An avian leukosis virus subgroup J isolate with a Rous sarcoma virus-like 5′-LTR shows enhanced replication capability

Yanni Gao,1 Xiaolu Guan,1 Yongzhen Liu,1 Xiaofei Li,1 Bingling Yun,1 Xiaole Qi,1 Yongqiang Wang,1 Honglei Gao,1 Hongyu Cui,1 Changjun Liu,1 Yanping Zhang,1 Xiaomei Wang1,2 and Yulong Gao1

1Division of Avian Infectious Diseases, State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin 150001, PR China
2Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Disease and Zoonoses, Yangzhou, 225009, PR China

Avian leukosis virus subgroup J (ALV-J) was first isolated from meat-producing chickens that had developed myeloid leukemia. However, ALV-J infections associated with hemangiomas have occurred in egg-producing (layer) flocks in China. In this study, we identified an ALV-J layer isolate (HLJ13SH01) as a recombinant of ALV-J and a Rous sarcoma virus Schmidt-Ruppin B strain (RSV-SRB), which contained the RSV-SRB 5′-LTR and the other genes of ALV-J. Replication kinetic testing indicated that the HLJ13SH01 strain replicated faster than other ALV-J layer isolates in vitro. Sequence analysis indicated that the main difference between the two isolates was the 5′-LTR sequences, particularly the U3 sequences. A 19 nt insertion was uniquely found in the U3 region of the HLJ13SH01 strain. The results of a Dual-Glo luciferase assay revealed that the 19 nt insertion in the HLJ13SH01 strain increased the enhancer activity of the U3 region. Moreover, an additional CCAAT/enhancer element was found in the 19 nt insertion and the luciferase assay indicated that this element played a key role in increasing the enhancer activity of the 5′-U3 region. To confirm the potentiation effect of the 19 nt insertion and the CCAAT/enhancer element on virus replication, three infectious clones with 5′-U3 region variations were constructed and rescued. Replication kinetic testing of the rescued viruses demonstrated that the CCAAT/enhancer element in the 19 nt insertion enhanced the replication capacity of the ALV-J recombinant in vitro.

INTRODUCTION

Avian leukosis viruses (ALVs) were originally isolated by V. Ellerman and O. Bang in 1908, and can be classified into two types: endogenous viruses (ALV-E) and exogenous viruses (ALV-A, -B, -C, -D and -J), based on their mode of transmission (Payne et al., 1991). ALVs were mainly associated with lymphocytoma until the emergence of ALV-J, which has primarily been associated with myeloid leukemia in broiler breeders (Payne et al., 1991). ALV-J causes more serious damage to broiler breeders than any other ALV subgroup (Fadly & Smith, 1999; Payne et al., 1992; Venugopal, 1999). In addition, recent studies found that ALV-J can induce various tumours and cause production problems in egg-producing (layer) chickens (Gao et al., 2010).

Retroviruses have high mutation rates due to the activity of the reverse transcriptase enzyme (Elena et al., 2000). In addition, the diploid nature of the retrovirus genome results in a high level of recombination (Hu & Temin, 1990a, b; Nichol, 1996). Recombination has been observed between exogenous viruses, between exogenous and endogenous viruses, and between exogenous virus and non-homologous cellular genes, e.g., a strain of ALV-J with a primer-binding site leader sequence that originated from other retroviruses (Wu et al., 2010), a naturally occurring recombinant ALV-B strain with a subgroup J-like long-terminal repeat (Gingerich et al., 2002; Lupiani et al., 2006), a recombinant that was composed of most of the ALV-J genome and part of the corresponding env sequence of ALV-E (Liu et al., 2011), and a recombinant ALV with ALV-A gp85 and the ALV-E LTR, which originated from contaminated Marek’s disease vaccines (Barbosa et al., 2008).

The overall genomic structure of ALV consists of 5′-LTR leader-gag-pol-env-trailer-3′-LTR. The LTRs play a vital role in the regulation of virus replication (Bizzel et al., 1984). The LTRs consist of U3, R and U5 regions, including promoters and enhancers for the growth of viruses, which regulate virus
replication (Bizub et al., 1984) and are prone to mutations during the process of virus replication (Shi et al., 2011). The replication capacity of ALV may influence the pathogenicity of the virus (Wang et al., 2012). gag, pol and env are the structural genes of ALVs, which encode gag, reverse transcriptase integrase and the envelope glycoprotein, respectively (Payne et al., 1993; Tsukamoto et al., 1985). The envelope glycoprotein determines the host range of virus infection and cross-neutralization patterns, which depend on the virus receptors on the cell membrane (Bova et al., 1986, 1988, 1991; Dorner et al., 1985).

