An active foamy virus integrase is required for virus replication

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Foamy viruses (FVs) make use of a replication strategy which is unique among retroviruses and shows analogies to hepadnaviruses. The presence of an integrase (IN) and obligate provirus integration distinguish retroviruses from hepadnaviruses. To clarify whether a functional IN is required for FV replication, a mutant in the highly conserved DD35E motif of the active centre was analysed. This mutant was found to be able to express Gag and Pol protein precursors and cleavage products and to generate and deliver cDNA. However, this mutant was replication-deficient. The junctions of individual foamy proviruses with cellular DNA were sequenced. The findings suggest that FV integration is asymmetrical, because the proviruses started with what is believed to be the U3 end of the free linear DNA to generate the conventional TG dinucleotide, while apparently two nucleotides from the U5 end were cleaved to create the complementary CA dinucleotide. Alignment of known FV genome sequences indicated that this mechanism of integration is not restricted to the two FV isolates from which integrates were studied, but appears to be a common feature of this retrovirus subfamily. In conclusion, with respect to the necessity of a functionally active IN for virus replication FVs behave like other retroviruses; their mechanism of integration, however, is probably unique.

Introduction

Foamy viruses (FVs) are retrovirus-like viruses which have a replication strategy different from that of retroviruses and hepadnaviruses (Weiss, 1996; Linial, 1999). Recent studies of various aspects of the FV replication cycle have led to the assumption that FVs represent a functional link between retroviruses and hepadnaviruses (Rethwilm, 1996; Linial, 1999). In this respect it is most important that large amounts of DNA have been found in FV particles (Yu et al., 1996) and that virion DNA appears to be the functionally relevant nucleic acid (Moebes et al., 1997; Yu et al., 1999). In addition, FV particle formation and Env protein function and particle incorporation are unique among retroviruses and also take some intermediate position between the retrovirus and hepadnavirus assembly process (Enssle et al., 1997; Fischer et al., 1998; Baldwin & Linial, 1998; Pietschmann et al., 1999). Despite these functional differences the overall structure of FV genomes is much more similar to that of typical complex retroviruses than that of hepadnaviruses (Rethwilm, 1995). In its provirus form the FV coding sequences are flanked by long terminal repeats (LTRs) (Rethwilm, 1995). Furthermore, FVs encode an integrase (IN) protein, which has been studied in some detail by in vitro experiments (Pahl & Flügel, 1993, 1995), and provirus integration has already been demonstrated for several FVs or FV vectors (Rethwilm et al., 1987; Schweizer et al., 1993; Renshaw et al., 1991; Russel & Miller, 1996; Tobaly-Tapiero et al., 1996; Neves et al., 1998).

Further characteristics which distinguish the replication strategy of retrovirus from that of hepadnavirus include the presence of an IN protein and obligate provirus integration (Coffin, 1996). Given the many analogies between FVs and hepadnaviruses we wanted to determine whether an active IN protein is essential for FV replication. In addition, we noticed a structural peculiarity in the primary nucleotide sequence of
the polypurine tract (ppt)–U3 LTR border in FVs (see below). Since this peculiarity may influence the process of integration, we first set out to determine the integration junctions of foamy proviruses with cellular DNA. We used the chimpanzee strain of FV, CFV/hu (previously called human foamy virus (HFV)), which was reported to be isolated from human material (Achong et al., 1971). However, this strain is almost identical to FV isolates from chimpanzees and evidence for natural HFV infections is lacking (Herchenröder et al., 1994, 1996; Schweizer et al., 1995; Ali et al., 1996).

Methods

Cells and viruses. Human 293T kidney cells (DuBridge et al., 1987), human KMST-6 skin fibroblastoid cells (Namba et al., 1985), baby hamster kidney cells (BHK-21), BHK/bel-1 (Biennasz et al., 1997) and BHK/LTRlacZ cells (Schmidt & Rethwilm, 1995) were cultivated in Eagle’s minimal essential medium (MEM) or Dulbecco’s modified MEM containing 5–10% foetal calf serum, antibiotics and 0.5 and 1 mg/ml G418 in the case of BHK/bel-1 and BHK/LTRlacZ cells, respectively. Plasmid-derived viruses were generated by calcium phosphate cotransfection of BHK-21, KMST-6 or 293T cells (Ausubel et al., 1987). Virus titrations were performed on BHK/LTRlacZ indicator cells as described (Schmidt & Rethwilm, 1995).

