Replication of a foamy virus mutant with a constitutively active U3 promoter and deleted accessory genes

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Introduction

Foamy viruses (FVs) are complex retroviruses which require for their replication the activity of a transcriptional trans-activator (Tas) as well as Tas-responsive elements in the viral promoters. A mutant of the chimpanzee FV strain, CFV/hu (previously called human FV), genome in which most of the U3 promoter of the CFV long terminal repeat was substituted by the constitutively active human cytomegalovirus immediate early gene enhancer/promoter was constructed. This plasmid (pTS12) and a derivative (pTS13), which has a deletion in the tas gene, gave rise to replication-competent virus. Compared with parental CFV, both mutants replicated only very poorly, with retarded growth kinetics and maximal cell-free virus titres reduced by approximately three orders of magnitude. Mutation of the DD35E motif of the CFV integrase to DA35E rendered the recombinant TS virus replication-deficient. This indicated that provirus integration is probably still required for this FV derivative, which had been converted from a complex regulated retrovirus into a simple one by incorporation of a constitutively active promoter from another virus which regularly does not integrate into the host cell genome.

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Introduction

In functional terms retroviruses have been divided into two subgroups: those having basically only gag, pol and env genes and those bearing in addition to these, genes regulating viral gene expression (Cullen, 1991). Murine leukaemia virus exemplifies the simple retroviruses, while human immunodeficiency virus (HIV) is the prototype of a complex retrovirus (Cullen, 1991). Complex retroviruses encode two kinds of proteins involved in regulating viral gene expression in trans at the transcriptional or post-transcriptional level. The Tat and Rev proteins of the lentiviruses are examples of these two categories of retroviral regulators (Cullen, 1991).

The foamy viruses (FVs) seem to be an exception to this rule, since they encode a transcriptional trans-activator (Tas/Bel-1) and appear to lack a regulator acting at the post-transcriptional level of gene expression (Baunach et al., 1993; Rethwilm, 1995). Tas acts at least in part by direct binding to sequences upstream of the FV start of transcription (He et al., 1996; Zou & Luciw, 1996). In addition to the conventional U3 long terminal repeat (LTR) promoter, FVs have a second internal promoter (IP) located in the env gene upstream of the accessory genes (Fig. 1) (Llöchelt et al., 1993). The activity of both promoters depends on Tas and both promoters are required for replication (Llöchelt et al., 1995; Rethwilm, 1995). At least the activity of the LTR promoter appears to be tightly controlled by Tas, since no gene expression from the LTR was found in the absence of either Tas or the Tas-responsive U3 elements (Rethwilm et al., 1990, 1991; Keller et al., 1991; Erlwein & Rethwilm, 1993; Lee et al., 1993). Furthermore, mutation of the tas gene demonstrated its essential role in virus replication (Baunach et al., 1993).

The conversion of the complex HIV into a simple retrovirus has been suggested as a way of virus attenuation (Temmin, 1993). In the case of another complex retrovirus, bovine leukaemia virus, a replicating virus lacking tas and rex genes has been constructed (Boris-Lawrie et al., 1997; Boris-Lawrie & Temmin, 1995). The replication strategy of FVs, however, appears to be unusual and differs significantly in some aspects...
from all other simple or complex retroviruses (Rethwilm, 1996; Weiss, 1996; Yu et al., 1996; Linial, 1999). Similar to hepadnaviruses, FVs reverse transcribe their RNA pregenome late in the replication cycle, which results in DNA being the functional relevant virion nucleic acid (Moebes et al., 1997; Yu et al., 1999). Given the many differences between FVs and the other retroviruses, we were interested in investigating the replication competence of a tas-negative FV genome with a constitutively active U3 promoter.

