RNA and Protein Requirements for Incorporation of the Pol Protein into Foamy Virus Particles

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Received 29 September 2004/Accepted 18 January 2005

Foamy viruses (FVs) generate their Pol protein precursor molecule independently of the Gag protein from a spliced mRNA. This mode of expression raises the question of the mechanism of Pol protein incorporation into the viral particle (capsid). We previously showed that the packaging of (pre)genomic RNA is essential for Pol encapsidation (M. Heinkelein, C. Leurs, M. Rammling, K. Peters, H. Hanenberg, and A. Rethwilm, *J. Virol.* 76:10069–10073, 2002). Here, we demonstrate that distinct sequences in the RNA, which we termed Pol encapsidation sequences (PES), are required to incorporate Pol protein into the FV capsid. Two PES were found, which are contained in the previously identified *cis*-acting sequences necessary to transfer an FV vector. One PES is located in the U5 region of the 5' long terminal repeat and one at the 3' end of the *pol* gene region. Neither element has any significant effect on RNA packaging. However, deletion of either PES resulted in a significant reduction in Pol encapsidation. On the protein level, we show that only the Pol precursor, but not the individual reverse transcriptase (RT) and integrase (IN) subunits, is incorporated into FV particles. However, enzymatic activities of the protease (PR), RT, or IN are not required. Our results strengthen the view that in FVs, (pre)genomic RNA functions as a bridging molecule between Gag and Pol precursor proteins.

Spumaretroviruses, or foamy viruses (FVs), are one of two subfamilies of retroviruses (30). A hallmark of the replication strategy of FVs is the Gag-independent expression of a Pol precursor protein from a spliced RNA (3, 6, 18, 25, 40). The consequences of this highly unusual strategy for the generation of a retroviral Pol protein are poorly understood. It has been shown that FV reverse transcriptase (RT) is much more processive than that of orthoretroviruses (31). Furthermore, it has been suggested that a very few molecules of Pol precursor are packaged into the FV capsid (4, 31). The FV means of expressing and encapsidating Pol protein has to be viewed in the context of reverse transcription, which appears to occur to a large extent in the virus-producing cell prior to budding (26, 32, 41). This leads to linear DNA, which is probably the virion nucleic acid and more relevant for infection than RNA (30).

The actual mechanism of FV Pol protein particle incorporation despite lack of a Gag-Pol precursor has remained obscure. It is evident that FVs must have found a means of Pol encapsidation that acknowledges that a Gag-Pol precursor protein, which in orthoretroviruses facilitates Pol incorporation into the viral particle, is not generated (35). We previously showed that the incorporation of (pre)genomic RNA is essential for Pol protein encapsidation (12). Here, we took the experiments a step further and asked whether specific sequences on the RNA which allow Pol protein incorporation can be identified and whether such sequences are different from signals needed to package RNA. Furthermore, we wanted to know what requirements exist on the protein level for encapsidating Pol protein.

Two *cis*-acting sequences (CAS), one located in the 5' un-

translated region (UTR) of the (pre)genomic RNA and one in the 3' region of the *pol* gene, have been empirically identified to be essential. Together with the long terminal repeats (LTRs) and adjacent sequences required for reverse transcription and integration, they are sufficient to allow efficient FV vector transfer (8, 11, 14, 15, 39). Thus, any RNA element required for (pre)genome packaging and for Pol protein encapsidation must be confined to these two CAS. By introducing deletions into the CAS elements and analyzing the protein compositions and RNA contents of FV vectors, we intended to identify those RNA sequence elements required for Pol protein incorporation.

MATERIALS AND METHODS

Recombinant DNA. Established recombinant DNA techniques were used (1, 33). The human cytomegalovirus immediate-early gene promoter-directed expression constructs for the prototypic FV (PFV) genes *gag* (pCIgag2), *pol* (pCpol2), and *env* (pCenv1); *gag/pol* with a mutated *pol* ATG start codon (pCgp/M54); the PFV transfer vector pMD9; the RT and integrase (IN) expression plasmids (pCRT2 and pCIN1); and the protease (PR) (M61), RT (M69), and IN (M73) active-site mutations in the context of a proviral PFV molecular clone or the pMH97 and pMH98 vectors carrying *gag/pol* have been described previously (7, 9, 11, 13, 14, 17, 21, 26). Deletions in CASI were introduced into pCgp/M54 as follows. pKP2 was generated by ligating a 2.4-kb pMH115 (15) MluI/SwaI fragment with an 8.5-kb pCgp1/M54 MluI/SwaI fragment. The insert contributed the deleted 5' UTR, while the larger fragment contributed the plasmid backbone. Similarly pKP3, -4, and -5 were made by ligating 2.5-kb pMH114 (15), 2.61-kb pMH93 (15), and 2.63-kb pKP1 MluI/SwaI fragments, respectively, with the 8.5-kb pCgp1/M54 MluI/SwaI-digested plasmid backbone. The intermediate construct, pKP1, has a 16-bp deletion in the PFV primer binding site (PBS). It was generated by recombinant PCR (16) followed by a four-fragment ligation. A detailed description of the pKP1 cloning procedure is available on request. The CASI constructs are shown schematically in Fig. 1A.

