Inefficient viral replication of bovine leukemia virus induced by spontaneous deletion mutation in the G4 gene

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Enzootic bovine leucosis is caused by bovine leukemia virus (BLV) infection, which is highly prevalent in several regions of the world and significantly impacts the livestock industry. In BLV infection, the proviral load in the blood reflects disease progression. Although the BLV genome is highly conserved among retroviruses, genetic variation has been reported. However, the relationship between proviral load and genetic variation is poorly understood. In this study, we investigated the changes in proviral load in BLV-infected cattle in Japan and then identified and analysed a BLV strain pvAF967 that had a static proviral load. First, examining the proviral load in the aleukaemic cattle in 2014 and 2015, cow AF967 showed a static proviral load, while the other cows showed significant increases in proviral load. Sequencing the provirus in cow AF967 showed a deletion of 12 nt located in the G4 gene. An in vitro assay system using BLV molecular clone was set up to evaluate viral replication and production. In this in vitro assay, the deletion mutation in the G4 gene resulted in a significant decrease in viral replication and production. In addition, we showed that the deletion mutation did not affect the viral transcriptional activity of Tax protein, which is also important for virus replication. The emergence of strain pvAF967 that showed a static proviral load, combined with other retrovirus evolutionary traits, suggests that some BLV strains may have evolved to be symbiotic with cattle.

INTRODUCTION

Bovine leukemia virus (BLV), which belongs to the family Retroviridae genus Deltaretrovirus, is the aetiologic agent of enzootic bovine leucosis (EBL). BLV is a ssRNA virus that is closely related to human T-cell leukemia virus type 1 (HTLV-1). Approximately one-third of BLV-infected cattle develop persistent lymphocytosis, characterized by non-malignant polyclonal B-cell expansion, while 1–5% of them develop B-cell leukemia/lymphoma after a long latency (Schwartz & Levy, 1994). Dairy economic loss because of BLV infection was estimated to be US$525 million annually in the USA, and the prevalence of BLV infection is high in several regions worldwide (Ott et al., 2003). Therefore, it is of great concern to reduce the economic losses caused by BLV.

After infecting white blood cells, BLV integrates into the host genome and is present as a provirus. During the first 2 weeks after an initial infection, BLV particles are produced and are detectable in the blood. After this viraemic period, viral particles are barely detectable (Kono et al., 1983; Pyeon & Splitter, 1999). The propagation of BLV in BLV-infected cattle mainly depends on mitosis of infected cells (Kettmann et al., 1982), while it is also thought that a small population of the BLV-infected cells produce virus (Gillet et al., 2007). The level of viral propagation in BLV-infected cattle can be estimated by the proviral load, defined as the
proviral copy number in blood that depends on the number of BLV-infected cells. It is believed that the proviral load increases with progression of disease and relates to BLV pathogenesis, because a high proviral load is generally detected in cattle developing EBL (Jimba et al., 2010; Somura et al., 2014). Thus, an understanding of the replication and production of BLV is very important for BLV pathogenesis.

The BLV proviral genome is c. 8.7 kbp, including LTRs at both ends of the genomic DNA, gag, pol and env and five non-structural genes (R3, G4, tax, rex and AS1) (Durkin et al., 2016). The BLV genome has been considered to be highly conserved because, similar to HTLV-1, viral replication mainly depends on mitosis (Yanagihara et al., 1995; Tajima et al., 1998). However, new genetic variations in BLV have recently been reported (Lee et al., 2016; Polat et al., 2016). The reports imply that the BLV genome has evolutionarily changed. In addition, previous studies reported that induced mutations by reverse genetic approach have been shown to change the BLV pathogenesis (Willems et al., 2000; de Brogniez et al., 2015). Thus, we thought that the evolutionary genetic variation induced alteration of viral properties related to BLV pathogenesis. However, the relationship between spontaneous mutation of BLV and the properties of the virus has not been studied. Here, we examined the changes in the proviral load in the BLV-infected cattle in Japan between 2014 and 2015. Using molecular biological techniques in vitro, we then identified and investigated the unique strain of BLV provirus that showed a static proviral load.