Methods

Recombinant DNA. All plasmid constructions were produced by established methods (Ausubel et al., 1987). The infectious molecular clone pcHSRV2 has been described previously (Moebes et al., 1997; Lindemann & Rethwilm, 1998). It consists of the human cytomegalovirus (CMV) promoter directing expression of the HSRV2 infectious variant of CFV/hu (Schmidt & Rethwilm, 1995; Schmidt et al., 1997). Using a subgenomic plasmid, the 3′ LTR of pcHSRV2 was deleted downstream from a BstEI site located at position -570 relative to the start of R, which resulted in a truncation of the bel-2 open reading frame (ORF) by 47 amino acids. Into this deletion a pHSRV2-derived 0-84 kb PCR amplicon was introduced covering the CMV promoter and the R region of the LTR. Nucleotide sequences of the PCR product were automatically determined using AmpliTaqFS and the ABI 310 sequence analysis system (Perkin Elmer) and revealed wild-type 0-7 kb CMV enhancer/promoter sequences (Boshart et al., 1985) followed by the CFV start of transcription and the 0-14 kb R region. Following establishment of the hybrid 3′ LTR, pTS12 was constructed by stepwise addition of the 5′ pHSRV2 sequences. Deletion of an internal 0-21 kb BamHI fragment in the tas gene of pTS12 and religation led to pTS13.

P TS16 was made by inserting a 1-15 kb cassette made up of the spleen focus-forming virus (SFFV) U3 region from -383 to +36, where +1 is the start of transcription (Baum et al., 1997), and directing the expression of green fluorescent protein (GFP) (Heinkelein et al., 1996), between the ActI site downstream of the tas gene and the EcoNI site upstream of the hybrid 3′ LTR. pTS17 is a derivative of this plasmid harbouring the integrase DD35E → DA35E mutation of pHSRV2-M73 (Enssle et al., 1999) and was made by exchanging a 2-31 kb PacI–KpnI fragment between pTS16 and pHSRV2-M73. This mutation was made by recombinant PCR and verified by DNA sequencing of the complete amplicon to exclude off-site mutations (Enssle et al., 1999).

To generate the CFV tas/bel-1 expression plasmid pCEP4/bel-1SDm, the tas gene of an infectious molecular clone which contains the tas splice donor mutation described by Venkatesh & Chinnadurai (1993) was excised as a 1-05 kb SphI fragment and inserted into the blunt HindIII site of pCEP4 (Invitrogen) downstream and in transcriptional direction of the CMV promoter.

P Tr5luci contains the HSRV2 U3R region (-777 to +195) which was inserted into pGL2-Basic (Promega) as a KpnI–BglII fragment derived from p5LTR (-777/+195) (Erwein & Rethwilm, 1993). P TS18luci is a corresponding lucerase expression plasmid containing the hybrid U3-CMV promoter and the CFV R region. It was made by inserting a 1-11 kb BglII–Cal (blunted) fragment excised from pTS12 into BglII– HindIII (blunted)-cut pGL2-Basic.

Analysis of viral protein expression. Plasmids (10 μg) were transfected using CaPO4 co-precipitation as described (Heinkelein et al., 1996; Lindemann & Rethwilm, 1998) into 2 × 10⁵ 293T cells (DuBridge et al., 1987) seeded in 6 cm dishes the day before transfection. Twenty-four hours following transfection expression from the CMV promoter was induced by addition of 10 mM sodium butyrate for 8 h. Forty-eight hours after transfection the cells were washed with PBS, subjected to one cycle of freezing (dry ice) and thawing (37 °C waterbath), and taken up into lysis buffer (Hahn et al., 1994). One-tenth of the volume was subjected to SDS–7-5 % PAGE as described (Hahn et al., 1994). Separated proteins were semi-dry blotted onto nitrocellulose (Schleicher & Schuell) and incubated with rabbit antiseria specific for CFV Gag (Hahn et al., 1994), Pol (Kögel et al., 1995) and Tas/Bet (Baunach et al., 1993) proteins. Blots were developed using the ECL detection system (Amersham).