Recombinant DNA. Conventional techniques were used to generate FV recombinants (Ausubel et al., 1987). The infectious FV plasmids pcHSRV2 and pcHSV2 and the replication-deficient FV vectors pMH4 and pMH5, which express green fluorescent protein (GFP) under the transcriptional control of the spleen focus-forming virus (SFFV) U3 region, have been described recently (Moebes et al., 1997; Fischer et al., 1998; Heinkelein et al., 1998; Lindemann & Rethwilm, 1998). The functional env inactivation mutant pcHSV2/M68 was made in a similar way to pHSV2/M68 (Moebes et al., 1997), by klenow enzyme fill-in of a MspI site at the beginning of env. IN mutants of the infectious plasmids were generated by recombinant PCR on a subgenomic 0.33 kb EcoRI–HindIII fragment (Higuchi, 1990). In pHSV2/M70 the DYIG motif was changed to DYTG, similar to a previous report (Pahl & Flügel, 1995), and in pHSV2/M73 and pHSV2/M73 the DD35E motif was changed to DA35E. The mutations were verified by automated DNA sequence analysis of the complete PCR fragment using AmpliTaqFS and the ABI 310 sequence analysis system (Perkin Elmer). Introduction of the IN protein. It involves the recognition and trimming of specific, rabbit antisera (Hahn et al., 1994, 1996; Schweizer et al., 1993). Proviral–cellular DNA junctions (A) total DNA was digested with EcoRI and AluI; for the amplification of U3–genomic DNA junctions (B) digestion was performed with AvrII and AluI; the DNA was cut only with AluI to amplify both junctions (C). DNA was self-ligated and subsequently recut with EcoRII (cases A and C) or SphI (cases B and C) to enhance the amplification from linear substrates. Inverse PCR was performed using the Panscript system (Boehringer Mannheim) and FV LTR-specific primers. A and B amplicons were purified by binding to glass milk and sequenced directly. C amplicons were sequenced following electrophoretic gel separation and purification.

FV protein analysis. pcHSV2 or mutant plasmids were transfected into BHK/bel-1 cells, and 4 days later a cellular lysate was prepared in detergent buffer as described (Hahn et al., 1994). Following separation in SDS–8% polyacrylamide gel and semi-dry blotting to nitrocellulose membranes (Schleicher & Schüll), the viral proteins were allowed to react with Gag-specific, and following stripping of the blot with RNaseH-specific, rabbit antiserum (Hahn et al., 1994; Kögel et al., 1995) and stained using the ECL system (Amersham).

Southern blot analysis. pHSV2, pcHSV2/M68 and pcHSV2/M73 were transfected into 293T cells. Four days later total cellular DNA was prepared, run on a 0.8% agarose gel, blotted onto a nylon membrane (Qiagen) and hybridized to an FV cRNA probe essentially as described previously (Moebes et al., 1997).

Analysis of FV cDNA delivery into recipient cells. 293T cells were transfected with 15 µg pcHSV2, pcHSV2/M68, pcHSV2/M73 or mock-transfected. Two days later the supernatants were collected, passed through a 0.45 µm filter and used to infect BHK-21 cells. One day after infection the low molecular mass DNA was extracted from the cells by using a modified Hirt method (Hirt, 1967). Briefly, low molecular mass DNA was separated from high molecular mass DNA by alkaline lysis of the cells and centrifugation in high salt, as usually done for bacterial plasmid preparation (Ausubel et al., 1987). DNA from the supernatant was extracted using the Qamp tissue kit (Qiagen). Viral DNA (one-twentieth of the original volume eluted from the preparation column) was amplified by PCR with pol gene-specific primers (#196 and #197) as described previously (Schmidt et al., 1997b). Amplicons were separated in ethidium bromide-containing agarose gels and photographed under UV light.

Single replication cycle assay. 293T cells were transfected with the replication-deficient FV vectors pMH5, pMH80 and pMH99 together with the env expression vector pcEnv-1, as described (Fischer et al., 1998; Heinkelein et al., 1998; Pietschmann et al., 1999). The supernatant of the transfected cells was used to transduce recipient BHK-21 and KMST-6 cells which were plated the day before at a density of 10^4 cells per 6 cm dish. The number of GFP-positive cells was determined over time by fluorescence-activated cell sorting (FACS) analysis on a FACScan using the LysisII and CellQuest software packages (Becton Dickinson) as reported (Lindemann et al., 1997; Heinkelein et al., 1998; Pietschmann et al., 1999).

