Generation of an improved foamy virus vector by dissection of cis-acting sequences

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In contrast to other retroviruses, foamy viruses (FVs) generate their Pol protein precursor independently of the Gag protein from a spliced mRNA. The exact mechanism of Pol protein incorporation into the viral capsid is poorly understood. Previously, we showed that Pol encapsidation critically depends on the packaging of (pre-) genomic RNA and identified two distinct signals within the cis-acting sequences (CASI and CASII), Pol encapsidation sequences (PESI and PESII), which are required for Pol capsid incorporation. Here, we investigated whether the presence of PESI and PESII in an FV vector is sufficient for Pol encapsidation and whether the rather extended CASII element can be shortened without loss of functionality. Our results indicate that (i) the presence of PESI and II are not sufficient for Pol encapsidation, (ii) prototype FV vectors with a shortened CASII element retain Pol incorporation and full functionality, in particular upon transducing fibroblasts and primary human mesenchymal stem cells, (iii) the presence of the central poly purine tract significantly increased the transduction rates of FV vectors and (iv) Pol encapsidation and RNA packaging can be clearly separated. In essence, we designed a new FV vector that bears approximately 850 bp less of CAS than previously established vectors and is fully functional when analysed to transduce cell lines and primary human cells.

INTRODUCTION

Spumaretroviruses or foamy viruses (FVs) constitute one of the two subfamilies of retroviruses (Linial et al., 2005). The replication pathway of FV differs from what has been established for orthoretroviruses (Linial, 2007; Rethwilm, 2005). Despite the conservation of the general physical order of the provirus with long-terminal repeat (LTR)–gag–pol–env–accessory genes–LTR virtually all aspects of the replication pathway have their own facets that separate FVs from orthoretroviruses (Rethwilm, 2003). Among these, the ways that FVs express and encapsidate their reverse transcriptase proteins are central and pose some unresolved questions. The FV pol gene is expressed independently of the gag gene essentially from a spliced mRNA and gives rise to a large precursor protein comprised of protease (PR), reverse transcriptase/RNaseH (RT) and integrase (IN) (Bodem et al., 1996; Enssle et al., 1996; Yu et al., 1996). While IN is cleaved from the rest of the protein, no cleavage of PR from RT occurs (Flügel & Pfrepper, 2003). The capsid protein-independent translation of the pol gene raises the question of how the Pol protein is incorporated into the newly formed capsid.

FV genomes bear two cis-acting sequences (CASI and II), which are required for transfer of a vector (Erlwein et al., 1998; Heinkelein et al., 1998; Wu et al., 1998). These elements are located in the 5′ region of the (pre-) genomic RNA extending some hundred nucleotides into the gag gene and in the 3′ pol genomic region (Heinkelein et al., 2000, 2002a; Russell et al., 2001; Trobridge et al., 2002). Both regions (together more than 2.5 kb) have complex functions in virus replication: (i) the RNA packaging signal of FVs is most likely contained within these regions (Heinkelein et al., 1998, 2000, 2002a). However, the packaging sequence has not been defined exactly; nor has the exact mechanism of RNA packaging been resolved. (ii) Pol precursor protein encapsidation appears to require the packaging of (pre-) genomic RNA as a kind of bridging molecule, which makes contacts to both Pol and Gag (Heinkelein et al., 2002b; Peters et al., 2005). Only the 127 kDa Pol precursor protein is encapsidated, the cleavage products of 85 (PR-RT) and 40 kDa (IN) are not packaged separately (Peters et al., 2005). This implicates cleavage of the Pol precursor to occur after encapsidation. Within the CAS elements the sequence required for Pol encapsidation has been identified (Peters et al., 2005). (iii) Within the 3′
pol genomic region an RNA element is contained that facilitates nuclear export of gag and pol mRNA via a novel mechanism involving the cellular proteins CRM1, HuR, ANP52A and ANP52B (J. Boden and others, unpublished data). (iv) The 3' pol genomic region contains a perfect duplication of the 3' purine tract (PPT) (Peters et al., 2008). Mutagenesis of this central (c) PPT showed influence on the effectiveness of virus vector transductions (Peters et al., 2008). The reason for this, however, remains unknown.