For the detection of Env protein, 7 × 10⁵ 293T cells were transfected with 5 μg plasmid DNA and the cells were radioactively labelled with

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**Fig. 1.** (A) CFV/hu constructs used in this study. pchSRV2 is an infectious CFV/hu genome in which only the first round of replication is directed by the CMV promoter (Lindemann & Rethwilm, 1998; Moebes et al., 1997). In the pTS series of plasmids the CMV promoter was also inserted into the 3′ U3 region of the LTR (downstream from a BstEI restriction site as indicated) and, therefore, was maintained in further rounds of virus replication. Except for a truncated bel-2 ORF the coding sequences of pTS12 are identical to pchSRV2. pTS13 has a small BamHI fragment deletion abrogating Tas/Bel-1 and Bet expression (Baunach et al., 1993). pTS16 and pTS17 harbour an internal expression cassette, consisting of the SFFV U3 promoter and the gene encoding GFP, in place of the bel-2 ORF. In pTS17 the highly conserved active centre of the integrase was mutated from DD35E to DA35E (Enssle et al., 1999). Start sites of transcription are indicated by arrows. IP, internal promoter. (B) Strategy to assay the replication competence of the TS viruses. p.t., Post-transfection; p.i., post-infection.
35S-Promix (Amersham) from 36–48 h following transfection. Harvest of the cells, preparation of the lysates, clearance of the lysates with normal rabbit serum, radioimmunoprecipitation (RIPA) with CVF surface protein–specific rabbit antisera and the separation was done as described recently (Lindemann & Rethwilm, 1998; Pietschmann et al., 1999).

**Analysis of virus replication.** 3 × 10^6 293T cells were transfected with plasmid DNA as described above. Two days after transfection a freeze–thaw lysate of the transfected cells was prepared, cellular debris was pelleted down by low speed centrifugation, the supernatant was passed through a 0.45 µm filter (Schleicher & Schüll) and 3 ml was used to inoculate 10^6 KMST-6 human fibroblasts (Namba et al., 1985). The cells were passaged regularly twice-weekly. Between 20 and 30 days following the first infection of KMST-6 cells, the cell-free supernatant was filtered (0.45 µm) and used for a second round infection experiment on KMST6 cells. Virus replication in round infected cells was monitored by indirect immunofluorescence using rabbit anti-CVF Gag antibody as described (Schliephake & Rethwilm, 1994). Determination of cell-free virus titres in the second round infected human fibroblasts was done using a modified infectious centre assay (Bauach et al., 1993). Briefly, 5 × 10^5 baby hamster kidney cells (BHK-21) were seeded per well in 48-well plates and following adherence inoculated with 10-fold serially diluted cell-free supernatant from the infected KMST-6 cells. Four days later the BHK-21 cells were fixed in cold methanol and reacted with Gag antibody. Following incubation with peroxidase-coupled second antibody the immunostain was developed with 3-amino-9-ethylcarbazole (ABC) (Sigma) as reported previously (Bauach et al., 1993). Foci were visualized using a light microscope and virus titres were calculated as described (Reed & Muench, 1938; Bauach et al., 1993). Syncytium formation in second generation infected KMST-6 fibroblasts was monitored by staining the cells with 0.1% crystal blue. GFP expression in cells transfected or infected with appropriate plasmids or viruses was monitored by fluorescence-activated cell sorting on a FACScan using the LysisII and CellQuest software packages (Becton Dickinson) as described recently (Heinekelein et al., 1998; Lindemann & Rethwilm, 1998).