The pMD9 vector was used for the introduction of deletions in CASII. The pKP16 plasmid was made by religation of SwaI/AfeI-digested pMD9. pKP17 was created by ligating the 8.6-kb SwaI/EcoRI-digested pMD9 vector with the 0.55-kb EcoRV/EcoRI pMD9-derived insert. In a similar fashion, pKP18 and pKP19 were generated by fusing 8.2-kb and 7.9-kb pMD9 fragments, which were obtained by digestion with EcoRV/BamHI and NheI/AfeI, with 1.5- and 1.0-kb pMD9-derived BamHI/HincII and NheI/HincII fragments, respectively. The

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FIG. 1. Mapping of a PES in CASI of PFV. (A) Deletions (dashed lines) were created in the wild-type pCgp1/M54 plasmid, and 293T cells were cotransfected with *pol*- and *env*-expressing plasmids and the constructs in question. The *pol* gene start codons in pCgp1/M54 and the pKP derivatives were mutated from ATG to CTG. Poly(A) signal derived from the bovine growth hormone (pA⁺) and the cytomegalovirus (CMV) enhancer/promoter are present in the plasmid vector backbone, which was the basis for all constructs (pcDNA from Invitrogen). The probe, which

CASII deletion constructs are depicted in Fig. 2A. The vector pMH66 used in some experiments is identical to the previously described vector pMH5/M54 (14) with respect to the viral coding sequence.

The *pol* expression plasmids with mutated active sites in PR (pCpol2/M61) and RT (pCpol2/M69) were generated by exchanging the 1.83-kb *SwaI*/*PacI* fragments of pcHSRV2/M61 and M69 for the equivalent fragment in pCpol2. To generate pCpol2/M83, which contains active-site mutations in RT and IN, we first created pcHSRV2/M83 by ligating 7.1-kb *NheI*/*MluI* pcHSRV2, 4.47-kb *MluI*/*PacI* pMH98, 1.34-kb *PacI*/*EcoRI* pMH97, and 3.26-kb *EcoRI*/*NheI* pcHSRV2 fragments. Finally, pCpol2/M83 was made by exchanging a 4.14-kb *SwaI*/*BspEI* fragment of pcHSRV2/M83 for the equivalent fragment in pCpol2. In a similar way, we created pCpol2/M73, mutated only in the IN active center, from pcHSRV2/M73.

The pSP65-based plasmid pSpHFV3 (11) was linearized with *XbaI* and used in vitro to transcribe the probe for the CASI deletions. This antisense probe is 349 bp long and protects a 334-bp fragment of the PFV *gag* RNA (11). The pKP20 plasmid was made by introduction of a 0.16-kb *BbsI*/*AvrII* pMD9-derived fragment into *BbsI*/*AvrII*-cut pSpHFV5 (12). Following linearization with *MfeI*, a 369-bp antisense probe was generated that can specifically protect a 297-bp pMD9-generated RNA fragment. For the detection of pMD9 RNA, we also made a probe that binds to the positive-sense RNA of the gene for enhanced green fluorescent protein (EGFP). EGFP is a marker inserted into the pMD9 vector (11). This probe was generated from pKP21. The pKP21 plasmid was generated by introduction of a 0.74-kb pMD9-derived *NotI* (Klenow enzyme-treated)/*BamHI* fragment into *EcoRI* (Klenow enzyme-treated)/*BamHI*-digested pSP65. In vitro transcription after linearization with *BstYI* resulted in a 237-bp antisense probe able to protect a 222-bp pMD9 RNA fragment.