Rous sarcoma virus (RSV) is significant amongst retroviruses as it was the first to be correlated with the formation of tumours. RSV has strong transcriptional promoter and enhancer elements within the U3 region of its LTRs (Cullen et al., 1985; Laimins et al., 1984; Luciw et al., 1983). Once viral DNA has been synthesized and integrated into host DNA, viral RNA transcription is performed by cellular factors (Majors, 1990). The 5'-terminal 100 nt region of the LTR, which contains a number of CCAAT/enhancer elements (Ryden et al., 1993), possesses enhancer activity; deletions in this region decrease LTR promoter function by 20- to 200-fold (Cullen et al., 1985; Gowda et al., 1988; Laimins et al., 1984; Norton & Coffin, 1987).

To date, ALV-J has been predominantly responsible for avian leukosis in layer chickens in China (Pan et al., 2011; Gao et al., 2012). In this study, an ALV was isolated from layer chickens with apparent tumours. Genomic sequence analysis revealed that this virus was a recombinant ALV with the RSV 5'-LTR region and the other genes of ALV-J. Furthermore, we found that this recombinant ALV-J isolate replicated faster than other ALV-J strains that were isolated from layer flocks and that enhanced virus replication capability was due to the substitution of the 5'-U3 (part of the 5'-LTR) region from the RSV. The key sites for the stronger replication capacity were further confirmed in the study.

RESULTS

Virus isolation and identification of ALV-J

A virus isolate was obtained from clinical samples of sick layer flocks with symptoms of hemangiomias of different sizes on the surface of the head, claw and wing as well as tumours in various organs, especially the liver, in Heilongjiang. Results from ELISA showed that this virus belonged to the ALV family (data not shown). Further analysis by indirect immunofluorescence assay (IFA) and multiplex PCR confirmed that the isolate was of the ALV-J subtype (data not shown). This strain was designated HLJ13SH01.

HLJ13SH01 is a recombinant with the ALV-J genome and the RSV 5'-LTR

The full-length proviral genome sequence of HLJ13SH01 (GenBank accession number KM376510) contained 7677 nt, which represented the typical genetic organization of replication-competent alpharetroviruses. Sequence analysis revealed that this new ALV-J strain encoded the env gene that shared 94% sequence identity with HPRS-103 – the ALV-J prototype virus (Payne et al., 1991). However, the 5'-LTR region of this isolate shared only 91.1% sequence identity with that of HPRS-103, but 99.1% sequence identity with that of the RSV Schmidt-Ruppin B (SRB) strain. Moreover, a novel 19 nt sequence, which exists in the 5'-LTR of the RSV-SRB strain, but not in the 5'-LTR of other ALV-J isolates, was found in the 5'-LTR of the HLJ13SH01 isolate (Fig. 1a). However, the env gene of the HLJ13SH01 isolate shared <25% sequence identity with the RSV-SRB strain and most of its other genes (gag, pol and 3'-LTR) shared high sequence identities with HPRS-103 except for a 205 bp deletion in the redundant transmembrane region. Therefore, the HLJ13SH01 strain was a recombinant of ALV-J with the replacement of the RSV-SRB 5'-LTR region (Fig. 1b).

HLJ13SH01 replicates faster than HuB09WH03, a typical ALV-J isolate from egg-type flocks, in DF-1 cells

DF-1 cells were used to evaluate the in vitro replication capacity of the recombinant HLJ13SH01 strain in comparison with previous ALV-J layer isolates, with HuB09WH03 (Gao et al., 2012) as a representative. DF-1 cell cultures were infected with equivalent amounts (m.o.i. 1) of the HLJ13SH01 and HuB09WH03 strains. Cell cultures were obtained after three rounds of freezing–thawing, and the supernatants were harvested periodically thereafter (1, 2, 3, 4, 5, 6 and 7 days post-infection) for titration of virus and assay of reverse transcriptase activity. As shown in Fig. 2, from day 1 post-infection, cells infected with the HLJ13SH01 strain had a higher titre than those infected with the HuB09WH03 strain. By day 6, the titres of both strains peaked. The results revealed that the HLJ13SH01 strain had a growth advantage over the HuB09WH03 strain (Fig. 2a). Additionally, we tested reverse transcriptase activity to determine retroviral replication (Ogert et al., 1996) and to confirm the titration results. As shown in Fig. 2(b), the reverse transcriptase activity of the culture supernatant samples from cells infected by HLJ13SH01 or HuB09WH03 strains supported the direct titration results. These results indicated that the HLJ13SH01 strain replicated faster than the HuB09WH03 strain in DF-1 cells.

