) does not occur under the conditions used.

Our results, however, do not rule out the possibility (i) that a frameshifting mechanism is utilized by HFV in different cells that were not analyzed here, (ii) that the half-life of the putative Gag-Pol fusion protein may be extremely short in HFV-infected cells, or (iii) that there are HFV genomes in an individual wt virus stock that contain insertions or deletions in the *gag-pol* overlap region different from the pHSRV13 sequence which could lead to a Gag-Pol fusion protein by readthrough or frameshifting.

The Gag-independent expression of Pro-Pol reported here explains why in cells transfected with wt provirus pHSRV13 and the PR-inactive provirus pHSRV-D/A, a *pol*-encoded protein of 120 kDa and not a 190-kDa Gag-Pol fusion protein was detected (19). Thus, there is no evidence for an HFV Gag-Pol precursor encoded by intact HFV genomes that is analogous to the Gag-Pol fusion proteins observed in oncovirus- and lentivirus-infected cells.

We conclude that the Met codon at position 9 of *pol*, which is conserved among sequenced foamy viruses, is used for initiation of HFV *pol* expression, since the mutation of this Met codon to Thr reduced Pol translation to very low levels. Minute amounts of Pro-Pol were synthesized by pHSRV-M/T proviruses, as indicated by residual product levels and the synthesis of very small numbers of infectious particles, as ACG codons may be used for translational initiation (15). The existence of a spliced *pol* transcript is highly unlikely, since HFV mRNA species of a size between that of the genomic RNA and that of the spliced *env* RNAs were not detected either in HFV or in simian foamy viruses (32, 34). In particular, spliced *pol* transcripts were not detectable by reverse transcription-coupled PCR with primers located at the beginning of the R region in the 51-nucleotide HFV leader RNA and at various sites of *pol* (22a). In addition, mutations that destroyed an apparent splice acceptor site in the *gag-pol* overlap region directly upstream of the first *pol* Met did not change *pol* gene expression or infectivity (see Materials and Methods). These observations rule out the possibility that *pol*-specific transcripts are formed by splicing and argue for alternative modes of *pol* expression. Presently, we favor the idea that *pol* expression is directed by the HFV genomic RNA and started by internal initiation. The first AUG codon of *pol* is in a sequence context that is not favorable for translational initiation (20) (Fig. 1B), and in particular, seven AUG codons are present in *gag* (about 2 kbp) between the first AUG of *gag* and the first Met codon in *pol*. Since some of these *gag* AUG codons are in a genetic context favorable for translational initiation, a leaky scanning mechanism to the *pol* Met seems unlikely (20). Therefore, mechanisms for translational initiation that are different from the scanning model and that do not use non-AUG codons as initiators have to be considered, particularly those already established for viral systems that are capable of decoding bicistronic mRNAs (36). In hepatitis B virus, for example, internal initiation possibly by a modified leaky scanning mechanism is used for the synthesis of Pol polyproteins (7, 8, 13, 22, 38). The mechanism of internal initiation shown to exist for cauliflower mosaic virus Pol expression is possibly due to a nonlinear ribosome migration or "relay race" that might be also valid for HFV (14, 36). The utilization of the *gag* start codon in murine leukemia viruses was recently shown to be enhanced by a mechanism relying on internal ribosomal entry sites (5, 41). This mechanism or modified versions of it cannot be ruled out for HFV Pro-Pol expression. Further experiments are required to determine what mechanisms of translation are used for HFV *pol* expression.

Our experimental data (Fig. 3 and 4) indicate that the HFV Pro-Pol protein initiated at the first Met of the *pol* gene has HFV PR activity even in the absence of HFV Gag proteins or preformed capsids present in the cytosol of infected cells (27). The distance of the amino-terminal Met of the HFV PR from its active center (AspSerGlyAla) is, within 23 residues, comparable to the spacing from the amino-terminal residue of the human immunodeficiency virus type 1 PR (40). Moreover, protein sequences directly downstream, but not sequences upstream, of the first Met of *pol* are highly conserved in known foamy viruses (23).

The mutant pHSRVΔFS protein was designed and engineered to serve as an HFV model Gag-Pol fusion protein that could be compared with the corresponding well-studied fusion proteins of oncoviruses and lentiviruses. The experiments showed that the engineered 190-kDa HFV Gag-Pol model fusion protein was processed by the HFV PR, as the double mutant pHSRVΔFS-D/A did not express the p74^{gag} or the 120-kDa Pro-Pol protein. The site cleaved by pHSRVΔFS is likely located in the carboxy terminus of Gag, which is also used to generate p74^{gag} in HFV-infected cells. The reason why other sites in Gag and Pol were not cleaved remains unknown.