Transfections and partial purification of particulate material from extracellular virus. 293T cells (5) were seeded in six 6-cm dishes at a density of 1.6×10^6 cells per dish 1 day prior to transfection. Unless otherwise stated, the cells were transfected using the Polyfect reagent (QIAGEN) with a total of 6 μ g of plasmid DNA per dish (2 μ g per plasmid in the case of three plasmids and 1.5 μ g per plasmid in the case of four plasmids). One day after transfection, transcription was induced by the addition of 8 mM Na-butyrate to the cell culture medium for 8 h. The supernatant was harvested 48 h following transfection, passed through a 0.45- μ m filter (Millipore), and layered onto 2 ml of a 20% sucrose cushion. Centrifugation was done in an SW41 rotor (Beckman) at 25,000 rpm and 4°C for 3 h. For the detection of viral proteins, the particulate material was suspended in protein-loading buffer, heated, and chilled on ice as described previously (11, 12, 14, 15). For the detection of viral RNA, the material was suspended in 70 μ l lysis buffer of the Direct Protect RPA Kit (Ambion). If not processed immediately, RNA and protein were stored at -80°C.

Immunoblotting. Cellular protein lysates from one of the plates were prepared by adding detergent-containing buffer on ice (11, 12, 14, 15), centrifugation of the lysates through a QIAshredder (QIAGEN), and addition of 2 $\times$ protein-loading buffer. After being heated and chilled, the samples were analyzed by immunoblotting or stored at -80°C. Protein samples from cellular lysates or virions were resolved by sodium dodecyl sulfate-containing 8% polyacrylamide gels, semidry blotted onto Hybond ECL nitrocellulose membranes (Amersham-Pharmacia), and incubated with mouse monoclonal antibodies (MAbs) directed against PFV Gag (SGG1) or Pol (15E10 and 3E11), as described previously (12). After incubation with a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Dako), the blots were developed with the ECLplus detection system (Amersham-Pharmacia).

RPA. Following linearization, plasmid DNA was transcribed in vitro into RNA. The reaction mixture of 10 μ l containing 1 to 2 μ g template DNA, RNA transcription buffer (Roche), 20 U RNase inhibitor (MBI Fermentas), 1 μ l biotin labeling mix (Roche), and 20 U SP6 polymerase (Roche) was incubated at 37°C

for 30 min. After digestion of the template DNA with RQ1 DNase (Promega) and precipitation, the sediment was suspended in 50 μ l lysis buffer contained in the Direct Protect Lysate RPA Kit (Ambion). For hybridization and RNase digestion, the protocol of the RNase protection analysis (RPA) kit (Ambion) was followed. The samples were separated on denaturing 5% polyacrylamide gels, electroblotted onto positively charged nylon membranes (Bright Star Plus from Ambion), and UV cross-linked (UV Stratalinker). The blots were developed using the North2South chemiluminescent hybridization and detection system, essentially as described by the manufacturer (Pierce), and horseradish peroxidase-conjugated streptavidin. The blots were exposed to X-ray films, and quantification was performed by ImageQuant software.

RESULTS AND DISCUSSION

Mapping of PES in CASI. For the identification of Pol encapsidation sequences (PES) located in CASI, deletions were introduced into the pCgp1/M54 *gag/pol* expression vector as shown in Fig. 1A. The largest deletion in pKP2 comprises all sequences between the R region of the LTR and the start of the *gag* gene. Only the U5 region in pKP3, 30 bp directly 5' of the PBS in pKP4, and the PBS in pKP5 were deleted. In pCgp1/M54, the *pol* gene ATG was mutated to CTG (11), and therefore, no Pol protein is expressed from this construct or its derivatives (6). Hence, we cotransfected 293T cells with the *pol* expression plasmid pCpol2 in addition to pCenv1 and the pCgp1/M54 or the pKP constructs in question.

The viral protein expression pattern was determined in lysates from transfected cells and from particulate material centrifuged through a sucrose cushion. We have previously shown that there is no significant difference in the viral protein detection pattern between lysates from virions prepared after centrifugation through sucrose cushions or after further purification by centrifugation through sucrose gradients (12). As shown in Fig. 1B, the viral Gag and Pol precursor proteins of 71- and 127-kDa apparent molecular mass (10, 27), respectively, were readily detected in all lysates from cells transfected with the respective constructs, indicating that gene expressions from the different plasmids were equally good.