5'-U3 region of HLJ13SH01 has stronger enhancer activity than that of HuB09WH03 due to a 19 nt insertion

The main difference between the HLJ13SH01 and HuB09WH03 strains was a 19 nt insertion in the 5'-U3 region of the 5'-LTR region of the HLJ13SH01 strain, which was not present in the HuB09WH03 strain. A Dual-Glo luciferase assay was performed to test the enhancer activity of these two different U3 regions. As shown in Fig. 3(a), the results indicated that the 5'-U3 region of the
The HLJ13SH01 strain had stronger enhancer activity than that of the HuB09WH03 strain. In addition, the luciferase assay results demonstrated that the 19 nt insertion was responsible for the increased enhancer activity of the U3 region in HLJ13SH01 (Fig. 3c).

A CCAAT/enhancer element plays a key role in the ability of the 19 nt insertion to improve enhancer activity

Analysis of the 19 nt sequence revealed the presence of a CCAAT/enhancer element in this region. CCAAT/enhancer elements have been shown to be important for the regulation of virus replication (Ryden et al., 1993). To investigate this issue, we constructed another luciferase vector, U3-M, which was similar to the 5'-U3 region of the HLJ13SH01 strain, but included a 2 nt mutation to destroy the CCAAT/enhancer element in the 19 nt insertion (Fig. 4a). Fig. 4(b) shows that the 2 nt mutation reduced the enhancer activity of the U3 region, indicating that the CCAAT/enhancer element played a key role in improving the enhancer activity of the 5'-U3 region of the HLJ13SH01 virus isolate.

Comparison of the replication kinetics of the three rescued viruses validates the function of the 19 nt insertion and the corresponding CCAAT/enhancer element

The luciferase assays demonstrated that the 19 nt insertion and the CCAAT/enhancer element increased the enhancer activity of the 5'-U3 region. To validate the direct relationship between the 19 nt insertion, the CCAAT/enhancer element and virus replication, pBlu-HLJ13SH01 (WT), pBlu-HLJ13SH01D (deletion of the 19 nt insertion) and pBlu-HLJ13SH01M (2 nt mutant in the 19 nt insertion which destroyed the CCAAT/enhancer element) recombinant plasmids were constructed, and the corresponding rHLJ13SH01, rHLJ13SH01D19 and rHLJ13SH01M infectious clones were rescued (Fig. 5b). After rescuing, the replication kinetics of the three rescued viruses were measured and compared. As shown in Fig. 5(b), rHLJ13SH01 had a significant advantage in replication over rHLJ13SH01D19, whilst rHLJ13SH01M exhibited lower replication capacity compared with rHLJ13SH01 and moderately higher replication capacity than rHLJ13SH01D19. These results were in
agreement with the results of the luciferase assays, which further confirmed that the 19 nt insertion and the CCAAT/enhancer element contributed to virus replication.

**DISCUSSION**

Following the initial isolation of ALV-J approximately two decades ago, this virus has become highly prevalent in
meat-producing chickens. ALV-J is associated with myeloid leukosis in chickens and has caused serious problems in the global poultry industry (Fadly & Smith, 1999). Furthermore, it was recently reported that ALV-J strains naturally infect egg-producing (layer) chickens and induce various tumours (Gao et al., 2010, 2012; Xu et al., 2004; Zhang et al., 2009). ALV-J strains from various countries continue to recombine and generate new viruses with novel pathogenicity (Wang et al., 2012). In this study, we identified a new ALV-J strain that was isolated from layer chickens with hemangiomas and myelomas. This strain was determined to be a recombinant of ALV-J and RSV. Additionally, this recombinant strain of ALV-J replicated more rapidly than other typical ALV-J strains isolated from layer chickens, as represented by the HuB09WH03 strain in this study, indicating a stronger replication capacity of the recombinant ALV-J strain compared with the typical ALV-J strain. This advantage may be the result of the natural evolution of the virus, which is aimed at virus spread (Ji et al., 2014; Zavala et al., 2007).