With respect to the synthesis of Pol proteins, there are several requirements that have to be met to ensure that infectious retrovirus particles are produced. Firstly, Pol proteins have to be targeted to the forming capsids in order to assemble within viral cores. The Gag-Pol fusion proteins of oncoviruses and lentiviruses place the enzymatic activities contained within the *pol* gene products into the forming virion. Foamy viruses likely developed a special mechanism for targeting Pol to virions that is different from that of the oncoviruses and lentiviruses, a subject for further studies. The second requirement is the fine control of the stoichiometry of Gag and Pol proteins. Both points are met by the quantitatively controlled transcriptional frameshifting or inefficient readthrough in oncoviruses and lentiviruses (9). Does the mechanism for HFV Pro-Pol expression control the stoichiometry of Gag and Pol proteins? Since significant amounts of the HFV 120-kDa Pro-Pol polyprotein are produced in infected cells, it seems that the lower specific

activities of the HFV RT and IN relative to those of the RTs and INs of avian myeloblastosis virus, murine leukemia viruses, and human immunodeficiency virus type 1 may require larger total concentrations of the mature and active HFV enzymes (17, 18, 35).

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REFERENCES

1. **Achong, B. G., P. W. A. Mansell, M. A. Epstein, and P. Clifford.** 1971. An unusual virus in cultures from a human nasopharyngeal carcinoma. J. Natl. Cancer Inst. **46:**299–307.
2. **Aguzzi, A.** 1993. The foamy virus family: molecular biology, epidemiology and neuropathology. Biochim. Biophys. Acta **1155:**1–24.
3. **Aronoff, R., A. M. Hajjar, and M. L. Linial.** 1993. Avian retroviral RNA encapsidation: reexamination of functional 5′ RNA sequences and the role of nucleocapsid Cys-His motifs. J. Virol. **67:**178–188.
4. **Bartholomä, A., W. Muranyi, and R. M. Flügel.** 1992. Bacterial expression of the capsid antigen domain and identification of native *gag* proteins in spumavirus-infected cells. Virus Res. **23:**27–38.
5. **Berlioz, C., and J.-L. Darlix.** 1995. An internal ribosomal entry mechanism promotes translation of murine leukemia virus *gag* polyprotein precursors. J. Virol. **69:**2214–2222.
6. **Chamorro, M., N. Parkin, and H. E. Varmus.** 1992. An RNA pseudoknot and an optimal heptameric shift site are required for highly efficient ribosomal frameshifting on a retroviral messenger RNA. Proc. Natl. Acad. Sci. USA **89:**713–717.
7. **Chang, L.-J., D. Ganem, and H. E. Varmus.** 1990. Mechanism of translation of the hepadnaviral polymerase (P) gene. Proc. Natl. Acad. Sci. USA **87:** 5158–5162.
8. **Chang, L.-J., P. Pryciak, D. Ganem, and H. E. Varmus.** 1989. Biosynthesis of the reverse transcriptase of hepatitis B viruses involves de novo translational initiation not ribosomal frameshifting. Nature (London) **337:**364–368.
9. **Coffin, J. M.** 1990. Replication of retroviridae, p. 1437–1500. *In* B. N. Fields and D. M. Knipe (ed.), Virology. Raven Press, New York.
10. **Cullen, B. R.** 1991. Human immunodeficiency virus as a prototypic complex retrovirus. J. Virol. **65:**1053–1056.
11. **Farabough, P. J.** 1995. Post-transcriptional regulation of transposition by Ty retrotransposons of Saccharomyces cerevisiae. J. Biol. Chem. **270:**10361–10364.
12. **Flügel, R. M., A. Rethwilm, B. Maurer, and G. Darai.** 1987. Nucleotide sequence analysis of the *env* gene and its flanking regions of the human spumaretrovirus reveals two novel genes. EMBO J. **6:**2077–2084.
13. **Fouillot, N., S. Tlouzeau, J.-M. Rossignol, and O. Jean-Jean.** 1993. Translation of the hepatitis B virus P gene by ribosomal scanning as an alternative to internal initiation. J. Virol. **67:**4886–4895.
14. **Fütterer, J., Z. Kiss-Laslo, and T. Hohn.** 1993. Nonlinear ribosome migration on cauliflower mosaic virus 35S RNA. Cell **73:**789–802.
15. **Gupta, K. C., E. Ono, E. V. Aritzia, and M. Inaba.** 1994. Translation initiation from non-AUG codons in COS1 cells is mRNA species dependent. Biochem. Biophys Res. Commun. **201:**567–573.
16. **Jacks, T.** 1990. Translational suppression in gene expression in retroviruses and retrotransposons. Curr. Top. Microbiol. Immunol. **157:**93–124.
17. **Kögel, D., M. Aboud, and R. M. Flügel.** 1995. Mutational analysis of the reverse transcriptase and ribonuclease H domains of the human foamy virus. Nucleic Acids Res. **23:**2621–2625.
18. **Kögel, D., M. Aboud, and R. M. Flügel.** 1995. Molecularbiological characterization of the human foamy virus reverse transcriptase and ribonuclease H domains. Virology **213:**97–108.
19. **Konvalinka, J., M. Löchelt, H. Zentgraf, R. M. Flügel, and H.-G. Kräusslich.** 1995. Active spumavirus proteinase is essential for virus infectivity but not for formation of the Pol polyprotein. J. Virol. **69:**7264–7268.
20. **Kozak, M.** 1991. Structural features in eukaryotic mRNAs that modulate the initiation of translation. J. Biol. Chem. **266:**19867–19870.
21. **Lee, S. I., J. G. Umen, and H. E. Varmus.** 1995. A genetic screen identifies cellular factors involved in retroviral −1 frameshifting. Proc. Natl. Acad. Sci. USA **92:**6587–6591.
22. **Lin, C.-G., and S. J. Lo.** 1992. Evidence for involvement of a ribosomal leaky scanning machanism in the translation of the hepatitis B virus Pol gene from the viral pregenome RNA. Virology **188:**342–352.