Proteolytic cleavage of the Gag precursor protein by the viral PR, which is part of the 127-kDa Pol precursor and of the 85-kDa PR-RT-RNase H (PR-RT-RN) cleavage product (10), was readily observed in lysates from cells transfected with the pCgp1/M54 positive control and in lysates from cells transfected with the pKP5 construct that bears a deletion of the PBS (Fig. 1B, lanes 1, 2, and 6). The pKP2, -3, and -4 constructs appeared to be severely handicapped in allowing this cleavage (Fig. 1B, lanes 3, 4, and 5). The analysis of particulate viral material from the supernatant revealed a similar picture. In FVs, the cotransfection of cells with an *env* gene expression

binds to the PFV *gag* gene and was used for the Fig. 1C RPA, is shown. R and U5 indicate the repeat and 5' unique regions, respectively, of the LTR. (B) Representative example of an immunoblot for PFV proteins with mouse MAbs directed against Gag (pr71 and p68) and Pol (p127^{prPol}, p85^{pr-RT-RN}, and p40^{IN}) molecules from cellular lysates (lanes 1 to 7) and particulate viral material from the supernatant (lanes 8 to 13). Cells were cotransfected with pCgp1/M54, pCpol2, and pcDNA (lanes 1 and 8); pCgp1/M54, pCpol2, and pCenv1 (lanes 2 and 9); pKP2, pCpol2, and pCenv1 (lanes 3 and 10); pKP3, pCpol2, and pCenv1 (lanes 4 and 11); pKP4, pCpol2, and pCenv1 (lanes 5 and 12); pKP5, pCpol2, and pCenv1 (lanes 6 and 13); and pcDNA (lane 7). (C) RPA of RNA incorporated into viral particles from the supernatant centrifuged through a 20% sucrose cushion with a 334-bp *gag* probe shown in Fig. 1A. The quantification of three independent experiments revealed that particles generated by the pKP2 to pKP5 constructs incorporate approximately as much RNA as the particles generated by pCgp1/M54. An example of an RPA is shown above the quantification. Probe treated with a cocktail of RNase A and RNase T1 (lane 1); material from cells transfected with pCgp1/M54, pCpol2, and pcDNA (lane 2); pCgp1/M54, pCpol2, and pCenv1 (lane 3); 50% of the material in lane 3 (lane 4); 20% of the material in lane 1 (lane 5); pKP2, pCpol2, and pCenv1 (lane 6); pKP3, pCpol2, and pCenv1 (lane 7); pKP4, pCpol2, and pCenv1 (lane 8); pKP5, pCpol2, and pCenv1 (lane 9). The undigested probe was run in every RPA; it is, however, not represented on this image.

FIG. 2. Mapping of a PES in CASII of PFV. (A) Deletions (dashed lines) were created in the wild-type pMD9 vector, and 293T cells were cotransfected with *gag*-, *pol*-, and *env*-expressing plasmids and the pKP16 to pKP19 constructs in question. The *gag* gene start codon in pMD9 and its derivatives were mutated from ATG to CTG. CASI and II, *cis*-acting sequences I and II required to transfer a PFV vector; SFFV U3,

plasmid is required to facilitate viral capsid egress (Fig. 1B, compare lanes 8 and 9) (reviewed in reference 20). Cleavage of the Gag precursor could hardly be detected in particulate material prepared from cells transfected with pKP2, -3, and -4, while it was readily visible in the sample of cells transfected with pKP5 (Fig. 1B, lanes 10 to 13). This observation coincided with the detectable particle incorporation of Pol protein. The 127-kDa Pol precursor and its cleavage products, the 85-kDa PR-RT-RN and the 40-kDa IN, were observed only in virions prepared from supernatants of cells transfected with pCgp1/M54 and pKP5, in addition to pCpol2 and pCenv1, but not with the other constructs (Fig. 1B, lanes 9 to 13).

Other fractions of the supernatants, which were used for the preparation and analysis of viral proteins in particulate material and which contained approximately equal amounts of viral protein, were processed in parallel to quantitate viral RNA. This analysis with a *gag* gene-specific probe (Fig. 1A) revealed that all pKP deletion constructs in CASI incorporated approximately wild-type levels of pregenomic RNA (Fig. 1C). Although the nonradioactive RPA may be regarded as only semi-quantitative, differences in band intensities reflected different specific amounts of the protected nucleic acid. This was revealed when only 50% or 20% of the positive control sample was loaded onto the gels. In these cases, the band intensities were on average 73% and 18%, respectively, of the wild-type reference (Fig. 1C).