We discovered that the key replication advantage for the recombinant strain was its stronger enhancer activity in the 5′-U3 region, which was a substitution of the RSV-SRB strain. The U3 region of the recombinant strain contained a 19 nt insertion in contrast to the U3 region of other ALV-J layer strains. It is important to monitor sequence changes in the U3 region of ALV-J because this region of avian retroviruses contains functional elements, such as promoter and enhancer elements, which are involved in key steps of the retroviral life cycle (Donzé & Spahr, 1992). In addition, sequence changes in the U3 region have been implicated in both the level of viral transcription and the oncogenic potential of ALVs (Cullen et al., 1985; Ryden et al., 1993). A comparison of the U3 sequences in ALV-J broiler strains and layer isolates indicated that the U3 region of ALV-J layer isolates has evolved rapidly (Hue et al., 2006; Gao et al., 2012; Wu et al., 2010). The results of this study suggested that the stronger enhancer activity of the U3 region that was substituted from RSV resulted in a faster replication rate of the recombinant ALV-J strain.

The CCAAT/enhancer element is a common cis-element in the promoters of many genes (Edwards et al., 1998; Kato, 2005; McNabb et al., 1995; Yazawa & Kamada, 2007). Several studies have demonstrated that the CCAAT/enhancer binding protein sites in the human immunodeficiency virus type 1 (HIV-1) LTR are crucial for HIV-1 replication in monocyte/macrophages and for the ability of IFN-β to inhibit ongoing active HIV replication in these cells (Henderson & Calame, 1997; Henderson et al., 1995, 1996; Ravimohan et al., 2010). Moreover, mutations in CCAAT/enhancer element are associated with an increased expression level of the HIV-1 LTR, which is closely related to the pathogenesis of HIV-1 infection (Henderson et al., 1995, 1996; Ravimohan et al., 2010). Therefore, it is essential to study the CCAAT/enhancer element of ALV-J strains to understand their pathogenicity and to develop effective strategies for their control.
enhancer elements have been found in the LTR region of RSV. This destruction of the consensus core sequence in CCAAT/enhancer elements reduced LTR-driven transcription and viral titres of RSV in fibroblasts (Ryden et al., 1993). In this study, to further investigate the mechanism of stronger enhancer activity in the 5'9-U3 region of HLJ13SH01 compared with that of other ALV-J isolates, a Dual-Glo luciferase assay was performed and the results confirmed that the CCAAT/enhancer element in the 19 nt insertion played an important role in increasing the enhancer activity of the 5'9-U3 region. These results further validated the significance of CCAAT/enhancer elements in virus replication.

As shown by results in this study, mutations in the CCAAT/enhancer elements reduced the enhancer activity of the 5'9-U3 region, but the enhancer activity of the mutant was still slightly higher than that without the 19 nt insertion. It is possible that the insertion may have changed the length of the 5'9-U3 region in the ALV-J strain, thereby readjusting its structure and ultimately influencing the cis-acting characteristics of the 5'9-U3 region. However, this hypothesis requires further investigation.

Based on the luciferase assay results, we further confirmed the ability of the 19 nt insertion and the CCAAT/enhancer element to enhance virus replication in the three rescued viruses. Several ALV-J layer isolates with similar substitutions have been reported in our previous studies (Pan et al., 2011), which suggests that similar recombinant ALV-J strains are currently circulating in flocks in China. ALV-J has caused significant economic losses in parent and commercial layer flocks in recent years in China (Gao et al., 2010). The results of molecular epidemiology indicated that the genome of ALV isolates shows obvious variations (Gao et al., 2012). Our previous study demonstrated that a 205 nt deletion in the 3'-UTR of ALV layer isolate can enhance the capacity of virus replication and contributes to increased pathogenicity (Wang et al., 2012). However, another ALV-J isolate with a 19 nt insertion in the leader sequence could contribute to virus replication in vitro, but did not influence its pathogenicity in vivo (Ji et al., 2014). These results suggested that the pathogenicity of ALV-J in chickens is extremely complicated. Accordingly, the prevalence of ALV-J in China is likely due to many factors that relate to both hosts and viruses, including virus replication rate. Further research is required to determine if the 19 nt insertion in this study would enhance virus pathogenicity in vivo.

