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Foamy virus reverse transcriptase is expressed independently from the Gag protein

(reverse transcriptase expression/ribosomal frameshifting/retrovirus/pararetrovirus)

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ABSTRACT **In the foamy virus (FV) subgroup of retroviruses the *pol* genes are located in the +1 reading frame relative to the *gag* genes and possess potential ATG initiation codons in their 5′ regions. This genome organization suggests either a +1 ribosomal frameshift to generate a Gag–Pol fusion protein, similar to all other retroviruses studied so far, or new initiation of Pol translation, as used by pararetroviruses, to express the Pol protein. By using a genetic approach we have ruled out the former possibility and provide evidence for the latter. Two down-mutations (M53 and M54) of the *pol* ATG codon were found to abolish replication and Pol protein expression of the human FV isolate. The introduction of a new ATG in mutation M55, 3′ to the down-mutated ATG of mutation M53, restored replication competence, indicating that the *pol* ATG functions as a translational initiation codon. Two nonsense mutants (M56 and M57), which functionally separated *gag* and *pol* with respect to potential frame-shifting sites, were also replication-competent, providing further genetic evidence that FVs express the Pol protein independently from Gag. Our results show that during a particular step of the replication cycle, FVs differ fundamentally from all other retroviruses.**

The expression of the *pol* gene, which encodes the reverse transcriptase, is a key event in the replication cycle of retroid elements (1). All retroviruses investigated so far have evolved mechanisms to express their Pol proteins initially as Gag–Pol fusion proteins from which the Pol proteins are autocatalytically cleaved by the virus proteinases (1–3). As shown for murine leukemia virus, Rous sarcoma virus, and yeast retrotranposons, translational stop codon suppression, −1 ribosomal frameshifting, and +1 ribosomal frameshifting, respectively, are used to generate the Gag–Pol fusion proteins (2, 4–7). The common feature of Gag–Pol fusion proteins among retroviruses has suggested an important role in the regulation of the amount of Pol protein expressed in relation to Gag protein and for the incorporation of the Pol protein into the virus particle (2, 3). In contrast, pararetroviruses express the reverse transcriptase independently from their Gag homologue, the core protein (8–11). The fundamental difference in Pol protein expression strategies between the two virus groups is reflected in differences in virus assembly, maturation, and reverse transcription (12–14).

Foamy viruses (FVs) are a distinct subgroup of exogenous mammalian retroviruses. They have a complex genome structure and encode for accessory proteins, at least one of which, the Bel-1 trans-activator, has regulatory functions (15, 16). The primary structure of the *gag–pol* overlap of FVs shows close similarities to pararetroviruses (see Fig. 1). In particular, the *pol* genes are located in the +1 reading frame relative to the *gag* genes and a potential ATG initiation codon is located in the 5′ region of the *pol* genes (see Fig. 1) (17–20). This genome organization has suggested that FVs may not synthesize Gag–Pol fusion proteins (16). On the other hand, it has been predicted that FVs perform +1 ribosomal frameshifting to generate such Gag–Pol fusion proteins characteristic of other retroviruses (21). Various theories on the potential mechanisms and sites of frameshifting in the *gag–pol* overlap have been made (18, 21, 22). However, previous studies have failed to demonstrate a Gag–Pol fusion protein of ≈200 kDa in human FV (HFV)-infected cells, while the Gag and Pol precursor molecules of 74 and 127 kDa, respectively, were readily detectable (19, 23, 24). Moreover, it has been shown recently that even an HFV protease-deficient mutant gave rise to these Gag and Pol molecules, whereas in other retroviruses the Gag–Pol precursor can be readily detected with such mutants (6). These findings indicated that the mechanism of Pol protein expression in FVs may differ from other retroviruses in that it does not involve the synthesis of a Gag–Pol precursor protein. This hypothesis prompted us to clarify the question by using a genetic approach similar to previous studies investigating the mechanism of *pol* expression in pararetroviruses (8–11).