In summary the mapping of PES in CASI revealed that a sequence in the 5' UTR is required for Pol protein encapsidation. This PES is probably confined to the 30-bp sequence deleted in pKP4 just upstream of the PBS. However, we cannot exclude the possibility that as a result of this deletion, alterations in the secondary structure were introduced into the highly ordered FV 5' UTR (28). However, the results obtained with pKP5 argue against this, since the deletion of the neighboring PBS in pKP5 did not result in a Pol encapsidation defect. That the PBS is dispensable for Pol protein incorporation has been shown previously (2). The PES identified in pKP4 is also contained within the larger deletions introduced into pKP2 and pKP3, with which we obtained similar results. Importantly, the deletions had no effect on PFV pregenome packaging. Thus, we can conclude that it is not RNA packaging per se that is essential for efficient encapsidation of the Pol protein but that a specific sequence on the RNA, which represents the likely start of reverse transcription, is required to facilitate Pol incorporation.

Mapping of PES in CASII. Compared to CASI, the second CAS that is located in the 3' region of the *pol* gene appears to be more extended. Approximately 2-kb CASIIs are optimal for FV vector transfer (14, 37). To get a better understanding of the role of CASII in Pol protein packaging, deletions were introduced using convenient restriction enzyme sites and in the same way as for the CASI deletions. However, this time we used a cotransfection system of 293T cells with four plasmids and employed the pMD9 vector and deletion constructs of this vector, as shown in Fig. 2A (12).

Based on the previously published observation that an internal 0.2-kb fragment is dispensable for PFV vector transfer (14), CASII was divided into three fragments, which were individually deleted from pMD9. In pKP17, the first 0.8 kb of CASII were eliminated; in pKP18, the deletion comprised an internal fragment of 0.2 kb; in pKP19, the 3' 1.0 kb of CASII, and in pKP16, the complete 2.0-kb CASII were deleted (Fig. 2A). Cells were cotransfected with the constructs under investigation and *gag*, *pol*, and *env* expression plasmids. Cellular lysates and partially purified virus preparations were analyzed for the presence of Gag and Pol molecules by immunoblotting.

As shown in Fig. 2B, the Gag and Pol precursor molecules were equally well detected in lysates from cells transfected with the respective expression constructs. However, cleavage of the Gag precursor was reduced in cells transfected with the pKP16, -17, and -19 plasmids (lanes 3, 4, and 6). In blotting experiments with extracellular viral particles, this finding was substantiated. Efficient Pol incorporation and cleavage of Gag was observed in particles produced by transfection of cells with pMD9 and pKP18 (Fig. 2B, lanes 9 and 12). The samples in question, however, showed reduced (pKP17) or hardly detectable (pKP16 and pKP19) Gag cleavage, as well as incorporation of pr127^{pol}, p85^{PR-RT-RN}, and p40^{IN} Pol proteins (Fig. 2B, lanes 10, 11, and 13). Consistent with previous results (14), only with the small deletion variant pKP18 did we obtain vector transfer rates comparable with those of the wild-type pMD9 vector (data not shown). Analysis of constructs in which deletions were introduced into the pMH66 backbone in such a way that the *gag* gene expression from this plasmid could be used for quantification of expression levels produced similar results (data not shown). This indicated that the levels of intracellular RNA generated from the different deletion constructs were not a limiting factor.

The RPA of these experiments is depicted in Fig. 2C. pKP18 and pKP19 showed amounts of the 297-bp protected fragment