Fig. 5. The 19 nt insertion and the CCAAT/enhancer element contribute to virus replication in DF-1 cells. (a) The structures of the three rescued viruses. The structures of rHLJ13SH01, rHLJ13SH01Δ19 and rHLJ13SH01-M were the same except for the 5'-U3 region. The 5'-U3 region of rHLJ13SH01 was a WT. The 5'-U3 region of rHLJ13SH01Δ19 lacked the 19 nt insertion compared with the WT. The 5'-U3 region of rHLJ13SH01 M had a 2 nt mutation which destroyed the CCAAT/enhancer element in the 19 nt insertion. A black triangle represents a mutation. (b) One-step growth curves for the three rescued viruses in DF-1 cells. The growth curves were drawn after assaying the viral titres. The viral titres at different intervals were calculated and expressed as TCID50 ml\(^{-1}\). Mean ± SD from three independent experiments. Significant differences were observed amongst the three rescued viruses according to Student’s t-test.
METHODS

Experimental samples, virus isolation and identification. In 2013, suspected cases of ALV-J infection erupted in layer flocks in Heilongjiang province in China. The clinical symptoms included haemorrhages in the skin of the phalanges and feather follicles. Several layer hens had hemorrhages of different sizes on the surface of the head, claw and wing as well as tumours in various organs, especially the liver. To confirm and analyse the pathogenesis of this disease, tumours were collected from the sick chickens. Filtered tumour homogenates were inoculated into DF-1 cells and the cells were incubated at 37 °C with 5% CO2 for 6–8 days for each passage (Maas et al., 2006). Uninfected DF-1 cells were used as a negative control. After three blind passages, the infected DF-1 cells were tested for the ALV group-specific antigen (p27) using an antigen-capture ELISA (Yun et al., 2013) and tested for the ALV-J-specific antigen by IFA with ALV-J-specific mAb, FE9 (Venugopal et al., 1997). The positive samples that were detected by ELISA and IFA were harvested for DNA extraction and further characterized using multiplex PCR for ALV-A/B/J with four specific primers (Gao et al., 2013).

DNA alignments and phylogenetic analysis. Proviral DNA was extracted from HLJ13SH01-infected DF-1 cells for use as a template for PCR amplification with three different pairs of overlapping primers (F1/R1, F2/R2 and F3/R3, shown in Table 1) for the full genome of ALV-J. The amplicons were gel-purified, subcloned into pMD-18T vectors and sequenced. The whole-genome sequences were assembled. Nucleotide and deduced amino acid sequences of the isolate and the ALV-J prototype HPRS-103, several other ALV-J layer isolates, and RSVs were aligned using CLUSTAL_W (version 5.06) and the MegAlign function in DNASTAR DNA analysis software.

Luciferase reporter plasmid construction. The luciferase expression vector pGL-Promoter vector was used as the parent vector for the 5′-U3 sequences of the HLJ13SH01 and HuB09WH03 strains and two variants from U3 region reporter analysis experiments. The 5′-U3 regions of these two strains of viruses (HLJ13SH01/U3 and HuB09WH03/U3) were amplified by PCR using cDNA from the proviral genome that was derived from infected DF-1 cells and primer pair U3F/U3R. The pGL-SH plasmid, which contained the U3 region of the HLJ13SH01 strain, was constructed by amplifying the pGL-SH plasmid using the primer pair SHU3MF/U3R. All of the amplicons were cloned into the pGL-Promoter vector with XhoI and ClaI restriction sites, which were added to the 5′ and 3′ primers (shown in Table 1). All of the constructs were confirmed by restriction enzyme digestion and sequencing.

Transfection and Dual-Glo luciferase assay. Transfection of the pGL-HuB09WH03, pGL-SH, pGL-SHA19, pGL-SHM and pRL-TK plasmids was performed in DF-1 cells using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s protocol. At 48 h, the cell medium was exchanged, the cells were harvested and the luciferase reporter activities were measured using a Dual-Glo Luciferase Assay System (Promega) according to the manufacturer’s protocol. The activities of the HLJ13SH01 5′-U3 enhancers and mutants were compared with those of the HuB09WH03 strain and the data represent the mean of three experimental replicates.

Construction of the rHLJ13SH01, rHLJ13SH01A19 and rHLJ13SH01M infectious clones. Proviral DNA was extracted from HLJ13SH01-infected DF-1 cells for use as a template for PCR amplification. The HLJ13SH01 genome was cleaved into three fragments using XbaI and ClaI restriction enzymes, which were amplified separately. The primer pairs F1/R1, F2/R2 and F3/R3 were used to amplify fragments I, II and III, respectively. The amplifications were gel-purified, subcloned into pMD-18T vectors and ligated into pBlueScript II KS (+) plasmids via Eag1/XhoI. The recombinant plasmid was named pBlu-HLJ13SH01. To delete the 19 nt insertion in the 5′-U3 region, fragment I was replaced with a product from a two-step PCR assay using the primer pairs SHU3SF1/R1 and F1-S-2/R1. Additionally, to obtain the mutant fragment I, the primers pair F1-M/R1 were used instead of F1/R1. Finally, the new fragments I, II and III were ligated into the pBlueScript II KS (+) plasmid as described above to obtain the pBlu-HLJ13SH01A19 and pBlu-HLJ13SH01M recombinant plasmids. The primers are shown in Table 1.