**Assay of replication competence of the chimeric viruses**

The strategy to analyse the replication competence of the TS viruses is outlined in Fig. 1(B). 293T cells were transfected with plasmids pTS12, pTS13, pchHSRV2 or mock-transfected. Two days later a freeze–thaw lysate was prepared from the transfected cells, passed through a 0.45 µm filter and used to inoculate KMST-6 fibroblasts. While a prominent cytopathic effect (CPE) was observed in cultures infected with HSRV2 lysate for 4 days, at that time point TS12- and TS13-infected cultures looked like the mock-infected control (data not shown). Indirect immunofluorescence with CVF Gag antibody on TS12- and TS13-infected KMST-6 cells seeded on chamber slides revealed, however, a few cells with typically positive-stained nuclei (Schliephake & Rethwilm, 1994), while numerous positive-stained cells were detected in HSRV2-infected cells (data not shown).

Filtered supernatant of these first generation KMST-6 cells infected with TS12 or TS13 for 20 to 30 days (three independent experiments) was used to infect fresh KMST-6 cells. Virus replication in second generation infected cells was
HSRV2-infected cultures usually reached titres in the range of $10^3$ to $10^4$, whereas growth kinetics compared with the parental virus. These results demonstrate that CFV requires a functional Tas. However, this does not appear to be a growth advantage for TS12 over TS13, which does not encode tas. To investigate whether TS12 deletes tas over time and to control for the identity of the TS13 tas deletion in long-term infected cultures, DNA extracted from KMST-6 cells infected in the second round for 40 days was analysed by PCR. As shown in Fig. 4(A) the lengths of the tas-deletion amplicons generated from the infected cells were identical to the lengths of the PCR products from original plasmids. To investigate whether Tas of TS12 was still functional, KMST-6 cells infected in the second round for 40 days were cocultured with BHK/LTRlacZ indicator cells (Schmidt & Rethwilm, 1995), which in turn were stained for β-gal expression. As shown in Fig. 5 stained cells were visible in cocultures with HSRV2- and TS12-infected cells, while in cocultures with TS13-infected cells only unstained syncytia were detected. This result indicated that TS12 after prolonged cultivation in cell culture is still able to express functional active Tas.

The stability of the hybrid LTR was analysed by PCR using DNA from KMST-6 fibroblasts infected in the second round for 20 and 40 days. As shown in Fig. 4(B), while DNA from two independent experiments with TS12 virus and one experiment (40 days of culture) with TS13 was of the expected length of 1264 bp, some minor U3 deletion variants were found in the other experiment (20 days of culture) with TS13 virus. This may indicate that there is a tendency for TS13 to rearrange the chimeric U3 promoter. Restriction enzyme analysis of the PCR products revealed that the deletions occurred in the CMV part of the hybrid promoter (data not shown).

The chimeric LTRs of TS12 and TS13 harbour approximately 200 nucleotides of upstream CFV/hu U3 sequences. Although previous studies did not identify major Tas-responsive elements in this region (Erlwein & Rethwilm, 1993; Lee et al., 1993), we investigated this topic by transient transfection of luciferase-encoding indicator gene plasmids. As shown in Fig. 4(C), the HSRV2 U3R region was stimulated by Tas approximately 600-fold. Transfection of the chimeric TS12/TS13 U3R region resulted in an indicator gene activity in the same range compared with the transactivated HSRV2 construct and, furthermore, Tas co-transfection did not lead to an increase in the activity. This result demonstrated that the gene activities of TS12 and TS13 promoters were independent of Tas.

**Analysis of a TS virus with mutated integrase**

We have demonstrated recently that CFV requires a functional integrase for virus replication (Enssle et al., 1999). Since CMV replicates from unintegrated DNA, we were interested to know how a TS virus, the replication of which relies on the hybrid CMV–U3 promoter, would behave in this respect. To analyse this, we constructed pTS16 and pTS17 (Fig. 1). Both plasmids are analogous to pTS12, with the exception of an internal expression cassette, consisting of the SFFV U3 promoter in front of the gene for GFP, in place of the bel-2 ORF. While the structural genes of pTS16 are of wild-type sequence, the DD35E motif of the pTS17 integrase was mutated to DA35E. Both plasmids were transfected into 293T cells with comparable transfection efficiencies, as determined

