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Complex effects of foamy virus central purine-rich regions on viral replication

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Abstract

Similar to the lentiviruses family of retroviruses, foamy viruses (FVs) contain purine-rich sequences located in the center of the genome. Their function on viral replication or vector transfer remains elusive, although dual initiation of plus-strand reverse transcription has been suggested. To elucidate the physical nature of the central region of the prototype FV (PFV) genome, we performed 3′ and 5′ RACE experiments. Our results revealed that the PFV genome contains a centrally located gap in the DNA plus-strand with no definite termination and start point and of variable length. We did not find evidence for a DNA flap region. The PFV isolate harbors four centrally located purine-rich elements (A–D). Only the D element is identical in sequence to the 3′ poly purine tract (PPT). We mutated these elements while conserving or altering the overlapping *pol* reading frame and analyzed the mutants for transient replication in an infectious or for vector transfer in a replication-deficient background. In addition, we determined the protein composition of the respective viral particles. The A and B elements appeared to play a role in Pol protein encapsidation, the C element is likely involved in regulating gene expression, while mutation of the D element resulted in an insignificant reduction in transiently replicating virus and an approximately 50% reduction in vector titer. The reason for this deficit remains to be elucidated.

Keywords: Foamy virus; Reverse transcription; Poly purine tract; Replication strategy; Polymerase encapsidation

Introduction

The lentivirus genus of exogenous retroviruses and the members of the FV subfamily harbor a central PPT (cPPT), in addition to the 3′ PPT upstream of the 3′ long terminal repeat (LTR) (Arhel et al., 2006; Kupiec et al., 1988). A cPPT is also present in endogenous Ty retroelements in yeast and in the plant pararetrovirus cauliflower mosaic virus (Heyman et al., 2003; Noad et al., 1998). PFV contains four motifs (A–D) of purine-rich sequences in the center of the genome (Fig. 1). Only the last of these purine-rich motifs shows sequence identity to the 3′ PPT (Fig. 1). It has been shown previously that unintegrated linear PFV DNA has a single-stranded gap at the duplicated PPT site (Kupiec et al., 1988; Tobaly-Tapiero et al., 1991). This finding was suggestive of dual initiation of plus-strand DNA synthesis (Kupiec et al., 1988; Tobaly-Tapiero et al., 1991).

The FV replication pathway diverges from the orthoretrovirus replication strategy in several aspects (for reviews, see Linial, 2007; Rethwilm, 2003, 2005). One prominent variation consists in the time point of reverse transcription, which in FVs appears to happen to a large extent late in replication before the progeny virus buds (Moebes et al., 1997; Roy et al., 2003; Yu et al., 1999). This results in FVs having a double-stranded DNA genome. However, there is evidence that plus-strand reverse transcription is not complete (Delelis et al., 2003; Roy et al., 2003; Schweizer et al., 1989; Yu et al., 1999). Since the single-stranded region of the genome has not been characterized exactly so far (Kupiec et al., 1988; Tobaly-Tapiero et al., 1991), we aimed to do so.

The lentivirus cPPT has been shown to function in delaying genomic RNA degradation at this site during minus-strand cDNA synthesis. Although the mechanism of resistance to degradation by RNaseH is not fully understood, the consequence is the generation of an RNA primer for plus-strand cDNA synthesis (for a review, see Rausch and Le Grice, 2004.

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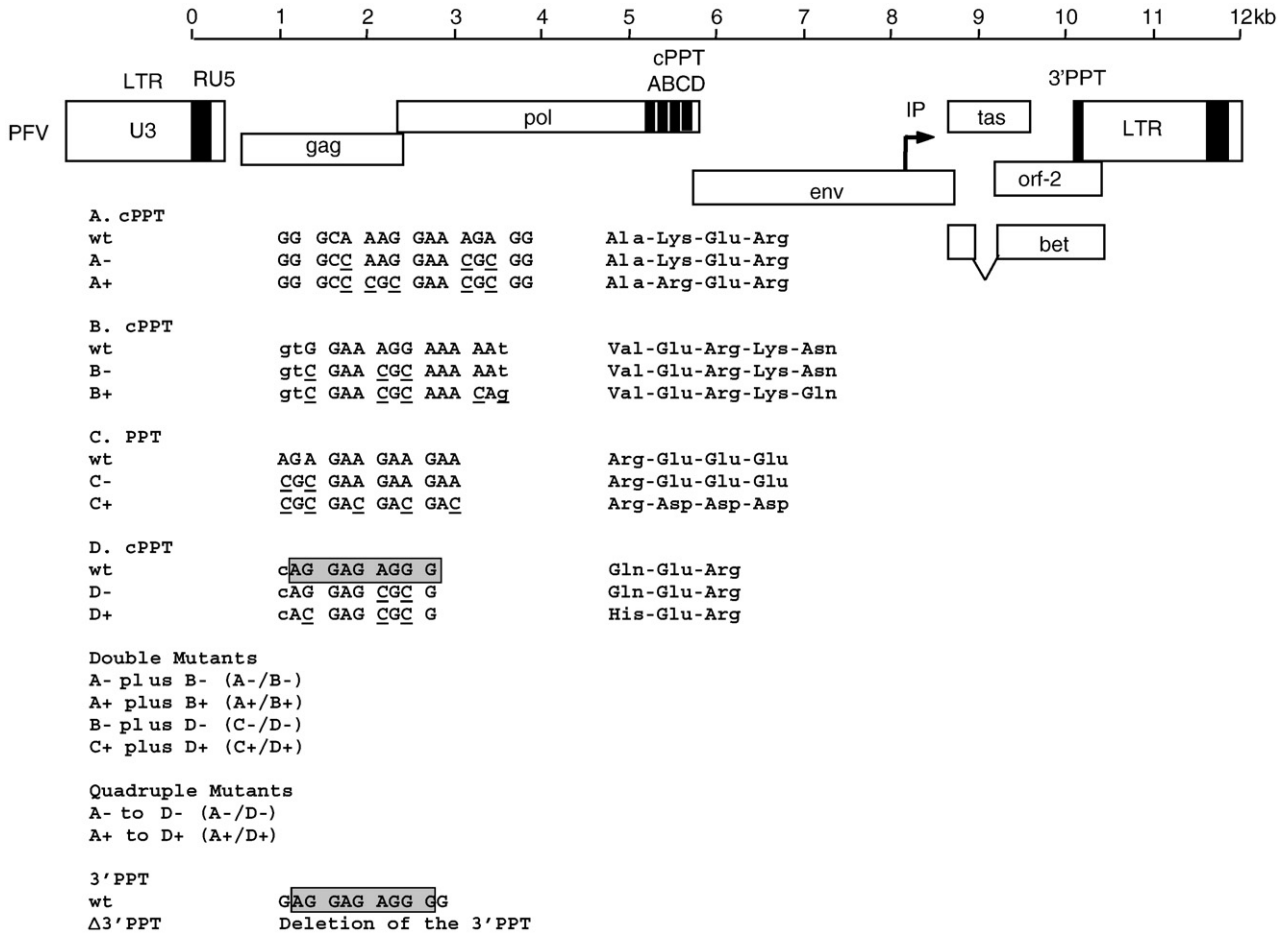

Fig. 1. Overview of PFV purine-rich elements and the mutants analyzed in this study. Upper panel: Genome organization of PFV and location of the purine-rich regions A–D in the center of the genome. Lower panel: The nucleotide sequences of the individual elements and the mutants are shown on the left side. Mutated nucleotides are underlined. The identical nucleotides between the element D and the 3′ PPT are highlighted. The IN open reading frame (ORF) and the altered amino acids in the + mutants are shown on the right side.