22a. **Löchelt, M.** Unpublished observations.

23. **Löchelt, M., and R. M. Flügel.** 1995. The molecular biology of primate spumaviruses, p. 239–292. *In* J. A. Levy (ed.), The Retroviridae, vol. 4. Plenum Press, New York.
24. **Löchelt, M., R. M. Flügel, and M. Aboud.** 1994. The human foamy virus internal promoter directs the expression of the functional Bel 1 transactivator and Bet protein early after infection. J. Virol. **68:**638–645.
25. **Löchelt, M., W. Muranyi, and R. M. Flügel.** 1993. Human foamy virus genome possesses an internal, Bel 1-dependent and functional promoter. Proc. Natl. Acad. Sci. USA **90:**7317–7321.
26. **Löchelt, M., S. F. Yu, M. L. Linial, and R. M. Flügel.** 1995. The human foamy virus internal promoter is required for efficient gene expression and infectivity. Virology **206:**601–610.
27. **Löchelt, M., H. Zentgraf, and R. M. Flügel.** 1991. Construction of an infectious DNA clone of the full-length human spumaretrovirus genome and mutagenesis of the *bel 1* gene. Virology **184:**43–54.
28. **Mahnke, C., M. Löchelt, B. Bannert, and R. M. Flügel.** 1990. Specific enzyme-linked immunosorbent assay for the detection of antibodies to the human spumavirus. J. Virol. Methods **29:**13–22.
29. **Matsufuji, S., T. Matsufuji, Y. Miyazaki, Y. Murakami, J. F. Atkins, R. F. Gesteland, and S.-I. Hayashi.** 1995. Autoregulatory frameshifting in decoding mammalian ornithine decarboxylase antizyme. Cell **80:**51–60.
30. **Maurer, B., H. Bannert, G. Darai, and R. M. Flügel.** 1988. Analysis of the primary structure of the long terminal repeat and the *gag* and *pol* genes of the human spumaretrovirus. J. Virol. **62:**1590–1597.
31. **Mergia, A., and P. Luciw.** 1991. Replication and regulation of primate foamy viruses. Virology **184:**475–482.
32. **Mergia, A., K. E. S. Shaw, E. Pratt-Lowe, P. E. Barry, and P. A. Luciw.** 1991. Identification of the simian foamy virus transactivator gene (*taf*). J. Virol. **65:**2903–2909.
33. **Merrick, W. C.** 1992. Mechanism and regulation of eukaryotic protein synthesis. Microbiol. Rev. **56:**291–315.
34. **Muranyi, W., and R. M. Flügel.** 1991. Analysis of splicing patterns of human spumaretrovirus by polymerase chain reaction reveals complex RNA structures. J. Virol. **65:**727–735.
35. **Pahl, A., and R. M. Flügel.** 1993. Endonucleolytic cleavages and DNA-joining activities of the integration protein of human foamy virus. J. Virol. **67:**5426–5434.
36. **Rothnie, H. M., Y. Chapdelaine, and T. Hohn.** 1994. Pararetroviruses and retroviruses: a comparative review of viral structure and gene expression strategies. Adv. Virus Res. **44:**1–67.
37. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Schlicht, H.-J., G. Radziwill, and H. Schaller.** 1989. Synthesis and encapsidation of duck hepatitis B virus reverse transcriptase do not require formation of core-polymerase fusion protein. Cell **56:**85–92.
39. **Schliephake, A. W., and A. Rethwilm.** 1994. Nuclear localization of foamy virus Gag precursor protein. J. Virol. **68:**4946–4954.
40. **Skalka, A. M.** 1989. Retroviral proteases: first glimpses at the anatomy of a processing machine. Cell **56:**911–913.
41. **Vagner, S., A. Waysbort, M. Marenda, M.-C. Gensac, F. Amalric, and A.-C. Prats.** 1995. Alternative translational initiation of the Moloney murine leukemia virus mRNA controlled by internal ribosomal entry involving the p57/PTB splicing factor. J. Biol. Chem. **270:**20376–20383.
42. **Yu, S. F., and M. L. Linial.** 1993. Analysis of the role of the *bel* and *bet* open reading frames of human foamy virus by using a new quantitative assay. J. Virol. **67:**6618–6624.