Results

Sequence determination of proviral–cellular DNA junctions

Retrovirus integration is a highly ordered process mediated by the IN protein. It involves the recognition and trimming of
21 cells with a replication-deficient CFV single FV proviruses obtained following transduction of BHK-determination of cellular DNA–U3 junctions of independent clones was originally derived from one provirus which was integrated into another CFV provirus and part of the junction sequences have been reported previously (Schweizer et al., 1993). The murine leukaemia virus (MLV) and HIV-1 sequences are shown for comparison. In HIV-1 the terminal base removed from the U5 end is the terminal A of the tRNA primer used to initiate reverse transcription (Whitcomb et al., 1990). All BHK-21 cell clones which were used for the determination of junction sequences showed single proviral integrations as revealed by inverse PCR analysis and ethidium bromide-containing agarose gel electrophoresis (data not shown). n, Any nucleotide; x, duplicated nucleotides at the MLV and HIV-1 proviral junctions; n.a., sequence is not available. (B) Strategy to generate BHK-21 cells harbouring single FV proviruses. pMH4 is an FV vector able to perform one round of replication (Heinkelein et al., 1998). ∆U3 is the U3 region of the HSRV2 LTR deletion variant (Schmidt et al., 1997a).

Fig. 1. (A) FV sequences at the ppt–U3 and U5–primer binding site (pbs) borders and proviral junctions to cellular DNA. Proximal 5′ U3 sequence of the pHSRV infectious molecular clones (Rethwilm et al., 1990; Lochelt et al., 1991; Schmidt et al., 1997a) and published FV genome sequences from CFV/hu (Flügel et al., 1987; Maurer et al., 1988), CFV (Herchenröder et al., 1994), simian FV type 1 (SFV-1) (Kupiec et al., 1991), SFV-3 (Renne et al., 1992), bovine FV (BFV) (Renshaw & Casey, 1994a, b) and feline FV (FFV) (Helps & Harbour, 1997; Winkler et al., 1997) were aligned. The sequences are arranged in such a way that the LTR borders can readily be compared with the proviral–cellular DNA junctions. The Vero-L cell line harbouring a single SFV-3 provirus and part of the junction sequences have been reported previously (Schweizer et al., 1993). The trimming of the U3 end appears to be unusual. The terminal dinucleotide and terminate with the subterminal CA for the joining reaction to host cell DNA. In CFV/hu integration were corroborated (Heinkelein et al., 1998). These findings on CFV/hu integration started with TGTG. Hence, the terminal dinucleotide of U3 was not removed during integration. In contrast, the sequence determination of the U5–cellular DNA junctions revealed, in all cases except clone F2 (Fig. 1A), that the two terminal nucleotides (AT) had been removed in order to provide the subterminal CA for the joining reaction to host cell DNA. In the case of provirus F2 the terminal AT dinucleotide was not removed from the right end of the unintegrated viral DNA, and this was probably reflected by only partial duplication of host cell sequences (Fig. 1A).
Fig. 2. Construction and properties of FV IN mutants. (A) Genome organization of FV and mutants in the pHSRV2 and pchSRV2 backbone. (B) Gag and Pol protein expression of pHSRV2 and M70 and M73. (C) Southern blot analysis to demonstrate the ability of the DD35E mutant M73 to generate cDNA. The lanes from transfected 293T cells were exposed for 3 days, and the control lanes (DNA from untransfected 293T and DNA from 293T cells spiked with pchSRV2 plasmid) were exposed for 14 days. (D) Detection of viral DNA in recipient BHK-21 cells exposed to virus produced by transient transfection of 293T cells with individual proviral plasmids (pchSRV2 background).

(Rethwilm et al., 1990; Löhchelt et al., 1991; Renshaw et al., 1991; Herchenröder et al., 1996; Tobaly-Tapiero et al., 1996; Schmidt et al., 1997a; Mergia & Wu, 1998).

As also shown in Fig. 1(A), FV integration apparently resulted in the duplication of four nucleotides of host cell DNA, as has been observed for feline and murine leukaemia virus integration (Brown, 1997). In the limited cases analysed G+C-rich sequences appeared to be the preferred target for FV integration, which may resemble previous observations with avian retrovirus IN protein (Kitamura et al., 1992). These results corroborate findings published recently (Neves et al., 1998).