The function of dual initiation of plus-strand synthesis is not precisely defined. It has been suggested that lentiviral reverse transcription may profit from two origins of plus-strand priming by speeding up plus-strand reverse transcription due to the length of the genome (Charneau et al., 1992; Hungnes et al., 1992). This view is compatible with a cPPT in FVs, which have the largest of all retroviral genomes (Linial, 2007; Rethwilm, 2003; Rethwilm, 2005). On the other hand, a cPPT has not been described for members of the epsilon genus of retroviruses, the genomes of which are considerably longer than those of lentiviruses (Goff, 2007). Furthermore, there exists only one start of reverse transcription of the DNA minus-strand. This makes the necessity of two primers for plus-strand synthesis questionable. In addition, in case the plus-strand cDNA reverse transcript initiating at an internal PPT is transferred to complete the reverse transcription reaction, it will not be able to generate replication-competent offspring because the attachment site at the left (5′) end of the unintegrated DNA will not be correctly presented to the viral integrase (IN) enzyme (Bowman et al., 1996). Dual initiation of plus-strand reverse transcription can also occur by chance and at minor frequencies in simple retroviruses (Wei Hsu and Taylor, 1982; Taylor et al., 1983).

Evidence has been provided that in lentiviruses the cPPT promotes plus-strand recombination by strand displacement (Fuentes et al., 1996). Recombination is a frequent event in infections by human immunodeficiency viruses (HIVs) (Charpentier et al., 2006; Nora et al., 2007; Shriner et al., 2004). Thus, the virus profits from this mechanism *in vivo* by being able to generate more virus variants that are resistant to antiviral drugs or immunity. However, FVs are known to be genetically extremely stable and do not generate viral variants *in vivo* (Switzer et al., 2005; Thümer et al., 2007). Instead of generating multiple variants as in the case of HIV, FV replication may profit from recombination by preserving the relatively large genome in the event of mutations in the RNA (pre)genome. The high rate of template jumping found recently (Boyer et al., 2007) upon characterization of the FV reverse transcriptase (RT) *in vitro* is in accordance with this view.

When the growing upstream plus-strand encounters the region of the cPPT in lentiviruses, RT continues to synthesize an additional 100 nts approximately and stops at a central termination sequence (CTS) (Charneau et al., 1994; Whitwam et al., 2001). Thus, an overlap or region of displacement of the downstream plus-strand, which originated at the cPPT, is

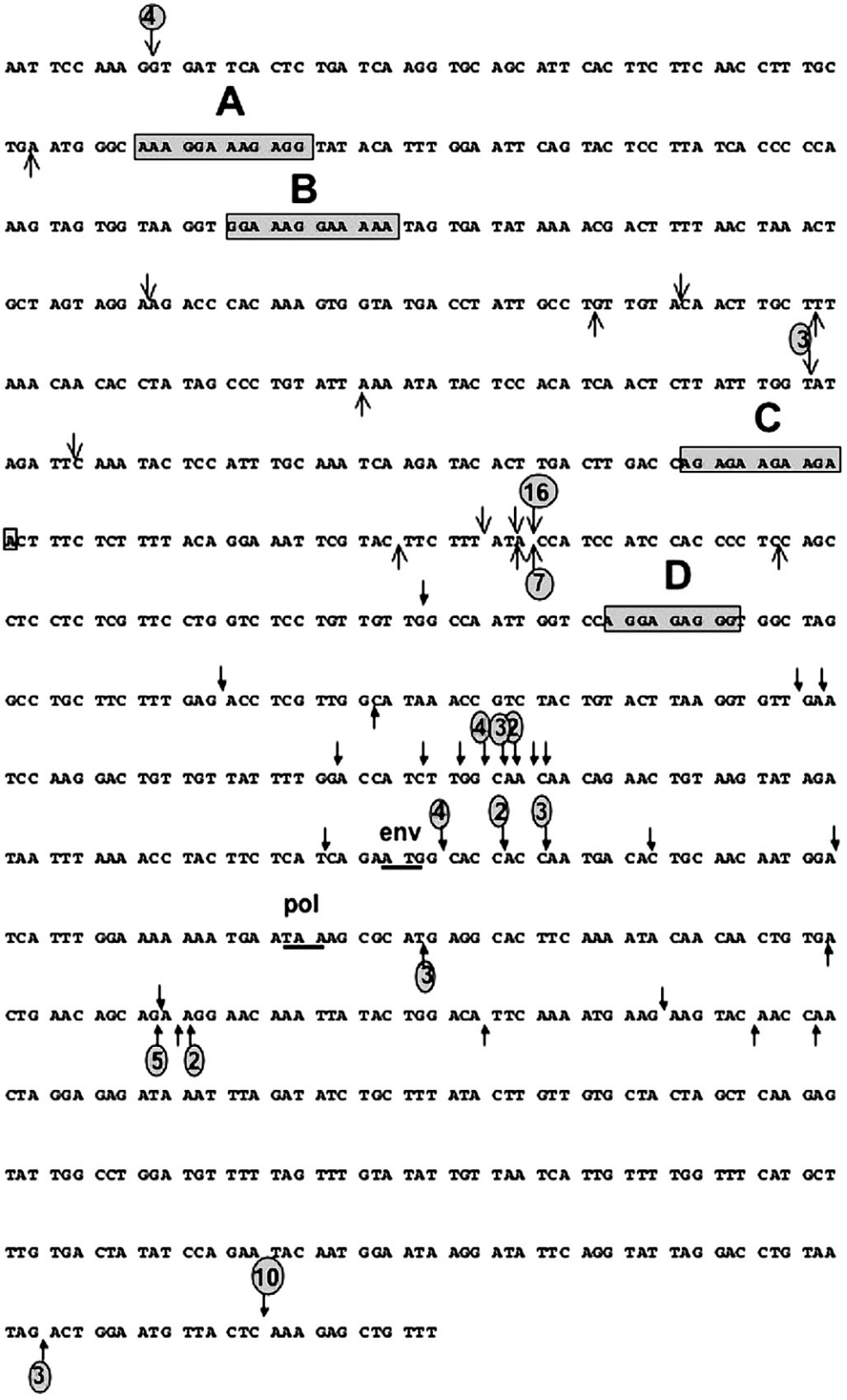

Fig. 2. Results of the 3′ and 5′ RACE experiments of virion DNA. RACE experiments were used to map the location of centrally terminated and initiated plus-strand reverse transcripts to the PFV genome. The amounts and positions of individual molecular clones are shown relative to the nucleotide sequence spanning the center of the PFV genome. An arrow without a number indicates one molecular clone. Results from wild-type virus are shown above the sequence (pointing down) and from the experiments with the D mutants below (pointing up). Thin arrows indicate results from 3′ RACEs and bold arrows from 5′ RACEs. The stop codon of the pol ORF and the start codon of the env ORF are underlined.

generated. This has been called the central DNA flap (Zennou et al., 2000). It has been suggested that the central DNA flap plays a role in lentiviral nuclear import of the pre-integration complex (PIC) (Zennou et al., 2000). However, others have disputed this view (Limón et al., 2002; Dvorin et al., 2002). Furthermore, a flap has not yet been demonstrated for FVs, which are also unable to infect truly resting cells (Bieniasz et al., 1995; Patton et al., 2004; Trobridge and Russell, 2004).