Although it is likely that the RNA regions fulfilling these functions overlap, evidence has also been presented that separate regions exist allowing (pre-) genome packaging and Pol encapsidation (Peters et al., 2005). The bimodal nature of the CAS elements and their total length are strong arguments in favour of this view. In particular, two motifs have been defined; the deletion of either element abrogated Pol encapsidation, while preserving RNA packaging (Peters et al., 2005). The two Pol encapsidation sequences (PES) are contained within the two CAS elements previously identified. PESI consists of 30 nt upstream of the primer-binding site (PBS) and PESII was mapped to the 3' half (approx. 1 kb) of CASII (Peters et al., 2005); CASII has a total length of approximately 2 kb (Fig. 1).

Here, we addressed the questions whether the previously identified PES are not only required, but are also sufficient for Pol encapsidation and whether the presence of the cPPT influences particle incorporation of the Pol protein. We also sought to dissect further the rather long CASII element in order to improve the safety and packaging capacity of currently available FV vectors using prototype FV (PFV) as a model.

**METHODS**

**Recombinant DNA.** Standard techniques of molecular cloning (Ausubel et al., 1987; Sambrook & Russell, 2001) were used for the generation of constructs. Vector viruses were abbreviated with the plasmid name lacking the ‘p’. In brief, we first generated the intermediate pKP32 by ligating a 1.33 kb EcoRl–NotI fragment from pMD9 (Heinkelein et al., 2002a) with a 7.954 kb EcoRI–NotI fragment from the same vector. Then, pKP33 was generated by ligating a 0.83 kb BamHI–HincII fragment of pKP32 with a 8.24 kb BamHI–EcoRI fragment of the same plasmid. The three-fragment ligation of the 7.8 kb Swal–MluI fragment of pKP33 with a 0.601 kb MluI–SacI pKP33 fragment and the annealed oligonucleotides #4129 (5'-CGGATAATCAATATAAAAATCCCATGACTTT-3') and #4131 (5'-AAAGTCATGGAATTGTGGTATTGATTATCCGAGCT-3') yielded pKP36. The vector pTW01 was derived from pKP33 by the introduction of the annealed oligonucleotides #4333 (5'-TGCGAGGAGGAGG-3') and #4334 (5'-CGCACCTGCTCTC-3') into the BsmI restriction site downstream of CAS. Likewise pTW02–pTW04 were generated using the oligonucleotide pairs #4335/#4336 (5'-TGCGACGAGCGCCG-5'/5'-CGCAGCGCGCTC-3'), #4337/#4338 (5'-TGTCGAGGCACGGCGG-5'/5'-CGCAGCGCGCTC-3') and #4339/#4340 (5'-TGTCGAGGGCCCTC-3'/5'-CGCAGGGGAGGA-3'), respectively. All vectors contain the gene encoding enhanced green fluorescent protein (EGFP) under the control of a constitutively active heterologous retroviral U3 promoter to enable the quantification of vector transfer rates and of packaged (pre-) genomic RNA by RNase protection analysis (Peters et al., 2005).

**Cell transfections and purification of supernatant.** HEK 293T cells (6 × 10⁶) (DuBridge et al., 1987) seeded into 10 cm dishes 1 day in advance were transfected with plasmid DNA using a polyethyleneimine transfection reagent (Polyscience) (Stange et al., 2005). The transfection mix contained 4 μg vector, gag, pol and env packaging plasmids (Stange et al., 2005). In cases when one of the above-mentioned plasmids was omitted, the total DNA amount in the transfection mix was adjusted to 16 μg using the empty pcDNA vector. One day after transfection, cellular transcription was induced by the addition of 10 mM sodium butyrate for 8 h. After 2 days, the supernatant was harvested, passed through a 0.45 μm filter (Millipore) and layered onto 6 ml of a sucrose cushion (20% in medium). The supernatant was centrifuged in a Surespin 630 rotor (Sorvall) at 25 000 r.p.m. and 4 °C for 3 h.