Construction and characterization of FV IN mutants

Provirus integration is required for retroviral gene expression and replication (Brown, 1997). To determine whether an active IN protein is essential for FV replication, two FV IN mutants were analysed (Fig. 2A). The mutant M70 contains an isoleucine to threonine substitution (DYIG → DYTG) around the first aspartic acid of the DD35E motif. This mutant was described previously as having a reduced endonuclease, a slightly elevated disintegrase and a largely impaired IN activity (reduction to less than 3% of wild-type activity) in in vitro assays (Pahl & Flügel, 1995). The mutant M73 contains an aspartic acid to alanine substitution (DD35E → DA35E) in the highly conserved catalytic centre present in all known IN proteins (Brown, 1997). Mutations introduced into this motif have previously been demonstrated to disable the integration and replication activity of a variety of retroviruses (Brown, 1997).

The expression of viral Gag and Pol proteins was analysed following transient transfection of proviral (pHSRV2 background) constructs into BHK/bel-1 cells which stably express the CFV/hu transcriptional trans-activator protein (Tas). As
Table 1. Development of cell-free virus titres/ml following transfection of BHK-21 and KMST-6 cells with pHSRV2 or mutants

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Table 1. Development of cell-free virus titres/ml following transfection of BHK-21 and KMST-6 cells with pHSRV2 or mutants

Plasmid DNA (5 µg) was transfected into the cells and cell-free virus titres were determined on BHK/LTRlacZ cells. The results represent the mean of two experiments with less than 20\% variation.

shown in Fig. 2(B), wild-type virus and the mutants expressed the p70/74 kDa Gag protein doublet, the p127 kDa Pol precursor protein and the p80 kDa RT. This demonstrated that gene expression from the proviral plasmids and the function of the pol-encoded protease in cleaving Gag and Pol precursor molecules (Konvalinka et al., 1995) were not affected by the mutations introduced.

We also investigated whether IN mutant M73 is able to generate cDNA. It has been demonstrated previously that FV reverse transcribes its RNA pregenome late in the replication cycle (Moebes et al., 1997). The generation of the DNA required an active polymerase and a correctly assembled capsid (Ensle et al., 1997; Moebes et al., 1997). To analyse this for M73, 293T cells were transfected with pHSRV2, pHSRV2/M68, in which env was inactivated, and pHSRV2/M73. DNA from the transfected cells was probed for the presence of linear HSRV2 DNA (Moebes et al., 1997). As shown in Fig. 2(C), M73 was found as able to synthesize cDNA as the unmutated virus. This result indicated that the replication of M73 up to this step was normal.

The delivery of viral DNA derived from M73 transfection into recipient cells was analysed by PCR. As shown in Fig. 2(D), we detected M73 DNA in the Hirt fraction of recipient BHK-21 cells, which indicated that IN mutant M73 was able to deliver virus DNA to fresh cells.

Analysis of the replication competence of FV IN mutants

The replication of the proviral mutants over time was analysed by transfection of BHK-21 and KMST-6 cells and determination of the virus titres in the cell-free supernatants. Wild-type virus replicated an average 5–10 times better compared to the M70 mutant (Table 1). In addition, cell-free infectious virus could be continuously transmitted to fresh cells from cultures infected with M70 (data not shown). It has been reported that even a replication-deficient human immunodeficiency virus (HIV) IN mutant reverted at a second site to regenerate a replication-competent virus (Taddeo et al., 1996).

We, therefore, amplified a 1.73 kb fragment spanning the complete IN gene from BHK-21 cells infected with HSRV2/M70 for 3 weeks. The DNA sequence of the IN gene was determined directly without further subcloning steps and revealed an identical nucleotide sequence as present in the original M70 mutant.

In contrast to M70 the M73 IN mutant did not replicate at all in BHK-21 cells (Table 1). A similar result was obtained.
when BHK/bel-1 cells were followed for a period of 4 weeks following transfection with pHSRV2/M73 (data not shown). However, when pHSRV2 and pHSRV2/M73 were transfected into 293T cells and the amounts of infectious virus were determined after transient production for 48 h, we obtained from five experiments mean cell-free titres of 3.1 × 10^3/ml and 26/ml for pHSRV2 and pHSRV2/M73, respectively.