MATERIALS AND METHODS

Recombinant DNA. All virus mutants were constructed in the background of the infectious HFV plasmid pHSRV2 (25) by using standard molecular cloning techniques (26). Subclone pGP-1 was constructed by inserting a 2.28-kb *Nco* I fragment spanning the *gag–pol* overlap of pHSRV2 into a pUC19 derivative in which the multiple cloning site had been substituted by an *Nco* I linker. Commercially purchased, chemically synthesized oligonucleotides were used for DNA mutagenesis. Site-specific mutagenesis of pGP-1 was performed by recombinant PCR (27) using Pwo DNA polymerase (Boehringer Mannheim) with a primer pair flanking a 0.35-kb *Dra* III–*Afl* II fragment and two inner primers leading to the virus mutants shown in Fig. 2. The recombinant amplicons were digested with *Dra* III and *Afl* II, inserted into similarly digested pGP-1, and completely sequenced (28) to verify the desired mutations and exclude off-site mutations. A 1.83-kb fragment from the mutant pGP-1 plasmids was then substituted for the respective fragment of pHSRV2 using the single-cutting restriction enzymes *Swa* I and *Pac* I. At this final level the DNA sequence of the relevant part was again determined in all virus mutants.

Determination of Virus Replication. U251-MG, 3T3 TK$^-$, baby hamster kidney (BHK-21), and BHK/LTRlacZ cells were cultivated in minimal Eagle's medium (MEM)/5–10% fetal bovine serum/antibiotics. U512-MG, 3T3 TK$^-$, and BHK-21 cells seeded in 12-well plates were transfected with 2 μg of plasmid DNAs as $Ca_3(PO_4)_2$ coprecipitates (29). Cell-free supernatant from each transfection (0.2-μm filtrate) was harvested on every other day following day 3 after transfection, and

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Abbreviations: FV, foamy virus; HFV, human FV; ORF, open reading frame.

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appropriately diluted virus was titered on BHK/LTRlacZ indicator cells seeded in 24-well plates by a blue cell assay as described (25). Similar results as those shown in Table 1 were obtained when, in some experiments, the β-galactosidase activity was determined biochemically as described (30).

Protein Analysis. BHK-21 cells seeded in 6-well plates were transfected with 1 μg of plasmid DNAs using DOTAP reagent (Boehringer Mannheim). Forty-eight hours after transfection the cells were metabolically labeled with [^{35}S]methionine (Amersham–Buchler) for 16 hr and further processed as described (19, 23). Cellular lysates were prepared, cleared with normal rabbit serum, and treated with rabbit serum directed against a recombinant HFV reverse transcriptase domain (31). After precipitation with protein A–Sepharose (Pharmacia), the supernatant was reacted with rabbit serum directed against recombinant HFV capsid protein (31). Precipitated and extensively washed proteins were resolved on SDS/11.5% polyacrylamide gels in a tricine buffer system (32). Gels were soaked in Amplify (Amersham–Buchler), dried, and exposed to x-ray film.

RESULTS

Analysis of HFV *pol* ATG Mutants. The strong conservation of a methionine codon at the 5′ end of all sequenced FV genomes (Fig. 1) indicated a specific role of this codon for virus replication. We therefore generated two ATG down-mutants, M53 and M54, of an infectious molecular clone of HFV (25), changing the ATG to ACG and CTG, respectively. The relevant parts of the mutants M53 and M54 and the corresponding amino acids changes in the *gag* and *pol* open reading frames (ORFs) are shown in Fig. 2. Mutant viruses were analyzed for virus replication and expression of Gag and Pol proteins.

Virus replication was scored by transfecting BHK-21, mouse fibroblasts (3T3 TK$^-$), and human glioma cells (U251-MG) and titrating cell-free virus from the primary transfected cultures on BHK/LTRlacZ indicator cells (25). It has been reported previously that this assay is the most sensitive for measuring replicating HFV, because the activity of β-galactosidase expressed in the BHK/LTRlacZ cells is invariantly correlated to the amount of the Bel-1 transcriptional transactivator expressed by the infecting virus (30, 33). Table 1 shows that virus replication was not detected with the two ATG mutants in the three different cell lines tested.