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**Fig. 2. Characterization of viral protein expression by pTS plasmids following transient transfection into 293T cells.**

Immunoblot with rabbit antisera directed against CFV Gag (A), Pol (B) and Tas/Bel-1 (D) and RIPA with an rabbit antiserum specific for the surface domain of Env (C).
Fig. 3. Detection of syncytium formation in human fibroblastoid cells infected in the second round with TS viruses. (A) Mock-infection, (B) acute infection of KMST-6 cells with HSRV2, (C) and (D) infection of KMST-6 cells for 45 days with TS12 and TS13, respectively. Cultures were stained with crystal blue and photographed at 125× magnification.

Fig. 4. Analysis of the stability of the tas gene and of the activity and stability of the hybrid LTR of the TS viruses. DNA was extracted from cultures infected with TS viruses in the second round for 20 or 40 days and used to amplify the tas gene (A) and the hybrid LTR (B). The tas amplicons from viruses in culture (40 days) were of the same length as the amplicons from the original plasmids. The LTR amplicons were of original length (40 days in second round infection) or showed, in addition, some smaller bands (TS13 virus for 20 days in second round infection). (C) 293T cells were either mock-transfected or co-transfected with luciferase-encoding reporter gene and effector plasmids as indicated. Forty-eight h later the luciferase activity in cellular lysates was determined and expressed in relative light units.
Fig. 5. Expression of functional Tas after prolonged cultivation of TS12. Approximately 10^4 KMST-6 cells either mock-infected (A), acutely infected with HSRV2 (B) or infected in the second round for 40 days with TS12 (C) or TS13 (D) were cocultured with 5 x 10^4 BHK/LTRlacZ indicator cells (Schmidt & Rethwilm, 1995). Two days later the cells were fixed and stained for β-Gal expression (Schmidt & Rethwilm, 1995). Blue-staining in (B) and (C) indicates Tas/Bel-1 activity, whereas in (D) only unstained syncytia were observed. Magnification is 125 x.

Table 1. Analysis of the infectivity of a TS virus with mutated integrase

(a) Transfection of 293T cells

293T cells were either mock-transfected or transfected with 10 µg plasmid DNA (pTS16 or pTS17 as shown in Fig. 1a) and the cells were analysed over time for GFP expression by FACS.

<table>
<thead>
<tr>
<th>Post-transfection (days)</th>
<th>Mock (%)</th>
<th>pTS16 (%)</th>
<th>pTS17 (%)</th>
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<tr>
<td>2</td>
<td>&lt; 0.1</td>
<td>42</td>
<td>58</td>
</tr>
<tr>
<td>13</td>
<td>0.2</td>
<td>92</td>
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<tr>
<td>19</td>
<td>0.2</td>
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</tr>
<tr>
<td>25</td>
<td>0.2</td>
<td>80</td>
<td>3.5</td>
</tr>
<tr>
<td>29</td>
<td>0.2</td>
<td>75</td>
<td>0.8</td>
</tr>
<tr>
<td>32</td>
<td>&lt; 0.1</td>
<td>79</td>
<td>1.2</td>
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</tbody>
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by measuring the percentage of GFP-positive cells 2 days post-transfection (Table 1a). KMST-6 cells were exposed to the supernatant of the transfected cells as described above and the percentage of GFP-expressing cells was followed over time by FACS analysis. As shown in Table 1a the TS16 virus was infectious with over 50% of GFP-positive cells at 3 weeks after infection, whereas TS17 virus-infected cells remained GFP-negative for an even longer period. When other measures for virus replication were used, such as CPE development, we

(b) Infection of KMST-6 cells

Forty-eight h following transfection the cell-free supernatant (0.45 µm filtrate) was used to inoculate KMST-6 fibroblasts, which in turn were monitored for GFP expression.