Whatever the exact function of the cPPTs in the individual reverse transcribing systems may be, it has been demonstrated that it is not essential for transposition of the Ty1 element (Heyman et al., 2003) and its mutagenesis delayed, but did not prevent, HIV replication in cell culture (Charneau et al., 1992). In contrast, the mutagenesis of the CTS signal in a proviral HIV context revealed that viral replication was more dramatically reduced, but also not prevented (Charneau et al., 1994). On the other hand, random mutagenesis that disabled the 3′ PPT failed to prevent viral replication completely, but the PPT quickly reverted to wild type (wt) (Miles et al., 2005).

Taken together, previous studies on the roles of cPPTs indicated some importance for viral replication without clarifying the mechanism. Since functional aspects of the FV cPPT have not yet been analyzed, we wanted to gain insight by using PFV and mutants thereof as a model.

Results

Characterization of PFV virion DNA

We first aimed to identify the proposed central gap region in the PFV virion DNA on the nucleotide level. To do this, we employed 3′ and 5′ RACE on viral DNA extracted from partially purified extra-cellular particles. In HIV, the upstream plus-strand terminates in a well-defined region downstream of the cPPT at the CTS (Charneau et al., 1994). In the PFV 3′ RACE experiments, 28 molecular clones were analyzed and 16 of these were found to terminate at one position downstream of the cPPT C (Fig. 2). Two further clones terminated in close proximity. The distribution of the other clones was more heterogeneous and covered a region from 5′ cPPT A to 5′ cPPT C (Fig. 2). In summary , the 3′ end of the upstream plus-strand appears to be located within a 370-nt region of the PFV genome.

The 5′ RACE experiments were designed to reveal the central start of transcription of the downstream plus-strand. Surprisingly, we did not identify a defined location, but the majority of the 42 molecular clones characterized in these experiments and potentially representing centrally initiated reverse transcripts started in three regions downstream of cPPT D (Fig. 2). The first being located 85–105 nt downstream of cPPT D, region II was identified between 145 and 170 nt downstream of cPPT D, and hotspot III was a single location 315 nt further 3′ (Fig. 2). In summary, the downstream plus-strand appeared to start in a region within approximately 500 nt 3′ of cPPT D since only one 5′ cDNA end was identified to be located upstream of this position.

The results presented in Fig. 2 were derived from five experiments for each DNA end. In some experiments, we digested the virion DNA with RNase to exclude any possibility of detecting RNA-derived ends. This treatment did not alter the results.

In conclusion, the RACE experiments revealed a centrally located gap in the PFV DNA that is approximately between 50 (the shortest distance identified between the individual cDNA ends) and 920 nt (the longest distance) in length. We find no evidence for a centrally located DNA flap, as has been suggested for HIV (Zennou et al., 2000).

Generation and analysis of proviral cPPT mutants

To analyze potential functions of the PFV cPPTs, we established several mutants of the infectious molecular clone pcHSRV2. The overlapping integrase (IN) frame only permitted minor alterations of the cPPTs without the introduction of IN amino acid changes (Fig. 1). Therefore, two mutants for each central purine-rich element were designed, one (the −mutant) was without change of the IN reading frame, while the other (the +mutant) introduced amino acid changes in IN and more severely altered the nucleotide composition of the respective element (Fig. 1). In addition, we constructed several combinatory mutants; these mutants combined A− and B− (A−/B−), A+ and B+ (A+/B+), C− and D− (C−/D−), C+ and D+ (C+/D+), A− to D− (A−/D−), and A+ to D+ (A+/D+), respectively (Fig. 1).

The constructs were tested for the expression of viral Gag and Pol proteins and for the generation of infectious virus following transient transfection of 293T cells. As shown in Fig. 3A, all mutants except C+ and derivatives containing C+ generated intracellular Gag and Pol proteins to steady state

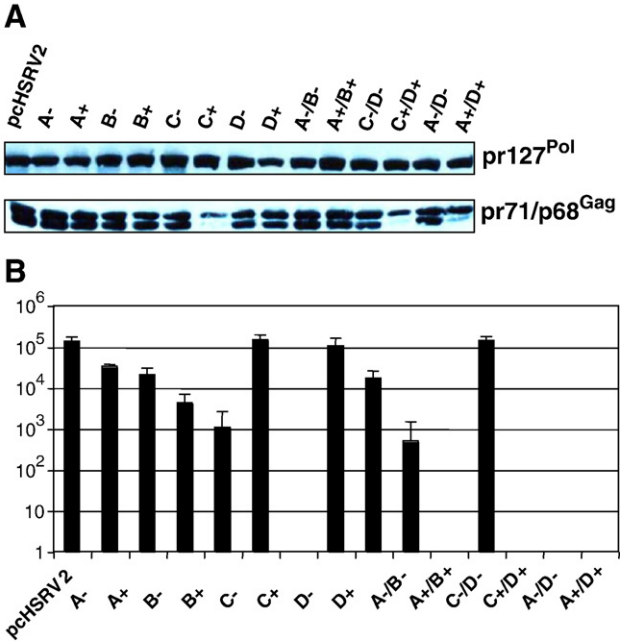

Fig. 3. Replication of cPPT mutants in the proviral context. The mutants shown in Fig. 1 were introduced into the proviral pcHSRV2 background and analyzed in panel A for the intracellular expression levels of Gag and Pol proteins following transient transfection of 293T cells and in panel B for the amounts of infectious cell-free virus secreted into the supernatant and scored by a blue-cell assay on indicator cells. The titer on the *y*-axis is in infectious units per ml.

levels comparable to non-mutated pcHSRV2. In addition, cleavage of the Gag precursor pr71^{gag} was hardly detectable with the C+ mutants, despite the expression of appreciable amounts of Pol (Fig. 3A).