constitutively active U3 promoter from spleen focus-forming virus, which drives the gene for EGFP and is present in the pMD9 vector. The probe that binds to the pMD9 CASI and that was used for the panel C RPA is indicated. (B) Immunoblot analysis for PFV proteins with mouse MAbs directed against Gag and Pol molecules from cellular lysates (lanes 1 to 7) and particulate viral material from the supernatant (lanes 8 to 13). Cells were cotransfected with pMD9, pCIgag2, pCpol2, and pcDNA (lanes 1 and 8); pMD9, pCIgag2, pCpol2, and pCenv1 (lanes 2 and 9); pKP16, pCIgag2, pCpol2, and pCenv1 (lanes 3 and 10); pKP17, pCIgag2, pCpol2, and pCenv1 (lanes 4 and 11); pKP18, pCIgag2, pCpol2, and pCenv1 (lanes 5 and 12); pKP19, pCIgag2, pCpol2, and pCenv1 (lanes 6 and 13); and pcDNA (lane 7). (C) RPA of RNA incorporated into viral particles from the supernatant centrifuged through a 20% sucrose cushion with a 369-nucleotide (nt) CASI probe shown in panel A (protected fragment of 297 nt). The quantification of three independent experiments revealed that particles generated by the pKP18 and pKP19 constructs incorporate approximately as much RNA as the particles generated by pMD9. An example of an RPA is shown above the quantification. Probe treated with RNase (lane 1); material from cells transfected with pMD9, pCIgag2, pCpol2, and pcDNA (lane 2); pMD9, pCIgag2, pCpol2, and pCenv1 (lane 3); 50% of the material loaded in lane 3 (lane 4); 20% of the material loaded in lane 1 (lane 5); pKP16, pCIgag2, pCpol2, and pCenv1 (lane 6); pKP17, pCIgag2, pCpol2, and pCenv1 (lane 7); pKP18, pCIgag2, pCpol2, and pCenv1 (lane 8); pKP19, pCIgag2, pCpol2, and pCenv1 (lane 9). The undigested probe is not represented on this image.

FIG. 3. Only full-length Pol precursor protein is incorporated into PFV particles. (A) Overview of the different expression constructs used for this analysis. pCpol2 encodes the wild-type Pol protein, pcRT2 the RT subunit, and pcIN1 the IN subunit; pCgag2 and pCenv1 are the *gag* and *env* expression constructs. A stop codon was inserted behind the RNase H (RN) domain of Pol in pcRT2, and an ATG start codon was introduced into pcIN1 at the beginning of the IN domain of Pol (17). (B) 293T cells were transfected with plasmid DNAs, and the cellular lysates (lanes 1 to 13) and partially purified virus from the supernatants (lanes 14 to 25) were analyzed for Gag and Pol proteins by immunoblotting with MAb. The cells were transfected with the *gag* expression plasmid (lanes 1 to 12 and 14 to 25), *env* expression plasmid (lanes 2, 3, 5, 6, 8, 9, 11, 12, 15, 16, 18, 19, 21, 22, 24, and 25), and pMD9 vector (lanes 1, 3, 4, 6, 7, 9, 10, 12, 14, 16, 17, 19, 20, 22, 23, and 25). The wild-type pCpol2 construct was included in lanes 1, 2, 3, 14, 15, and 16. In lanes 4, 5, 6, 17, 18, and 19, cells were transfected with the expression construct for the p85^{RT} protein (pcRT2); in lanes 7, 8, 9, 20, 21, and 22 with the expression construct for the p40^{IN} protein (pcIN1); and in lanes 10, 11, 12, 23, 24, and 25 with both constructs (pcRT2 plus pcIN1). The lysate in lane 13 was from cells transfected with pcDNA vector plasmid. The DNA amounts in all transfections were adjusted to 7.5 μ g with vector DNA. Only cells transfected with the plasmid encoding the full-length pr127 (127-kDa) Pol precursor protein gave rise to detectable amounts of particle-associated Pol (lane 3 for the cellular lysates, and lane 16 from extracellular virus).

from the CASI region that were similar to wild-type pMD9 levels, while the RNA contents of particles produced in the presence of pKP16 and pKP17 were reduced almost to background levels. When a probe protecting a different region of the RNA (the gene for EGFP) was used, identical results were obtained (data not shown). Thus, within the region of the deletion in pKP19, an element has been identified that is irrelevant for (pre)genome RNA packaging but important for the efficient encapsidation of Pol proteins.

The sequence deleted in pKP16 and pKP19 is still present in pKP17 and probably allows the particle incorporation of tiny amounts of Pol protein that are undetectable by immunoblotting and that result in some residual Gag cleavage. Therefore,

the results obtained by analyzing CASII deletion mutants appear to be less definitive than the results obtained with the CASI deletions. However, our findings suggest a role for the more upstream region of CASII, which was deleted in pKP17, in RNA packaging or conferring stability on the packaged RNA. Furthermore, the clear reduction in Gag cleavage and Pol incorporation seen with this construct probably result from the failure to incorporate significant amounts of (pre)genomic RNA. Thus, our data support a model in which CASII can be subdivided into at least two elements with different functions. Between these elements there is a small sequence to which we cannot yet assign a distinctive *cis*-acting function.