When the expression of Gag and Pol proteins was analyzed by radioimmunoprecipitation after transient transfection of BHK-21 cells (Fig. 3), the recently identified pr74 Gag precursor molecule and its p70 cleavage product were readily detected in addition to the pr127 Pol precursor and the p80 reverse transcriptase cleavage product in cultures transfected with pHSRV2 (6, 19, 23). In contrast, both ATG mutants expressed only the pr74 Gag antibody-reactive protein but no detectable Pol protein (Fig. 3). The p70 and the p80 molecules are cleaved from the respective precursors by the virus protease that is encoded in the *pol* ORF (6, 19). The inability to detect p70 in lysates from M53- and M54-transfected cells further indicates that the Pol protein is not expressed in these mutants.

The result of this experiment left two likely explanations: Pol expression and virus replication might have been abrogated in M53 and M54 by the introduction of nucleotide changes at a site potentially involved in ribosomal frameshifting, or perhaps because the methionine initiation codon was down-mutated. To further elucidate this question we introduced a new ATG codon in M53 immediately downstream of the wild-type ATG position as shown in Fig. 2. When this mutant (M55) was analyzed for replication competence and expression of Gag and Pol precursor proteins and their cleavage products, these functions were restored (Table 1 and Fig. 3), which strongly suggested that the 5′ *pol* ATG functions as a translational initiation codon.

Exclusion of the Requirement of *gag–pol* Ribosomal Frameshifting for HFV Replication. To rule out the requirement of ribosomal frameshifting and the expression of a Gag–Pol fusion protein for HFV replication, we constructed two other mutants as shown in Fig. 2. In M56 the *pol* ORF was terminated upstream of the *pol* ATG. Any ribosomal frameshifting essential for the generation of the Pol protein would have to occur in the 54-nt *gag–pol* overlap downstream of this mutation. Potential +1 ribosomal frameshift sites, postulated to exist in FVs and involving either purine- or pyrimidine-rich sequences, were left unaffected in the M56 mutant (18, 21). We therefore constructed the M57 virus in which the *gag* ORF was truncated by a nonsense mutation upstream of the M56 *pol* mutant (Fig. 2). If ribosomal frameshifting is required for the expression of Pol protein, it would have to occur in the 15-nt *gag–pol* overlap upstream of this mutation. Thus, M56 and M57 are exclusive mutants with respect to a potential ribosomal frameshifting site.

Functional analysis revealed that both mutants were replication-competent and expressed Gag and Pol proteins (Table 1 and Fig. 3). Whereas these proteins were of wild-type size in M56, the pr74 Gag precursor protein of the weakly replicating M57 mutant was 2–3 kDa smaller, reflecting the truncation of the 20 C-terminal amino acids of Gag.

New restriction sites for *Sfa*NI, *Mse* I, and *Bsa*AI were introduced at the mutagenized sites in M55, M56, and M57, respectively. The genetic stability of the mutants on further replication in tissue culture was demonstrated by PCR amplification of the *gag–pol* overlap region of the viral DNA harvested from infected cells and restriction enzyme digestion (data not shown).