When the viral titers were determined, only C−, D−, and the combinatory mutant C−/D− were able transiently to produce infectious virus in the range of pHSRV2, while the other mutants were found to be more or less reduced in this capacity (Fig. 3B). We observed a gradual decline in the replication competence from A− to B+. Replication of the D+ mutant was reduced by the factor of 10. Consistent with the very low Gag expression levels, a titer of less than 1/ml was found for the C+ mutation and all derivatives containing C+. The other combinatory mutants showed an additive effect of the single mutations. A−/B− was severely restricted in replication and generated a virus titer two to three orders of magnitude reduced when compared to non-mutated virus; A+/B+ and A−/D− were found to be replication incompetent (Fig. 3B).

Analysis of Pol protein functionality

We next determined the influence of the introduced mutations on Pol protein function since the experiments with the proviral mutants raised the possibility that alterations of the IN frame in the +mutants would alter the Pol protein function and result in the observed replication defects. To determine this more precisely, we inserted the mutations into the Pol protein expression plasmid pCpol-2 (Fig. 4). Any replication defect due to the alteration of the IN frame of the +mutants should lead to a drop in transduction efficiency of vector supernatant produced by transfection of 293T cells with the respective mutants together with plasmids specifying PFV Gag and Env proteins and a PFV vector. We analyzed enhanced green fluorescent protein (EGFP)—encoding vector transfer by FACS 48 h after transduction of recipient HT1080 cells (Fig. 5). As expected, the transduction rates of all −mutants were in the range of those obtained with wt pCpol-2. A pronounced defect was revealed by the B+, C+, and D+ and all mutants derived from them (A+/B+, C+/D+, and A+/D+). This indicated the inability of the respective mutants to express functional Pol protein. Thus, the results obtained with these six +mutants in the above-described

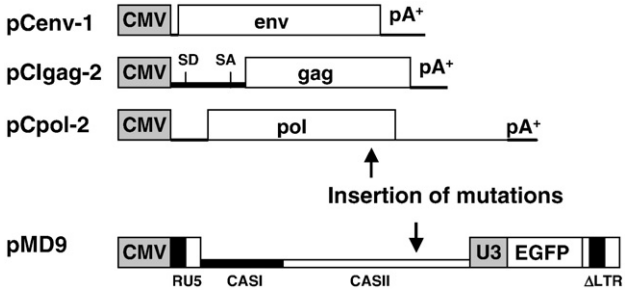

Fig. 4. Strategy to analyze mutant central purine-rich elements. The mutants shown in Fig. 1 were introduced into the *pol* expression plasmid pCpol-2 and into the EGFP-encoding PFV vector pMD9. The use of wt Gag- and Env-specifying constructs together with pMD9 vector, in case of *pol* mutants, and wt pCpol-2, in case of pMD9 mutants, allowed for the characterization of the mutant plasmids on the protein and nucleic acid level.

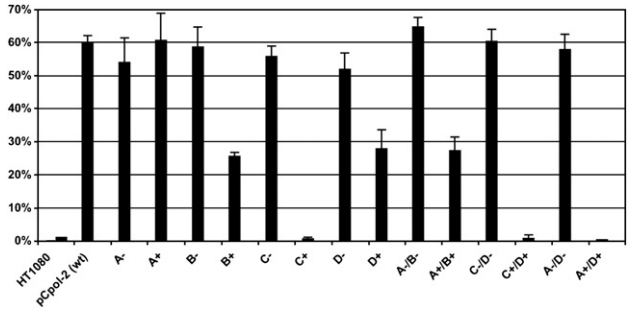

Fig. 5. Transduction rates using pCpol-2 mutants. HEK 293T cells were cotransfected with wt EGFP-encoding pMD9 vector, Gag- and Env-specifying plasmids, and pCpol-2 mutant plasmids. Vector in the cell-free supernatant was used to transduce recipient HT1080 fibroblasts. The transduction rates were determined by FACS analysis.

experiment with the proviral constructs may be due to disabling the viral Pol enzyme, namely the IN function.

Analysis of viral vectors with cPPT mutations

To rule out any effect of the introduced mutations on viral protein function and to determine the fate of the mutations at the nucleic acid level, we inserted them into the pMD9 vector (Fig. 4). Since viral proteins are not generated by pMD9 (Heinkelein et al., 2002), any defect in vector transfer detected with the pMD9 derivatives should be a direct consequence of alterations in nucleic acid elements. Viral supernatant was produced by cotransfection of HEK 293T cells with EGFP-encoding vector DNA and wt expression plasmids specifying PFV Gag, Pol, and Env proteins (Fig. 4). As depicted in Fig. 6A, only the transfection of cells with the C− and C+ derivatives of pMD9 resulted in amounts of vector virus, which were similar (C−) or even slightly above (C+) the wt level. All other single or combinatory mutants resulted in transduction efficiencies, which were approximately half (A−, A+, D−, D+, C−/D−, and C+/D+) the wt level or even more severely reduced (B−, B+, A−/B−, A+/B+, A−/D−, and A+/D+). In particular, the combinatory mutants A−/D− and A+/D+ were in the range of Δ3′ PPT that lacks the 3′ PPT and was highly dysfunctional in vector transfer (Fig. 6A).

To elucidate potential mechanisms responsible for the vector transfer reduction of the affected mutants, we determined the composition of partially purified extra-cellular viral particles with respect to PFV Gag and Pol proteins (Fig. 6B). Interestingly, we found that all mutants in the A and B elements and their derivatives, which were low in vector transfer, were also restricted in incorporating appreciable amounts of Pol proteins detectable by our antibodies (Fig. 6B). This deficiency did not result from lower cellular expression levels, as all mutants generated wt levels of intracellular Pol precursor protein (Fig. 6C). Furthermore, cleavage of the Gag precursor protein by the pol-encoded protease was detectable in all mutants. Such cleavages likely occurred intracellularly by catalytically active protein. However, we did not find, for instance, any particle-associated IN incorporated by the respective mutants (Fig. 6B). The mutants in the D element showed an approximately 50% reduction in vector titer without