The results favor the view of an extended nature of CASII

FIG. 4. Demonstration that active Pol protein is not required for FV particle encapsidation. (A) Overview of the different *pol* expression constructs used to transfect 293T cells: pCpol2 harbors the wild-type *pol* gene; in pCpol2/M69, the active center of RT was changed from YVDD to GAAA; pCpol2/M73 contains a mutation of the active site of IN, which changes the DD35E motif to DA35E; pCpol2/M83 combines the mutations of M69 and M73; and in pCpol2/M61, the active site of PR was changed from DSG to DAG. The mutation of the enzymatic activity is depicted as a downward-pointing arrow. (B) Immunoblot of cellular lysates (lanes 1 to 13) and viral particle preparations (lanes 14 to 25) obtained after transfection of cells with PFV constructs deficient in Pol enzymatic functions for Gag and Pol antigens. The cells were transfected with the wild-type *pol* expression plasmid (lanes 1 to 4 and 14 to 17), pCpol2/M69 (lanes 5, 6, 18, and 19), pCpol2/M73 (lanes 7, 8, 20, and 21), pCpol2/M83 (lanes 9, 10, 22, and 23), pCpol2/M61 (lanes 11, 12, 24, and 25); lane 13 was derived from cells transfected with pcDNA vector. The pMD9 vector was included in lanes 1, 3, 4, 6, 8, 10, 12, 14, 16, 17, 19, 21, 23, and 25. The transfection cocktail contained the *env* expression plasmid in all lanes except 1, 13, and 14 and the *gag* expression plasmid in all lanes except 3, 13, and 16. In all cases in which cells were transfected with the complete cocktail, Pol protein was detected in particulate viral material regardless of the functionality of the Pol PR, RT, or IN enzyme.

(14, 37). Interestingly, it has been reported for the simian FV, SFV_{mac}, that two downstream *cis*-acting sequences are located in the *pol* genomic region and that these are required for efficient vector transfer (29). These elements are approximately 1.5 kb apart; one is located in the first half of the *pol* gene, and the other is in the 3' *pol* region. The functions of the sequences have not yet been analyzed. However, it is tempting to speculate that these two elements serve functions for SFV_{mac} analogous to those that the two elements in CASII serve for PFV.

Protein requirements for the encapsidation of Pol protein. To analyze whether the PR-RT-RN or the IN protein of the cleaved Pol precursor may be sufficient for particle incorporation, we transfected cells with eukaryotic expression constructs for these subunits (Fig. 3A). As shown in Fig. 3B (lanes 3 and 16), the cotransfection of cells with the complete cocktail con-

sisting of pMD9 vector and wild-type *gag*, *env*, and *pol* expression plasmids resulted in Gag cleavage and detectable amounts of Pol in viral particles. When the Pol precursor-specifying plasmid was replaced by pCpol2 or pCIN1, either individually (lanes 6, 9, 19, and 22) or together (lanes 12 and 25), expression of the respective proteins was readily detected in cellular lysates (lanes 6, 9, and 12). However, we were not able to identify these proteins in particulate extracellular viral material (lanes 19, 22, and 25), nor did we detect Gag protein cleavage following transfection of cells with the plasmid encoding the 85-kDa protein, which contains the viral PR enzyme.

To address the question of whether active enzymes are necessary for encapsidation, we used *pol* expression plasmids in which the active sites of PR, RT, and/or IN had been disabled. As shown in Fig. 4 (lanes 6, 8, 10, 19, 21, and 23), Gag cleavage, the Pol precursor protein, and the Pol subunits were found to

FIG. 5. Overview of CAS and PES elements in the FV genome. The CAS elements are those sequences that, in addition to the LTRs and adjacent sequences, are essential and sufficient to transfer an FV vector genome. The actual RNA-packaging sequences have not yet been determined. The PES elements, as shown in this study, are parts of these CAS. They are involved in encapsidating the FV Pol proteins. iPPT, internal polypurine tract; IP, internal promoter driving the expression of the accessory genes; tas, transcriptional *trans*-activator gene; orf-2, accessory open reading frame 2, which is expressed from a multisplliced mRNA, designated bet (24); 3'PPT, polypurine tract upstream of the 3' LTR. The numbers specify the borders of the CAS and PES elements relative to the PFV start of transcription according to GenBank accession number NC_001736. U3 is the 3' unique region of the LTR.

be FV particle associated in all cases except for the protease mutant M61 (lanes 12 and 25). For that mutant, only the precursor molecule could be incorporated into the particle and no Gag cleavage was expected.