**Immunoblotting.** Analysis of viral protein expression was done essentially as described previously (Peters et al., 2005). In brief, lysates were prepared from the partially purified vector supernatant and from transfected cells by suspension in detergent-containing buffer. Viral proteins were reacted with Gag (Heinkelein et al., 2002a) and Pol (Imrich et al., 2000) mouse monoclonal antibodies (mAbs) after separation in 8% SDS-PAGE and semi-dry blotting onto Hybond membrane (Pharmacia). Protein bands were detected by using a horseradish peroxidase-coupled secondary antibody (Dako) and employing the enhanced chemiluminescence detection system (Pharmacia).

**Vector transfer.** After clarification the vector-containing supernatant was also assayed functionally by transfer to 1.5 × 10⁶ recipient HT1080 fibroblastsoid cells or to 2 × 10⁶ primary human mesenchymal stem cells (MSCs). The expression of EGFP was monitored by fluorescence-activated cell sorting (FACS) 48 h after transduction. Primary MSCs were obtained from bone marrow of two human donors undergoing total hip replacement surgery after informed consent and as approved by the institutional review board of Würzburg University. MSCs were isolated by adherence of cells drained from the patient’s spongiosa to plastic and maintained as described previously (Nöth et al., 2002). The vector transfer assays were done at least three times with different plasmid preparations.

**RNase protection assay (RPA).** A 237 nt antisense probe able to protect a 222 nt sense transcript from the vector-encoded gene for
EGFP was transcribed from pKP21 with SP6 polymerase as reported previously (Peters et al., 2005). RNA was prepared from the partially purified virus and non-radioactive RPA was performed essentially as described previously (Peters et al., 2005).

RESULTS

To address the questions on Pol encapsidation and FV vector improvement raised above, we constructed the two vectors shown in Fig. 2(a) and analysed them in functional assays (Fig. 2b-d). Vector pKP33 contains the complete CASI element, the 5′ half of CASII, that has been shown previously to be required for RNA incorporation (Peters et al., 2005), and a fragment of the 3′ half of CASII with a shortened PESII element. The establishment of this vector was the result of introducing consecutive deletions from both ends of the 3′ half of CASII in the pMD9 reference vector (Heinkelein et al., 2002a). Further deletions of CASII, than those shown in

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**Fig. 2.** Analysis of pKP33 and pKP36 vectors. (a) Genetic maps of the pKP33 and pKP36 vectors used in this study in relation to the established pMD9 vector. Enhancer/promoter of the human cytomegalovirus immediate early gene (CMV), constitutively active spleen focus forming virus U3 promoter (SFFV U3), gene encoding enhanced green fluorescent protein (EGFP), internally deleted U3 region of the 3′ LTR (ΔU3). (b) RNA encapsidation of KP33 and KP36 vector viruses. An example of RPA is shown in the upper panel and the mean intensity of the protected 222 bp RNA fragment is shown below. To the left the total amount and 50 and 20 % of viral vectors particles produced by transfection of cells with pMD9 were assayed. (c) Detection of PFV Gag and Pol proteins in cells cotransfected with vectors and gag- and pol-encoding plasmids (GP) or gag-, pol- and env-encoding constructs (GPE) or in partially purified viral particles produced by these cells. (d) Transduction rates of fibroblastoid cells by KP33 and KP36 vector viruses in relation to MD9. Fluorescence of HT1080 human fibroblastoid cells transduced with MD9, KP33 and KP36 vector viruses produced by cotransfection of 293T cells with gag- and pol-encoding (GP) or gag-, pol- and env-encoding (GPE) plasmids and the respective vector DNA. The mean ± SD values from three independent assays are shown.
pKP33 (Fig. 2a), led to a gradual decline of vector functionality (data not shown). The vector pKP36 is basically identical to pKP33. However, it harbours only the previously identified PESI element and no further sequences from CASII are contained in this plasmid. Using pKP33 we investigated whether a shortened PESSI element retains it functionality, and using pKP36 we wanted to know whether the presence of PESI is sufficient for Pol encapsidation, provided we could demonstrate the functionality of pKP33, and thus a shortened version of CASII.