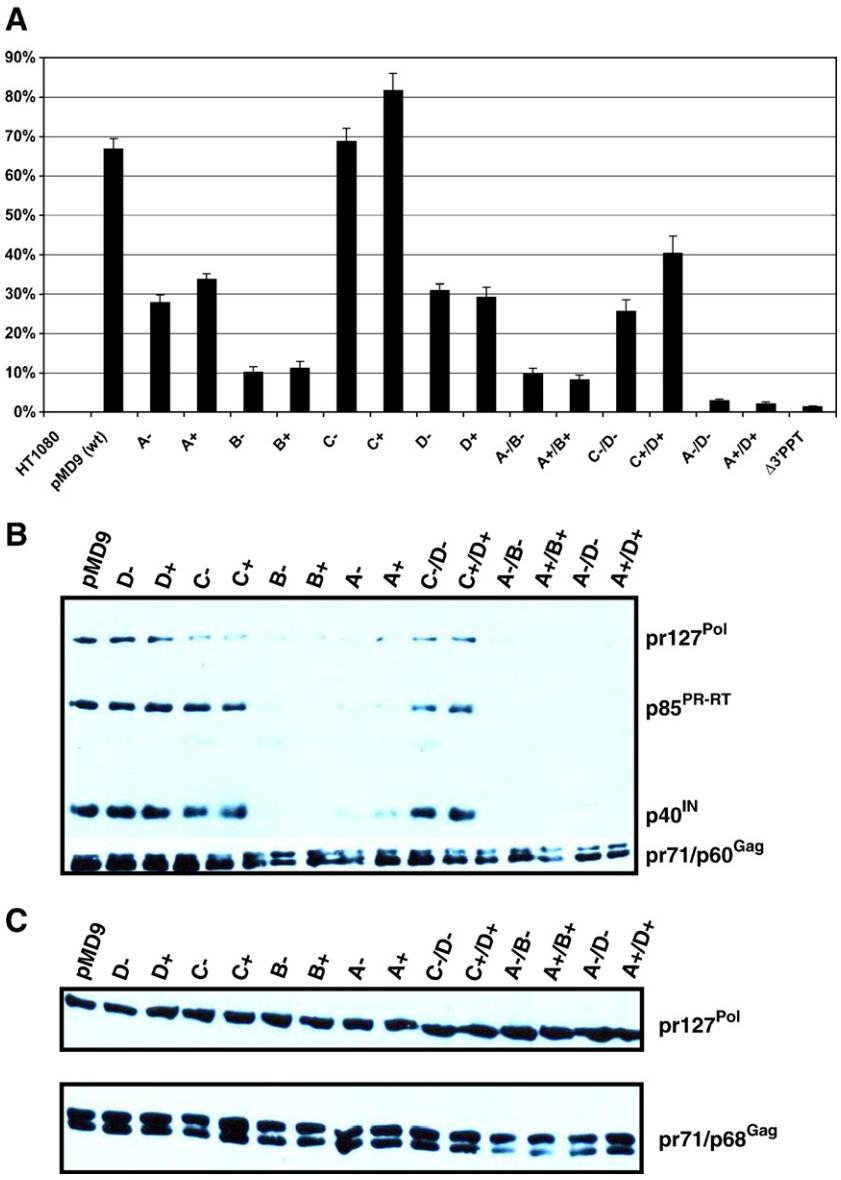

Fig. 6. Transduction rates using pMD9 mutants and pMD9 mutant virus protein composition. In panel A, the transduction rates on HT1080 recipient cells were determined following the generation of vector by cotransfection of 293T cells with plasmids specifying wt Gag, Pol, and Env proteins together with mutant pMD9 plasmids. In panel B, we determined the amounts of Gag and Pol proteins that could be detected in vector particles partially purified by centrifugation through a sucrose cushion. For comparison, the viral Gag and Pol proteins expressed in the transfected cells are shown in panel C.

evidence of reduced Pol protein encapsidation and prompted further analyses.

AZT inhibition test

One of the distinguishing features of the FV replication strategy compared to orthoretroviruses concerns the time point of reverse transcription that is "early" in orthoretroviruses, while it is to a large extent "late" in FVs (Moebes et al., 1997). This results in FVs having a DNA genome (Roy et al., 2003; Yu et al., 1999). One of the most stringent ways to assay this is by producing and titrating virus in the presence or absence of the RT-inhibiting drug AZT (Moebes et al., 1997; Roy et al., 2003). However, except for an expected reduction in viral titers, the analysis of the D element mutants in the proviral context did not reveal significant changes in the levels of "late" versus "early" reverse transcription in relation to the unmutated virus (data not shown).

Determination of the cDNA gap region in the D element mutants

We finally investigated whether the centrally located gap region in the plus-strand that was identified in the experiment shown in Fig. 2 was altered in pcHSRV2/D− and /D+ compared to unmutated virus. Transfection of cells with both plasmids resulted in appreciable amounts of replication-competent virus. The RACE analysis of DNA from D− and D+ virions (altogether 14 3′ RACE molecular clones and 19 5′ RACE molecular clones) did not reveal significant differences with

respect to the location of plus-strand transcript termination and start sequences compared to the unmutated virus (Fig. 2). However, while the majority of downstream plus-strand reverse transcripts initiated at hotspot I in the unmutated virus, this region appeared to be spared in the D element-mutated clones.

Discussion

The roles for viral replication of the central purine-rich regions within the PFV genome appear to be complex. The results are suggestive of different functions for the different motifs.

As shown in the pMD9 vector transfer experiment, mutations of the A and B elements resulted in defects to encapsidate Pol protein (Fig. 6). Both elements are located in a region that has been implicated in packaging the Pol protein (Peters et al., 2005). Although the exact mechanism of FV Pol encapsidation is still unknown, the findings with the A and B mutants are highly suggestive of having altered motifs that are directly or indirectly involved in facilitating RNA–Pol interactions.

The C element is obviously relevant in *cis* to allow for the generation of reasonable amounts of Gag protein (Fig. 3B). This became evident only with the C+ mutant that also affected Pol function (Fig. 5). Although FV gene expression involves a variety of spliced subgenomic RNAs, any virally encoded transactivator acting at the post-transcriptional level has not been identified (Linial, 2007; Rethwilm, 2005). However, Wodrich et al. (2001) previously demonstrated that PFV bears an RNA element permitting the expression of HIV Gag independently of the Rev-RRE interaction. Interestingly, the purine-rich C element is contained within the region identified by these authors. An RNA transport element relevant for regulating FV gene expression post-transcriptionally has not yet been identified. It is possible that with our C+ mutant, we disturbed an FV RNA sequence fulfilling this function, namely nuclear export of unspliced RNA. However, the function of this hypothetical element was certainly not ablated since residual Gag expression could be detected (Fig. 3A). If the assumption of the C element acting in *cis* on capsid protein expression was correct, C− and C+ should not negatively influence vector transfer in the pMD9 background because in the four-plasmid vector system, the Gag protein is specified by a different expression plasmid and expression of the indicator gene is directed by a different expression cassette (Heinkelein et al., 2002). This was exactly what we observed (Fig. 6A).

Concerning the D element, our results highlight the importance of this motif for viral replication and vector transfer. It has been noted already (Kupiec et al., 1988) that the 3′ PPT is perfectly mirrored in the cPPT D element (Fig. 1). Furthermore, a cPPT with sequence identity to the 3′ PPT is a conserved feature of all sequenced FV genomes (Fig. 7). This strongly suggests functional importance of this motif.