From these experiments, we conclude that only the 127-kDa Pol precursor protein is encapsidated and that cleavage into its subunits occurs after association with Gag. However, the enzymatic activity of the FV Pol protein does not seem to be essential for particle incorporation, at least as far as PR, RT, and IN activities are concerned. The dispensability of an active PR for PFV Pol encapsidation has been shown previously (2).

Compilation and consequences of the PES mapping experiments. In this study, we defined RNA elements which are of minor or negligible significance for the incorporation of (pre)genomic RNA into FV capsids. However, they appeared to be important for the encapsidation of the Pol protein. An extended PES of approximately 1.0 kb is probably located in the 3' region of the *pol* gene, and another PES, which can be as short as 30 bp, is probably located just 5' to the PBS. Attempts to dissect the downstream PES in more detail were not conclusive, leaving the possibility of a large domain on the RNA for interaction with Pol (data not shown). The results are summarized in Fig. 5. The identification of the PES elements is compatible with a model in which the (pre)genomic RNA serves as a bridging molecule for the interaction with Pol on the one hand and Gag on the other. In keeping with this model, we suggest several explanations that are not mutually exclusive. In the first scenario, Pol initially interacts with the downstream PES and is transferred by an as-yet-unidentified mechanism to the upstream PES for the initiation of reverse transcription. Another possibility could be that initial binding of Pol to the upstream PES is lost if not stabilized by the downstream PES. Complex RNA secondary structures are likely to be involved in both reactions. They may even facilitate close proximity of the proximal and distal PES elements, which could form a conformational epitope for Pol binding. Another explanation is that Pol binds to the upstream PES, followed by minus strong stop cDNA synthesis, while the downstream PES is involved in second-strand cDNA synthesis. This would be in accordance with the presence of the second polypurine tract (PPT) within CASII (14, 19, 36). The role of this internal PPT has yet to be experimentally addressed. Following this explanation, both PES would place the RT in the vicinity where its

enzymatic activity is required. However, the independence of Pol encapsidation from the presence of the PBS and the downstream PPT may argue against this possibility. Clearly, the way in which FVs encapsidate their Pol proteins is different from the orthoretroviral strategy. Biochemical studies of the RNA-Pol interaction will be instrumental in the elucidation of the mechanism of Pol incorporation into FV particles and the mode of reverse transcription.

Previously, it was reported that a functional homologue of a constitutive RNA transport element may be contained in the downstream FV element identified here as a PES (38). This conclusion was drawn on the basis of human immunodeficiency virus *gag* gene expression, which was facilitated by a sense, but not by an antisense, FV construct. We did not find any evidence for a constitutive RNA transport element being operative in the FV system. Deletion of the downstream PES resulted in as much RNA particle incorporation, and consequently, cytoplasmic RNA transport as in the wild-type pMD9 vector construct. However, as described above, the downstream PES was found to be critical for Pol protein encapsidation.

We did not address the question of whether Gag-Pol interactions take place in addition to RNA-Pol interactions. It has been reported recently that such protein-protein interactions may indeed take place in FV particle assembly (34). However, the results of that study are also fully compatible with our findings, since the investigated Gag constructs, which were deficient in Pol incorporation, were also found to be deficient in RNA packaging or to be unable to generate Pol protein (34). Furthermore, the fact that the Gag protein C terminus is dispensable for Pol protein encapsidation has been documented (2).

We did not address the question of the packaging signal of the (pre)genomic RNA. The upstream element in CASII, which was found to be important for (pre)genome packaging, may serve several functions, from a role in packaging and stabilization of the packaged RNA to nuclear export. All of these can influence the efficiency of genome packaging. The upstream CASII element is unlikely to be the sole packaging signal, since it is located on the subgenomic *pol* transcript and the (pre)genomic RNA. Additional elements, which may be located in the 5' *gag* region, are probably also required for the discrimination of both RNAs (14, 22, 23). A closer inspection

of the mode of FV particle assembly, Pol protein packaging, and initiation of reverse transcription is likely to reveal additional surprising and distinctive features of this retroviral subfamily.

ACKNOWLEDGMENTS

We thank Michael Bock, Jochen Bodem, Dirk Lindemann, and Myra O. McClure for critical reviews of the manuscript.

This work was supported by grants from the DFG (RE627/6-3 and SFB479) to A.R.

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