The analysis revealed that KP36 was unable to incorporate RNA as well as the Pol protein, while KP33 was shown to be fully competent in these assays (Fig. 2b and c). We consistently observed the lack of Gag cleavage in RNA-deficient particles (Heinkelein et al., 2002b; Peters et al., 2005) in contrast to a recent publication in which intracellular Gag cleavage was observed despite a claimed absence of particle-associated Pol protein (Lee & Linial, 2008). These results indicated that the presence of PESI is insufficient to incorporate Pol protein and that further sequences within CASII are required for RNA packaging as well as for Pol encapsidation as long as essential sequences of CASII are present in the vector. However, when we determined the vector transfer capability of KP33, we found this vector to be repeatedly and significantly inferior in comparison to the results obtained with the MD9 reference vector virus (Fig. 2d).

Therefore, we generated a series of pTW plasmids as derivatives of pKP33 (Fig. 3a). pTW01 is identical to pKP33 with the exception of the presence of a small oligonucleotide overlapping the cPPT sequence and pTW02–pTW04 are derivatives of pTW01 with less (pTW02 and pTW03) or more severe (pTW04) alterations of the cPPT sequence.

These vectors were assayed for protein composition of the virus particle, RNA content and vector transfer rates. Most remarkable was the phenotype of pTW01. In terms of RNA and Pol incorporation pTW01 was not superior to pKP33 (Fig. 3b and c). However, pTW01 vector transduced recipient fibroblastoid cells as efficiently as the pMD9 reference vector, suggesting that the presence of a small cPPT sequence is sufficient to confer full functionality to an FV vector provided that additional essential cis-elements are present (Fig. 3d).

When the pTW02–pTW04 vectors were functionally characterized, we observed only a slight reduction in capacity to encapsidate RNA and Pol in comparison to TW01, pKP33 or MD9 (Fig. 3b and c). However, the capacity of these vectors to transduce recipient fibroblastoid cells with the EGFP marker was even more reduced as with the parental pKP33 vector (Fig. 3d). This finding suggests that the restoration of the full functionality of the pTW01 vector compared with pKP33 depends on the integrity of cPPT.

One of the advantages of FV vectors is their exquisite ability to transduce stem cells of various origins (Gharwan et al., 2007; Leurs et al., 2003). To address this for the newly established pTW01 vector, we analysed its ability to transduce primary human MSCs. As shown in Fig. 3(e), pTW01 behaved in an identical manner to the established pMD9 vector in this respect, while the other vectors showed the reduced capability already observed when fibroblastoid cells were transduced. We observed no significant differences upon transduction of MSCs from the two different donors.

**DISCUSSION**

The CAS required for transfer of an FV vector include (from 5′ to 3′): R-U5-CASI-CASII-3′PPT-ΔU3-R. R-, U5- and ΔU3-sequences are derived from LTRs and contain elements necessary for genome dimerization, reverse transcription and integration. The 3′PPT is also essential for reverse transcription in initiating plus-strand DNA synthesis. The miracle of FV vectors lies in the presence of CASI and II elements (Erlwein et al., 1998; Heinkelein et al., 1998; Wu et al., 1998). Whereas the location and length of CASI (approx. 460 bp) at the 5′ end of the RNA is conventional among retroviruses, the requirement of a pol gene-located CASII as such is highly unusual for retroviral vectors. Moreover, with CASII being approximately 2 kb in length it is rather long.