In the transient replication assay, both D element mutants failed to reveal significant differences to the unmutated virus. However, the blue-cell assay we employed for titration measures the activity of the viral Tas transcriptional transactivator, the expression of which is probably independent of proviral integration. The necessity for integration and a functional IN protein becomes relevant only upon virus cultivation (Enssle et al., 1999; Meiering et al., 2000). Therefore, it will be interesting to analyze the pcHSRV2/D− mutant upon long-term viral replication in future experiments.

	cPPT	3'PPT
PFV (D)	AGGAGAGGG	AGGAGAGGG
SFVcpz	AGGAGAGGG	AGGAGAGGG
SFVora	AGGAGAGGG	AGGAGAGGG
SFVmac	AGGAGAGGG	AGGAGAGGG
SFVagm	AGGAGAGGG	AGcAGAGGG
SFVspm	AGGAGAGGG	AGGAGAGGG
FFV	AGGAGAGGG	AGGAGAGGG
BFV	AGGAGAGGG	AGGAGAGGG
EFV	AGGAGAGGG	AGGAGAGGG

Fig. 7. Conservation of cPPT sequences among FV genomes. An element corresponding to the D motif of PFV and identical to the 3′ PPT is almost perfectly conserved in all FV genomes sequenced to date. Sequences were taken from PFV (GenBank accession number: Y07725) and from FVs from chimpanzee (SFVcpz; U04327), orangutan (SFVora; AJ544579), macaque (SFVmac; X54482), African green monkey (SFVagm; M74895), spider monkey (SFVspm; EU010385), felines (FFV; AJ564746), bovines (BFV; U94514), and equines (EFV; AF201902).

Evidence has been presented for a role of the flap structure at the cPPT of HIV in nuclear entry of the reverse transcript (Arhel et al., 2006; Arhel et al., 2007). Our RACE experiments did not reveal a flap structure in PFV DNA. PFV vectors have the ability to transduce rarely dividing cells, such as hematopoietic stem cells, with high efficiency (for a review, see Rethwilm, 2007). It may be that access of the FV genome to the host chromatin in these cells is mediated by the cPPT. This can be analyzed using the pMD9 vector derivatives described in this study.

We also determined the PFV single-stranded region consisting only of the minus-stranded DNA. We did not found evidence for a definite stop or for a start of transcription. Instead, the RACE experiments revealed the gap region to be relatively flexible in length. Our finding of an extended gap region is in accordance with some “early” reverse transcription occurring in the target cell (Delelis et al., 2003). However, since we analyzed separately only the internal termination and start points of plus-strand reverse transcripts, we cannot exclude a shorter gap region than 920 bp. Taking the locations of the majority of terminating and initiating (hotspot I) plus-strand reverse transcripts into consideration, a medium gap region of approximately 170 nts appears probable. This is in accordance with previous suggestions of an approximately 200 nts FV gap region (Kupiec et al., 1988; Tobaly-Tapiero et al., 1991).

In particular, the lack of a definite start downstream plus-strand reverse transcripts was surprising since we expected these to originate at or a few nts 3′ of the cPPT D. Shuffling of the cPPT D within the pMD9 genome and reverse transcript mapping studies should reveal whether location of cPPT D is essential for replication and may point to its function. In addition, future experiments employing more quantitative techniques

should show whether there exist differences in this respect between unmutated and cPPT-mutated virus.

Materials and methods

Cells and viruses

HEK 293T, BHK/LTR(PFV)LacZ, and HT1080 cells were cultivated as described (Heinkelein et al., 2002; Schmidt and Rethwilm, 1995). Virus was obtained by transient transfection of 293T cells with pcHSRV2-derived (Moebes et al., 1997) proviral constructs employing the Polyfect (Qiagen) transfection protocol (Heinkelein et al., 2002). For the generation of viral vector-containing supernatants, we used a polyethyleneimine (PEI) transfection (Mannigel et al., 2007) protocol of 293T cells with separate expression plasmids for Gag, Pol, Env, and a PFV vector specifying EGFP (Heinkelein et al., 2002). Supernatants were clarified through 0.45-μm pore-size filters and used for virus titrations on BHK/LTR(PFV)LacZ cells (Schmidt and Rethwilm, 1995) or the determination of vector transfer rates on HT1080 cells by fluorescence-activated cell sorting (FACS) (Peters et al., 2005). In addition, cellular lysates were prepared from transfected cells and analyzed by immunoblotting with PFV Gag- and Pol-specific monoclonal antibodies, as described (Peters et al., 2005). Extra-cellular virus was partially purified by centrifugation (in a Sorvall TH641 rotor at 25,000 rpm and 4 °C for 3 h) through a cushion of 20% sucrose and then used in immunoblotting experiments (Peters et al., 2005). All experiments were done at least three times.

AZT inhibition assay

293T cells were transfected with plasmid DNA and viral titers in the supernatant were analyzed on the indicator cells. Virus production and titration were done either in the absence or presence of AZT (zidovudine), as described (Moebes et al., 1997; Roy et al., 2003).

Rapid amplification of cDNA ends (RACE)

Nucleic acids were extracted from partially purified virus using the MinElute Virus Spin kit (Qiagen). The RACE experiments were performed with 100 ng of virion DNA, either left untreated or treated with RNase (10 mg/ml at room temperature for 15 min) following extraction. We employed different primer combinations and C- or T-tailing in RACEs to enable the detection of minor species of reverse transcripts.

5′ RACE was carried out by elongating 100 pmol of primers #312 or #4192 that were hybridized to the downstream DNA plus-strand in a linear PCR-reaction with Pwo-polymerase (Peqlab) for 35 cycles. Following purification of the PCR product with the PureLink PCR Purification kit (Invitrogen), the DNA was C- or T-tailed, respectively, with 80 U terminal deoxynucleotidyl transferase (MBI Fermentas) and 200 mmol TTP or dCTP, respectively. After phenol–chloroform extraction and precipitation, one-fourth of the tailed DNA was subjected to a standard PCR reaction with 100 pmol of each primer #4192 and #4127 (oligo-dG primer) or #312 and #4194 (oligo-dA primer), respectively, and Pwo polymerase. After purification of the amplimer, a nested PCR was performed with 1/50 of the DNA from the first reaction and with 100 pmol of each primer #4127 and #4193 or using the combination #326 and #4194 and Pwo polymerase. The final elongation step was performed after addition of Taq polymerase (MBI Fermentas). After purification, the amplimers were inserted into the pDrive or pGEM-T Easy cloning vectors (Qiagen and Promega, respectively). The ligation reaction was used to transform Top10F′ bacteria (Invitrogen) that were selected for blue and white colonies with isopropylthio-β-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). DNA from white colonies was prepared, diagnostically analyzed after digestion with Alw44I, and sequenced using M13 forward and reverse primers.

The 3′ RACE was performed in an analogous way, omitting the initial linear PCR reaction and by directly adding the C- or T-tail. Of the nested PCR, the first reaction was performed with primers #1849 and #4127 or with the primer combination #1447 and #4194, while the second reaction was done with primers #4191 and #4127 or with primers #1448 and #4194, respectively. The DNA from bacterial colonies was diagnostically screened by *Sca*I digestion before sequencing.

The primers sequences are shown online under http://viminfo.virologie.uni-wuerzburg.de/onlinematerial/Peters.pdf.