<table>
<thead>
<tr>
<th>Post-infection (days)</th>
<th>Mock (%)</th>
<th>TS16 (%)</th>
<th>TS17 (%)</th>
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<tbody>
<tr>
<td>11</td>
<td>&lt; 0.1</td>
<td>2.4</td>
<td>0.2</td>
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<tr>
<td>14</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
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<tr>
<td>17</td>
<td>0.1</td>
<td>15.1</td>
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<tr>
<td>20</td>
<td>&lt; 0.1</td>
<td>20</td>
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<tr>
<td>23</td>
<td>0.1</td>
<td>53</td>
<td>0.1</td>
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<td>27</td>
<td>0.1</td>
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<tr>
<td>30</td>
<td>0.1</td>
<td>11</td>
<td>0.1</td>
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</table>

FACS analysis. As shown in Table 1b the TS16 virus was infectious with over 50% of GFP-positive cells at 3 weeks after infection, whereas TS17 virus-infected cells remained GFP-negative for an even longer period. When other measures for virus replication were used, such as CPE development, we
Discussion

We demonstrate in this study that it is possible to generate a replication-competent trans-activator-negative primate FV by substituting the CMV promoter for the Tas-dependent U3 promoter. To construct the TS viruses, it was essential to modify the U3 regions of the 5' and the 3' LTR, since transfection of a plasmid, in which only the 3' LTR was modified, constantly resulted in virus with original HSRV2 U3 sequences (data not shown). This observation was similar to recombination events following plasmid transfections which were described in a previous report on the reconstruction of the authentic full-length CFV/hu LTR (Schmidt et al., 1997).

The TS viruses replicated only very poorly. Their growth kinetics were slowed down and the maximal cell-free virus titres reduced by approximately three orders of magnitude. TS viruses do not express a full-length Bet protein. The comparison of HSRV2 with a bet-mutated derivative suggested that Bet promotes higher extracellular virus titres (Yu & Linial, 1993). The observed Bet effect was at maximum tenfold and is, therefore, unlikely to explain the much higher difference in virus titres observed between HSRV2 and the TS viruses. In line with this assumption, we did not achieve significant higher virus titres compared with cell-free supernatants when freeze-thaw lysates were prepared from TS virus-infected cells (data not shown).

The CMV promoter is among the strongest promoters known and widely used in transient and stable gene expression assays (Boshart et al., 1985). The upstream CFV/hu U3 region, which is present in the hybrid promoter of the TS viruses, was reported to have some negative regulating effect on the Tas-induced gene activity of the HSRV2 LTR (Erlewin & Rethwilm, 1993). Transient transfections of a reporter plasmid containing the hybrid promoter region resulted in equally high or even higher levels of expression compared with a plasmid harbouring the HSRV2 U3 region and co-transfected with a Tas expression plasmid (Fig. 4C). This argues against the role of the putative negative regulating element in slowing down TS virus replication. However, it is possible that the incorporation of the CMV promoter into the CFV/hu U3 region in the backbone of a complete viral genome somehow modified its general high activity.

The gene expression of wild-type CFV is complex, regulated with an initial activity of the IP giving rise to some Tas protein which in turn switches on the IP and the U3 promoter (Rethwilm, 1995; Linial, 1999). The concerted action of two promoters and one transcriptional trans-activator guarantees optimal virus replication. We assume that disturbing this rather delicate system by the introduction of a constitutively active U3 promoter, although still resulting in replication-competent virus, is the main reason for the poor titres obtained with the TS viruses.

TS virus required an intact integrase for virus replication, indicating that provirus integration was essential for this virus to replicate. Physiologically the CMV promoter is active from unintegrated, probably circular viral DNA, while retroviral LTRs require integration into the host cell genome for activity (Mocarski, 1996; Brown, 1997). We assume that either in the context of a retroviral LTR the CMV promoter becomes integration-dependent or that there are properties in the CFV genome other than the LTR activity which require integration to result in virus replication.

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