However, the most advanced FV vectors are pMD9 from our group (Heinkelein et al., 2002a) and pΔφ from David W. Russell, Seattle, WA, USA (Trobridge et al., 2002). Both vectors have a similar basic design, they harbour the 2 kb CASII, and are of similar performance in transducing recipient cells (David W. Russell & A. Rethwilm, unpublished results). Here, we attempted to dissect CASII and to assign different functions to different genomic sections. Furthermore, we asked whether the previously identified PES elements, which have been shown to be required for Pol protein encapsidation (Trobridge et al., 2002), are sufficient to incorporate Pol. Our results show that this is not the case. While the presence of the PES elements is clearly required, the experiment with pKP36 demonstrated that they are not sufficient to encapsidate Pol.

As the main focus of the work, we dissected CASII and established the pKP33 vector in which large parts of CASII were deleted. pKP33 was able to incorporate RNA and Pol as efficiently as the pMD9 reference vector. However, in contrast to pMD9 the transduction rates using pKP33 were always slightly reduced. To restore these we speculated that the incorporation of the cPPT sequence might be favourable. This was indeed the case, since pTW01 that harbours the wild-type cPPT was able to transduce the recipient cell as efficiently as the reference pMD9 vector. The usefulness of pTW01 was not only shown in transducing an established fibroblastoid cell line, but also primary MSCs, which may be more critical for vector application. The experiments with pTW02–pTW04 vector viruses excluded the possibility that other features of the introduced sequences than the identity to the cPPT were responsible for the observed effect.
In human immunodeficiency virus (HIV), the functional role of the cPPT sequence is controversial. The vast majority of publications indicate an enhancing effect on vector transductions, if the HIV cPPT is present (Dardalhon et al., 2001; Follenzi et al., 2000; Van Maele et al., 2003). However, the reason for this and the role of the cPPT sequence in nuclear entry of the viral preintegration complex is debated (Arhel et al., 2006a, b; Limón et al., 2002; Skasko & Kim, 2008). In addition, structural differences, which probably result in functional differences between the FV and the HIV

Fig. 3. Analysis of the pTW vectors. (a) Genetic maps of the pTW series of vectors bearing cPPT sequences or alterations thereof. (b) RNA incorporation of pTW vectors. (c) Pol protein encapsidation. (d) Transduction rates of human HT1080 fibroblastoid cells by pTW vector viruses in relation to KP33 and MD9. (e) Transduction rates of MSCs by pTW vector viruses in relation to MD9 and KP33. Primary human MSCs were transduced with vector viruses produced by cotransfection of 293T cells with gag- and pol-encoding (GP) or gag-, pol- and env-encoding (GPE) plasmids and the respective vector DNA. The mean ± SD values from three independent assays are shown.
cPPT, have been pointed out recently (Peters et al., 2008). As outlined above, the centrally located PFV sequences adopt different functions in viral replication. The 2 kb CASII element can be dissected as follows: 5′ sequences (approx. 800 bp) are probably involved in RNA packaging (Heinkelein et al., 1998; Peters et al., 2005), this is followed by a stretch of RNA irrelevant for vector transfer, 3′ to this a sequence required for Pol packaging (approx. 350 hp) is located, eventually a small sequence identical to cPPT has to be added to provide full functionality to an FV vector. Interestingly sequences 3′ to this, which may be required for nuclear export of the unspliced gag and the single-spliced pol mRNA in the proviral context (J. Bodem and others, unpublished data), are not essential for PFV vector transfer. The identified domains are summarized in Fig. 4. Probably, a similar dissection as done here for CASII can be carried out for CASI, which may result in an even more advanced PFV vector.

Although we have not elucidated mechanistic aspects of CASII function, such as the mode of RNA and Pol packaging, as well as the cPPT function in viral replication, the dissection of CASII into different functional domains may help do so in future experiments. In addition, we have improved the current PFV vectors by the identification of approximately 850 bp of sequence that can be deleted from the vector without the loss of function. This adds to the safety of PFV vectors as well as to their packaging limit.

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REFERENCES