Recombinant DNA

The PFV *pol* gene was amplified from pcHSRV2 (Moebes et al., 1997) with primers #312 and #1957 and inserted into the pCRII-TOPO vector (Invitrogen) prior to DNA sequencing to verify the wt gene. This plasmid was used for mutagenesis of the cPPTs employing the 16 oligonucleotides #1967 to #1970, #1973, #1974, and #1977 to #1986 (all primer sequences are shown online under the abovementioned Web address) and the QuickChange Site-Directed Mutagenesis kit (Stratagene). Following DNA sequencing, the altered 2.3-kb *Pac*I/*Bsp*EI fragments were inserted into the 13.86- and 8.9-kb fragments of *Pac*I/*Bsp*EI digested pcHSRV2 and pCpol-2 (Heinkelein et al., 1998; Moebes et al., 1997), respectively. This resulted in mutants A− to D+ in Fig. 1 (M126 to M133). The pcHSRV2 double mutants A−/B− (M134) and A+/B+ (M135) were made by inserting the 1.34-kb *Pac*I/*Eco*RI fragments from M126 and M127 and the 0.96-kb *Eco*RI/*Bsp*EI fragments from M128 and M129 into the 13.86-kb fragment of *Pac*I/*Bsp*EI digested pcHSRV2 via a three fragment ligation. The pcHSRV2 double mutants C−/D− (M136) and C+/D+ (M137) were constructed in a similar way by joining the 1.68-kb *Pac*I/*Mfe*I fragments from M130 and M131, the 0.62-kb *Mfe*I/*Bsp*EI fragments from M132 and M133, and the large 13.86 kb *Pac*I/*Bsp*EI fragment of pcHSRV2. The pCpol-2 double mutants were made by exchanging the 2.3-kb *Pac*I/*Bsp*EI wt fragment for the respective fragment of pcHSRV2 mutants M134 to M137. The quadruple mutants A−/D− (M138) and A+/D+ (M139) were first generated in the pCpol-2 background by fusing the 0.2-kb *Bsg*I/*Sfc*I fragments of pCpol-2 M134 and M135 with the 0.8-kb *Sfc*I/*Bsp*EI fragments of pCpol-2 M136 and M137 and the 10.2-

kb fragment of pCpol-2 digested with *Bsg*I and *Bsp*EI. The proviral pcHSRV2 derivatives M138 and M139 were created by exchanging the 2.3-kb *Pac*I/*Bsp*EI fragment from pCpol-2 M138 and M139, respectively. The pMD9 (Heinkelein et al., 2002) vector derivatives were generated via a three-fragment ligation of the 8.8-kb *Bmg*BI/*Bsm*BI pMD9 fragment with the 0.82-kb *Bmg*BI/*Afl*II and the 0.28-kb *Afl*II/*Bsm*BI fragments derived from the respective pCpol-2 variants. The Δ3′ PPT construct (M140) was made by exchanging a 0.76-kb *Not*I/*Fsp*AI fragment, where *Not*I cuts 5′ of the 3′ PPT and *Fsp*AI in the U3 region of the LTR, for a 0.69-kb PCR fragment. This amplimer was generated with primers #4086 and #4087. Primer #4087 introduced a *Not*I restriction site immediately 5′ of the U3 region of the LTR.

All constructs were DNA sequenced at the final level in the altered or exchanged parts to exclude unwanted mutations with oligonucleotide primers available online under the above-mentioned Web address.

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References

Arhel, N., Munier, S., Souque, P., Mollier, K., Charneau, P., 2006. Nuclear import defect of human immunodeficiency virus type 1 DNA flap mutants is not dependent on the viral strain or target cell type. J. Virol. 80, 10262–10269.

Arhel, N.J., Souquere-Besse, S., Munier, S., Souque, P., Guadagnini, S., Rutherford, S., Prevost, M.C., Allen, T.D., Charneau, P., 2007. HIV-1 DNA Flap formation promotes uncoating of the pre-integration complex at the nuclear pore. EMBO J. 26, 3025–3037.

Bieniasz, P.D., Weiss, R.A., McClure, M.O., 1995. Cell cycle dependence of foamy retrovirus infection. J. Virol. 69, 7295–7299.

Bowman, E.H., Pathak, V.K., Hu, W.S., 1996. Efficient initiation and strand transfer of polypurine tract-primed plus-strand DNA prevent strand transfer of internally initiated plus-strand DNA. J. Virol. 70, 1687–1694.

Boyer, P.L., Stenbak, C., Hoberman, D., Linial, M., Hughes, H.S., 2007. Fidelity of the prototype primate foamy virus (PFV) RT compared to HIV-1 RT. Virology 367, 253–264.

Charneau, P., Alizon, M., Clavel, F., 1992. A second origin of DNA plus-strand synthesis is required for optimal human immunodeficiency virus replication. J. Virol. 66, 2814–2820.

Charneau, P., Mirambeau, G., Roux, P., Paulous, S., Buc, H., Clavel, F., 1994. HIV-1 reverse transcription. A termination step at the center of the genome. J. Mol. Biol. 241, 651–662.

Charpentier, C., Nora, T., Tenaillon, O., Clavel, F., Hance, A.J., 2006. Extensive recombination among human immunodeficiency virus type 1 quasispecies makes an important contribution to viral diversity in individual patients. J. Virol. 80, 2472–2482.

Delelis, O., Saïb, A., Sonigo, P., 2003. Biphasic DNA synthesis in spumaviruses. J. Virol. 77, 8141–8146.

Dvorin, J.D., Bell, P., Maul, P., Yamashita, G.G., Emermen, M., Malim, M.H., 2002. Reassasment of the roles of integrase and the central DNA flap in human immunodeficiency virus type 1 nuclear import. J. Virol. 76, 12087–12096.

Enssle, J., Moebes, A., Heinkelein, M., Panhuysen, M., Mauer, B., Schweizer, M., Neumann-Haefelin, D., Rethwilm, A., 1999. An active foamy virus integrase is required for virus replication. J. Gen. Virol. 80, 1445–1452.

Fuentes, G.M., Palaniappan, C., Fay, P.J., Bambara, R.A., 1996. Strand displacement synthesis in the central polypurine tract region of HIV-1 promotes DNA to DNA strand transfer recombination. J. Biol. Chem. 271, 29605–29611.

Goff, S., 2007. Retroviridae: the retroviruses and their replication, In: Kniepe, D.M., Howley, P.M. (Eds.), 5th Ed. Fields Virology, vol. 2. Lippincot Williams & Wilkins, Philadelphia, pp. 1999–2069. 2 vols.

Heinkelein, M., Schmidt, M., Fischer, N., Moebes, A., Lindemann, D., Enssle, J., Rethwilm, A., 1998. Characterization of a *cis*-acting sequence in the Pol region required to transfer human foamy virus vectors. J. Virol. 72, 6307–6314.

Heinkelein, M., Dressler, M., Jarmy, G., Rammling, M., Imrich, H., Thurow, J., Lindemann, D., Rethwilm, A., 2002. Improved primate foamy virus vectors and packaging constructs. J. Virol. 76, 3774–3783.

Heyman, T., Wilhelm, M., Wilhelm, F.X., 2003. The central PPT of the yeast retrotransposon Ty1 is not essential for transposition. J. Mol. Biol. 331, 315–320.