Virus rescue and identification. Highly purified pBlu-HLJ13SH01, pBlu-HLJ13SH01A19 and pBlu-HLJ13SH01M DNA were obtained using Qiagen Plasmid midi kits according to the manufacturer’s instructions. The purified plasmid DNA (4 μg) from the pBlu-HLJ13SH01, pBlu-HLJ13SH01A19 or pBlu-HLJ13SH01M clone was introduced into DF-1 cells using Lipofectamine 2000 and blind-passaged into secondary DF-1 cells. The rescued viruses that were generated from the pBlu-HLJ13SH01, pBlu-HLJ13SH01A19 and pBlu-HLJ13SH01M plasmids were named rHLJ13SH01, rHLJ13SH01A19

Table 1. The primer sequences for the luciferase reporter plasmid constructs and infectious clones

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<tr>
<th>Primer</th>
<th>Sequence (5′→3′)</th>
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<tr>
<td>U3F</td>
<td>GCCGGTACCTGATGTTATGCAATACCTC</td>
</tr>
<tr>
<td>U3R</td>
<td>TACCTCGAGGTTTAAGTTGATCGCTAGG</td>
</tr>
<tr>
<td>SHU3SF1</td>
<td>GCGCTATGGTGAAGATGAGT</td>
</tr>
<tr>
<td>SHU3SF2</td>
<td>GAAAGTACCTGATGTTATGCAATACCTCTTTATGTAAGATGAGT</td>
</tr>
<tr>
<td>SHU3MF</td>
<td>GAGATGGTACCTGATGTTATGCAATACCTCTTTAGTAAACATACCT</td>
</tr>
<tr>
<td>F1</td>
<td>TATCGGCCGTGATGTTATGCAATACCTCTTTGTA</td>
</tr>
<tr>
<td>R1</td>
<td>GCGCATTTTTCTATGCTGATT</td>
</tr>
<tr>
<td>R2</td>
<td>GGCAGGGAAATGGAATCT</td>
</tr>
<tr>
<td>R3</td>
<td>CAGCAGCGATCGGATTTCTTTCTAC</td>
</tr>
<tr>
<td>F1-S-2</td>
<td>CAAGGCGGCTGATGTTATGCAATACCTCTTTAGTAAACATACCT</td>
</tr>
<tr>
<td>F1-M</td>
<td>GAACGCGGGTTATGCAATACCTCTTTAGTAAACATACCT</td>
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and rHLJ13SH01M, respectively. To determine the specificity of the rescued viruses, the secondary DF-1 cell supernatants were analysed using an ELISA kit with the ALV group-specific antigen p27. IFA was performed using virally infected DF-1 cells and ALV-J-specific mAb FE9 (Venugopal et al., 1997). Non-infected DF-1 cells were used as a negative control.

Replication kinetics and reverse transcriptase activity of HLIJ13SH01 and HuB09WH03 and the three rescued viruses. The titres of the HLIJ13SH01 and HuB09WH03 strains and the three rescued viruses were measured using ELISA and were presented as TCID₅₀ ml⁻¹ according to the Reed–Muench method (Reed & Muench, 1938). DF-1 cells were plated in 35 mm dishes 1 day before infection. Just prior to infection, the cells were washed twice with PBS and mock infected or infected with the HLIJ13SH01 strain, the HuB09WH03 strain or the three rescued viruses at m.o.i. 1. Time was set to zero after 2 h of adsorption. The supernatant was removed and the cells were washed twice with PBS to remove unattached virus before adding 2 ml DMEM medium containing 10% FBS to each well. The cells were cultured at 37 °C with 5% CO₂ in an incubator. Cell morphology was monitored every day, and the infected cells were harvested separately on days 1, 2, 3, 4, 5, 6 and 7 post-infection at −80 °C following the collection of 500 μl culture supernatants, which were used for the quantification of reverse transcriptase activity in a colorimetric reverse transcriptase assay. After three freeze–thaw cycles, the cell lysates were tested for an ALV group-specific antigen (p27) by ELISA to determine the replication rates as described above.

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