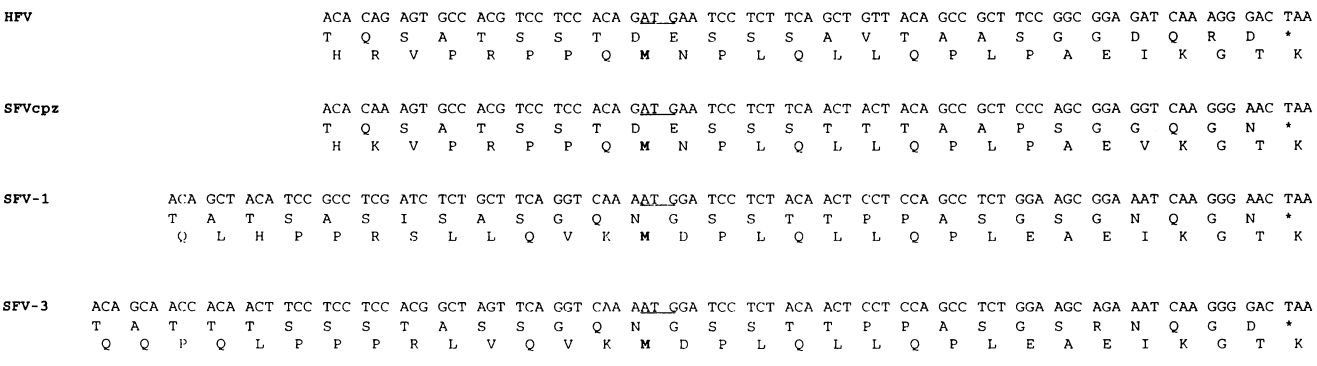

FIG. 1. Genomic regions and corresponding amino acid sequences of the *gag–pol* overlaps of four sequenced FV genomes. The *pol* ORFs are located in the +1 reading frames relative to the *gag* ORFs and have potential ATG translational initiation codons (underlined) in their 5′ regions. The single-letter code is used to abbreviate amino acids, and the first Pol methionine is in boldface type. The second Pol methionine is located 197, 197, 45, and 93 codons downstream of the first ATG codon in the genomes of HFV, SFVcpz, SFV-1, and SFV-3, respectively. The star indicates the *gag* termination codon. Sequences were taken from refs. 17–20.

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WT   ACA CAG AGT GCC ACG TCC TCC ACA GAT GAA TCC TCT TCA GCT GTT ACA GCC GCT TCC GGC GGA GAT CAA AGG GAC TAA
     T   Q   S   A   T   S   S   T   D   E   S   S   S   A   V   T   A   A   S   G   G   D   Q   R   D   *     gag
      H   R   V   P   R   P   P   Q   M   N   P   L   Q   L   L   Q   P   L   P   A   E   I   K   G   T   K     pol

M53  ACA CAG AGT GCC ACG TCC TCC ACA GAC GAA TCC TCT TCA GCT GTT ACA GCC GCT TCC GGC GGA GAT CAA AGG GAC TAA
     T   Q   S   A   T   S   S   T   D   E   S   S   S   A   V   T   A   A   S   G   G   D   Q   R   D   *     gag
      H   R   V   P   R   P   P   Q   T   N   P   L   Q   L   L   Q   P   L   P   A   E   I   K   G   T   K     pol

M54  ACA CAG AGT GCC ACG TCC TCC ACA GCT GAA TCC TCT TCA GCT GTT ACA GCC GCT TCC GGC GGA GAT CAA AGG GAC TAA
     T   Q   S   A   T   S   S   T   A   E   S   S   S   A   V   T   A   A   S   G   G   D   Q   R   D   *     gag
      H   R   V   P   R   P   P   Q   L   N   P   L   Q   L   L   Q   P   L   P   A   E   I   K   G   T   K     pol

M55  ACA CAG AGT GCC ACG TCC TCC ACA GAC GAT GCC TCT TCA GCT GTT ACA GCC GCT TCC GGC GGA GAT CAA AGG GAC TAA
     T   Q   S   A   T   S   S   T   D   D   A   S   S   A   V   T   A   A   S   G   G   D   Q   R   D   *     gag
      H   R   V   P   R   P   P   Q   T   M   P   L   Q   L   L   Q   P   L   P   A   E   I   K   G   T   K     pol

M56  ACA CAG AGT GCC ACG TCC TTA ACA GAT GAA TCC TCT TCA GCT GTT ACA GCC GCT TCC GGC GGA GAT CAA AGG GAC TAA
     T   Q   S   A   T   S   L   T   A   E   S   S   S   A   V   T   A   A   S   G   G   D   Q   R   D   *     gag
      H   R   V   P   R   P   *       M   N   P   L   Q   L   L   Q   P   L   P   A   E   I   K   G   T   K     pol