**Analysis of FV IN mutants in a single replication assay**

To determine the effect of the IN mutants in a single replication assay, we took advantage of indicator-gene-expressing FV vectors. Similar analyses have been performed previously using HIV-1 IN mutants (Masuda et al., 1995; Wiskerchen & Muesing, 1995; Liu et al., 1997). To do this we used vectors pMH99 and pMH80, shown in Fig. 3. BHK-21 and KMST-6 cells were transduced with cell-free FV vectors, which were produced by transient co-transfection of vector DNA and an Env expression plasmid, and the percentage of GFP-positive cells was monitored over time. Wild-type pMH5 vector transduced recipient cells to a similar extent, as described recently (Heinkelein et al., 1998), while only very low, if any, transduction was obtained with pMH80 (Fig. 3). Transduction following transfection of the pMH99 construct showed the GFP expression level to be reduced by 20–50% compared with the pMH5 wild-type vector (Fig. 3), which was in good agreement with the result of the infection experiment with this mutant (Table 1).

**Discussion**

We demonstrate in this study that FV, which is highly divergent from all other retroviruses, essentially requires an active IN for replication. Episomal replication of FVs, which could have been assumed following the discovery of a variety of analogies between FVs and hepadnaviruses appears to be negligible or non-existent. The few blue cells observed in our most sensitive assay (infection of BHK/LTRlacZ indicator cells with transiently produced virus in 293T cells) indicated that either there exists a very weak residual activity that expresses FV tass from unintegrated DNA or that the FV DNA may express genes from occasional and unspecific integrations into the host cell genome. A very weak replication of IN-mutated retroviruses and unspecific integration of such mutants has been reported previously (Panganiban & Temin, 1984; Schwartzberg et al., 1984; Hagino-Yamagashi et al., 1987; Wiskerchen & Muesing, 1995; Gaur & Leavitt, 1998).

With respect to the M70 mutation (DYIG → DYTG) our results indicate that this mutant, in contrast to what has been expected from in vitro integration assays (Pahl & Flügel, 1995), behaves in a very similar way to unmutated virus. Except for FVs the threonine (in HIV-1 IN at position 66) is highly conserved in retroviral IN proteins (Engelman & Craigie, 1992). Substitution of the HIV-1 IN threonine at position 66 by alanine, while having moderate effects on in vitro IN activities, had no effect on virus replication in an established T-cell line (Engelman & Craigie, 1992; Toddeo et al., 1994). We show here that mutation of the isoleucine, which is in the similar position in FVs compared with the threonine in other retroviral IN proteins (Engelman & Craigie, 1992), also had only a moderate effect on virus replication, provided that it was mutated to threonine.

Another part of our study highlighted a further difference between FVs and the other retroviruses. Our sequencing of individual proviruses revealed that their left ends were identical to what is believed to be the start of the U3 region (Flügel et al., 1987), in contrast to other retroviruses, in which two bases from U3 are lost during integration (Fig. 1; Brown, 1997). This finding suggests that either the left end of the unintegrated FV DNA remains unprocessed during integration or that the processing is unusual and involves cleavage of RNA–DNA hybrid sequences originating from the ppt and remaining attached to the U3 end of the linear viral DNA. Such untidy ends have been found previously in other retroviral systems and in particular in the yeast retrotransposons (Miller et al., 1997; Mules et al., 1998). Remarkably, the latter preferentially integrate blunt-end substrates (Brown, 1997).

A high percentage of uncleaved proviral right and left ends has been recently claimed for visna virus integrates (List & Haase, 1997). However, the reported sequences were not derived from single proviruses, which makes interpretation of the findings difficult. Furthermore, as deduced from the primary sequences, visna virus allows and probably requires cleavage of the terminal nucleotides from both ends of linear DNA for efficient integration (Sonigo et al., 1985).

There is a major discrepancy between our results and a previous study on the in vitro activity of purified recombinant FV IN. Pahl & Flügel (1993) reported that CFV/hu IN was able to cleave the end of what they called a ‘wild-type’ U3 oligonucleotide. However, to obtain this result, two non-templated nucleotides, which were complementary to the terminal U5 dinucleotide, were added to the U3 substrate (Pahl & Flügel, 1993). In addition, their introduction would destroy the bet reading frame overlapping the ppt–U3 border in all FVs (Flügel et al., 1987; Kupiec et al., 1991; Renne et al., 1992; Baunach et al., 1993; Hahn et al., 1994; Renshaw & Casey, 1994; Herchenröder et al., 1994; Helps & Harbour, 1997; Winkler et al., 1997). Therefore, we suggest that the report of Pahl & Flügel (1993) showed that FV IN is able to cleave some kind of elongated U3 end, which may arise through the generation of untidy ends during reverse transcription. However, such a substrate, which remains to be identified, would probably be different from the one used in the study in question (Pahl & Flügel, 1993).

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References


Miller, M. D., Farnet, C. M. & Bushman, F. D. (1997). Human


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