Hungnes, O., Tjotta, E., Grinde, B., 1992. Mutations in the central polypurine tract of HIV-1 result in delayed replication. Virology 190, 440–442.

Kupiec, J.J., Tobaly-Tapiero, J., Canivet, M., Santillana-Hayat, M., Flugel, R.M., Peries, J., Emanoil-Ravier, R., 1988. Evidence for a gapped linear duplex DNA intermediate in the replicative cycle of human and simian spumaviruses. Nucleic Acids Res. 16, 9557–9565.

Limón, A., Nakajima, N., Lu, R., Ghory, H.Z., Engelman, A., 2002. Wild-type levels of nuclear localization and human immunodeficiency virus type 1 replication in the absence of the central DNA flap. J. Virol. 76, 12078–12086.

Linial, M., 2007. Foamy viruses, In: Kniepe, D.M., Howley, P.M. (Eds.), 5th Ed. Fields Virology, vol. 2. Lippincot Williams & Wilkins, Philadelphia, pp. 2245–2262. 2 vols.

Mannigel, I., Stange, A., Zentgraf, H., Lindemann, D., 2007. Correct capsid assembly mediated by a conserved YXXLGL motif in prototypie foamy virus gag is essential for infectivity and reverse transcription of the viral genome. J. Virol. 81, 3317–3326.

Meiering, C.D., Comstock, K.E., Linial, M.L., 2000. Multiple integrations of human foamy virus in persistently infected human erythroleukemia cells. J. Virol. 74, 1718–1726.

Miles, L.R., Agresta, B.E., Khan, M.B., Tang, S., Levin, J.G., Powell, M.D., 2005. Effect of polypurine tract (PPT) mutations on human immunodeficiency virus type 1 replication: a virus with a completely randomized PPT retains low infectivity. J. Virol. 79, 6859–6867.

Moebes, A., Enssle, J., Bieniasz, P.D., Heinkelein, M., Lindemann, D., Bock, M., McClure, M.O., Rethwilm, A., 1997. Human foamy virus reverse transcription that occurs late in the viral replication cycle. J. Virol. 71, 7305–7311.

Noad, R.J., Al-Kaff, N.S., Turner, D.S., Covey, S.N., 1998. Analysis of polypurine tract-associated DNA plus-strand priming in vivo utilizing a plant pararetroviral vector carrying redundant ectopic priming elements. J. Biol. Chem. 273, 32568–32575.

Nora, T., Charpentier, C., Tenaillon, O., Hoede, C., Clavel, F., Hance, A.J., 2007. Contribution of recombination to the evolution of human immunodeficiency viruses expressing resistance to antiretroviral treatment. J. Virol. 81, 7620–7628.

Patton, G., Erlwein, O., McClure, M.O., 2004. Cell-cycle dependence of foamy virus vectors. J. Gen. Virol. 85, 2925–2930.

Peters, K., Wiktorowicz, T., Heinkelein, M., Rethwilm, A., 2005. RNA and protein requirements for incorporation of the pol protein into foamy virus particles. J. Virol. 79, 7005–7013.

Rausch, J.W., Le Grice, S.F., 2004. 'Binding, bending and bonding': polypurine tract-primed initiation of plus-strand DNA synthesis in human immunodeficiency virus. Int. J. Biochem. Cell. Biol. 36, 1752–1766.

Rethwilm, A., 2003. The replication strategy of foamy viruses. Curr. Top. Microbiol. Immunol. 277, 1–26.

Rethwilm, A., 2005. Foamy viruses, In: Mahy, B.W.J., ter Meulen, V. (Eds.), 10th Ed. Topley & Wilson's Microbiology and Microbial Infections—Virology, vol. 2. Hodder Arnold, London, pp. 1304–1321.

Rethwilm, A., 2007. Foamy virus vectors: an awaited alternative to gamma retroviral and lentiviral vectors. Curr. Gene Ther. 7, 261–271.

Roy, J., Rudolph, W., Juretzek, T., Gärtner, K., Bock, M., Herchenröder, O., Lindemann, D., Heinkelein, M., Rethwilm, A., 2003. Feline foamy virus genome and replication strategy. J. Virol. 77, 11324–11331.

Schmidt, M., Rethwilm, A., 1995. Replicating foamy virus-based vectors directing high level expression of foreign genes. Virology 210, 167–178.

Schweizer, M., Renne, R., Neumann-Haefelin, D., 1989. Structural analysis of proviral DNA in simian foamy virus (LK-3)-infected cells. Arch. Virol. 109, 103–114.

Shriner, D., Rodrigo, A.G., Nickle, D.C., Mullins, J.I., 2004. Pervasive genomic recombination of HIV-1 in vivo. Genetics 167, 1573–1583.

Switzer, W.M., Salemi, M., Shanmugam, V., Gao, F., Cong, M.E., Kuiken, C., Bhullar, V., Beer, B.E., Vallet, D., Gautier-Hion, A., Tooze, Z., Villinger, F., Holmes, E.C., Heneine, W., 2005. Ancient co-speciation of simian foamy viruses and primates. Nature 434, 376–380.

Taylor, J.M., Cywinski, A., Smith, J.K., 1983. Discontinuities in the DNA synthesized by an avian retrovirus. J. Virol. 48, 654–659.

Thümer, L., Rethwilm, A., Holmes, E.C., Bodem, J., 2007. Complete nucleotide sequence of a New World simian foamy virus. Virology 369, 191–197.

Tobaly-Tapiero, J., Kupiec, J.J., Santillana-Hayat, M., Canivet, M., Peries, J., Emanoil-Ravier, R., 1991. Further characterization of the gapped DNA intermediates of human spumavirus: evidence for a dual initiation of plus-strand DNA synthesis. J. Gen. Virol. 72, 605–608.

Trobridge, G., Russell, D.W., 2004. Cell cycle requirements for transduction by foamy virus vectors compared to those of oncovirus and lentivirus vectors. J. Virol. 78, 2327–2335.

Wei Hsu, T., Taylor, J.M., 1982. Single-stranded regions on unintegrated avian retrovirus DNA. J. Virol. 44, 47–53.

Whitwam, T., Peretz, M., Poeschla, E., 2001. Identification of a central DNA flap in feline immunodeficiency virus. J. Virol. 75, 9407–9414.

Wodrich, H., Bohne, J., Gumz, E., Welker, R., Krausslich, H.G., 2001. A new RNA element located in the coding region of a murine endogenous retrovirus can functionally replace the Rev/Rev-responsive element system in human immunodeficiency virus type 1 Gag expression. J. Virol. 75, 10670–10682.

Yu, S.F., Sullivan, M.D., Linial, M.L., 1999. Evidence that the human foamy virus genome is DNA. J. Virol. 73, 1565–1572.

Zennou, V., Petit, C., Guetard, D., Nerhbass, U., Montagnier, L., Charneau, P., 2000. HIV-1 genome nuclear import is mediated by a central DNA flap. Cell 101, 173–185.