M57  ACA CAG AGT GCC ACG TAA TCC ACA GAT GAA TCC TCT TCA GCT GTT ACA GCC GCT TCC GGC GGA GAT CAA AGG GAC TAA
     T   Q   S   A   T   *                                                                                 gag
      H   R   V   P   R   N   P   Q   M   N   P   L   Q   L   L   Q   P   L   P   A   E   I   K   G   T   K     pol
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FIG. 2. HFV mutants generated to exclude ribosomal frameshifting for the FV Pol expression strategy. The wild-type (WT) sequence is shown for comparison. The *pol* ATG codon is underlined. Nucleotide and corresponding amino acid changes in the *gag* and *pol* ORFs are in boldface type. In M53 and M54, the *pol* ATG was mutated to ACG and CGT, respectively. In M55 a methionine codon was restored in the M53 background one codon 3′ to the wild-type *pol* ATG position. In M56, a nonsense mutation was introduced into the *pol* ORF two codons upstream of the *pol* ATG. In M57, the gag ORF was truncated by the introduction of a nonsense codon upstream of the M56 *pol* mutation.

DISCUSSION

The expression of a Gag–Pol fusion protein is a general rule applying to all retroviruses studied so far (1–3). We report here that HFV, and probably all FVs, break this rule. Two lines of evidence were obtained indicating that HFV expresses Pol independently from Gag. The analysis of the *pol* ATG mutants (M53, M54, and M55) demonstrated the importance of this codon for translational initiation since we could restore the replicating phenotype by introduction of an ATG at a novel site in an ATG down-mutant. The synthesis of a Gag–Pol fusion protein was further excluded by the

Table 1. Development of extracellular virus titers in cell cultures transfected with pHSRV2 or virus mutants

Cell line	DPT	Exp.	pUC19	pHSRV2	M53	M54	M55	M56	M57
BHK-21	Day 3	1	0	8.0×10^3	0	0	5×10^3	2.0×10^3	2.0×10^2
		2	0	6.0×10^3	0	0	8×10^3	1.4×10^3	1.2×10^2
		3	0	8.0×10^3	0	0	8×10^3	6.0×10^2	1.3×10^2
	Day 5	1	0	6.5×10^4	0	0	1.4×10^4	2.6×10^4	1.1×10^3
		2	0	1.3×10^4	0	0	4.2×10^4	7.5×10^4	3.0×10^2
		3	0	2.2×10^4	0	0	3.7×10^4	5.0×10^4	2.2×10^3
	Day 7	1	0	2.3×10^5	0	0	6.5×10^4	7.6×10^4	2.4×10^4
		2	0	8.5×10^4	0	0	3.2×10^5	1.3×10^5	2.6×10^4
		3	0	1.6×10^5	0	0	2.7×10^5	8.2×10^4	1.8×10^4
3T3 TK$^-$	Day 3	1	0	24	0	0	1.0×10^2	4	8
		2	0	24	0	0	27	4	2
		3	0	28	0	0	30	8	2
	Day 5	1	0	1.1×10^2	0	0	1.7×10^2	40	12
		2	0	1.1×10^2	0	0	1.4×10^2	30	4
		3	0	1.6×10^2	0	0	1.2×10^2	50	5
	Day 7	1	0	2.4×10^2	0	0	2.6×10^2	80	60
		2	0	4.0×10^2	0	0	2.0×10^2	1.3×10^2	24
		3	0	4.8×10^2	0	0	1.8×10^2	2.1×10^2	28
	Day 9	1	0	3.0×10^2	0	0	3.5×10^2	1.2×10^2	76
		2	0	5.2×10^2	0	0	3.8×10^2	1.5×10^2	32
		3	0	6.4×10^2	0	0	2.0×10^2	2.2×10^2	43
U-251 MG	Day 3	1	0	0	0	0	0	0	0
		2	0	0	0	0	0	0	0
		3	0	0	0	0	0	0	0
	Day 5	1	0	2	0	0	2	0	0
		2	0	2	0	0	4	2	0
		3	0	4	0	0	2	1	0
	Day 7	1	0	30	0	0	30	3	2
		2	0	52	0	0	60	12	1
		3	0	65	0	0	55	8	5
	Day 9	1	0	1.2×10^2	0	0	92	42	25
		2	0	1.6×10^2	0	0	3.2×10^2	78	19
		3	0	2.8×10^2	0	0	1.8×10^2	65	68

Virus titrations were performed by inoculating BHK/LTRlacZ indicator cells with serial 10-fold dilutions of supernatant from the primary transfected cells and counting blue foci after staining with chromogen 48 hr later as described (25, 33). DPT, days posttransfection; Exp., results from three independent experiments.

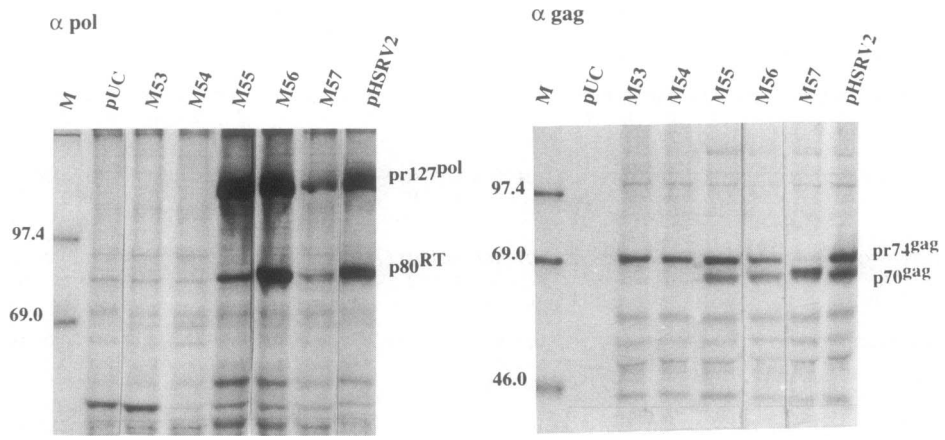

FIG. 3. Radioimmunoprecipitation of Gag and Pol proteins in BHK-21 cells transiently transfected with pHSRV2 or mutant viruses. Gag and Pol precursor molecules (pr74^{gag} and pr127^{pol}) as well as the cleavage products (p70^{gag} and p80^{pol}) are indicated. The 74-kDa band observed in the M53 and M54 lanes was of roughly the same intensity compared with the 74-kDa band in the pHSRV2 lane, indicating similar transfection and Gag expression efficiencies of the different provirus plasmids. The Gag precursor protein in M57-transfected cells was 2–3 kDa smaller due to the truncation of the gag ORF by 20 amino acids in this mutant; therefore pr74^{gag} and the p70^{gag} cleavage product comigrate as a doublet in the M57 lane. The Gag radioimmunoprecipitation was exposed for 1 day while the Pol RIPA was exposed for 3 days.

demonstration of two viable virus mutants (M56 and M57), in which the *gag* and *pol* genes were functionally separated. With respect to the latter experiment, one could argue that HFV may theoretically make use of two alternative frameshifting sites, one downstream of the M56 and one upstream of the M57 mutation. However, the result of the experiment with the ATG mutants negates this unlikely possibility.

Gag-independent translation of the Pol protein in FVs is reminiscent of the replication strategy of pararetroviruses (12–14, 34–36). Other common features between FVs and pararetroviruses have been reported recently and concern the structure and function of the capsid protein (16, 35). Thus, in functional terms, FVs share aspects of the replication strategies of retroviruses and pararetroviruses. However, it remains to be seen whether or not this functional relationship of FVs to both virus groups reflects a true evolutionary relationship or merely results from coevolution of certain aspects of gene expression strategies. Regardless of this, our findings broaden the understanding of the flexibility that retroviruses have gained to enable replication of their genomes.

There are several important questions arising from our study. (*i*) What is the mechanism of FV Pol expression and how is the Pol expression level regulated? (*ii*) How is Pol incorporated into the FV particle? (*iii*) What consequences result from Gag-independent expression of Pol with respect to FV particle assembly and reverse transcription? With respect to the first question, we have obtained recent evidence that FVs transcribe a spliced *pol* mRNA required for *pol* translation, indicating that the amount of Pol protein is regulated at the level of mRNA splicing (unpublished data). Elucidation of the other questions will be the task of future experiments. The answers may reveal that the life cycle of FVs is even more similar to the pararetrovirus life cycle than is evident at present.

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