RESULTS

Static proviral load in a cow infected with BLV strain pvAF967 and analysis of the pvAF967 genome

Sixty-six cows were tested for the presence of anti-BLV antibody in blood using an agar gel immunodiffusion (AGID) test, and six cows (cows AF019, AF153, AF438, AF805, AF967 and AF982) were diagnosed with BLV infection. These cows were kept on the same farm from 2014 to 2015. In addition, antibody titres of the six cows were measured by AGID test. Only cow AF967 had the titre of 1, while other cows had the titre of more than 2 (the antibody titres of cows AF019, AF153, AF438, AF805 and AF982 were 2, 2, 2, 4 and 8, respectively) on 3 April 2015. Next, the proviral loads of these cows on 6 August 2014 and 3 April 2015 were measured by quantitative PCR (qPCR). Only cow AF967 showed no change in proviral load during this period, while the other five cows showed significant increases (Fig. 1a). The whole genomes of the proviruses present in cow AF967 (pvAF967) in 2014 and 2015 were sequenced and analysed. The whole genome of the provirus present in the fetal lamb kidney persistently infected with BLV (FLK-BLV) cell line (pvFLK) was also sequenced to be used as a BLV reference genome. The results showed that the genome sequences of pvAF967 in August 2014 and April 2015 were 100 % identical and that the genome sequence of pvAF967 was 99.22 % identical to that of pvFLK. A deletion of 12 nt at positions 7140–7151 relative to the proviral genome sequence was the major mutation found in the pvAF967 genome (Fig. 1b). Subsequently, we used BLAST to search the public database at the National Center for Biotechnology Information for this 12 nt deletion and performed PCR and sequencing analysis of the proviral genomes in the other BLV-infected cows (cows AF019, AF153, AF438, AF805 and AF982) kept on the same farm (Fig. S1a, b, available in the online Supplementary Material). However, the same deletion mutation was not detected in either analysis. Therefore, the deletion mutation in pvAF967 was considered to be unique among BLV strains.

This deletion mutation was located in the G4 and AS1 genes of the BLV genome, which overlap on the sense and antisense DNA strands, respectively (Fig. 1b). The G4 protein is a small protein (105 aa, molecular weight 11.6 kDa), and its function is not fully understood (Lairmore, 2014). The deletion mutation in the G4 gene seemed to result in the deletion of amino acids RLPL, which are located 48–51 amino acids from the N terminus of the G4 protein. Previous studies showed that the deficiency of the G4 gene in an infectious molecular clone resulted in a low proviral load (Dequiedt et al., 1997; Florins et al., 2008). In contrast, the AS1 gene is a newly identified gene encoded on the antisense strand. While the AS1 gene probably encodes a protein (87 aa, predicted molecular weight 9.4 kDa), its function is not known (Durkin et al., 2016). In the AS1 gene, the deletion mutation seemed to lead to result in the deletion of amino acids AEAA, which are located 22–25 amino acids from the N terminus of the AS1 protein. These results together with those of previous studies suggest that the deletion mutation is likely to affect the function of the G4 protein.

In vitro assay of virus replication and production using BLV molecular clones

An in vitro assay system using BLV molecular clones was prepared to examine virus production by different strains of BLV. The molecular clones, pBLV-AF967 and pBLV-FLK, were constructed from the pvAF967 and pvFLK genomes, respectively (Table 1). pBLV-FLK was used as the positive control. First, pBLV-AF967 and pBLV-FLK were transfected into CC81 cells and both caused syncytium formation in the transfected cells (Fig. 2a), since the CC81 cell line can form syncytia after BLV infection (Ferrer et al., 1981). Second, we examined the viral protein expression of these clones in the transfected cells by immunostaining and demonstrated the expression of the nucleocapsid protein p24 in the syncytia transfected with both clones (Fig. 2a). Thus, the clones constructed in this study would be infectious and could be used for further in vitro experiments.

Next, we transfected HeLa cells with the clones and quantified the virus copy number in the culture supernatants of
the cells by qPCR. The quantity of viral RNA for both clones reached a peak 2 days post-transfection, then gradually decreased and was not detected thereafter (Fig. 2b). The change in viral RNA quantity seemed to be similar in the supernatants of the cells transfected with both clones. However, the cells transfected with pBLV-AF967 showed significantly lower quantity of viral RNA 2 days post-transfection, and the period during which viral RNA could be detected in the cells transfected with pBLV-AF967 (2–4 days) was shorter than that for the cells transfected with pBLV-FLK (2–6 days). Thus, the ability of pBLV-AF967 to replicate viral RNA was weaker than that of pBLV-FLK.

Next, the infectious viruses produced from the HeLa cells transfected with the clones were measured by the syncytium assay. In the syncytium assay, CC81 cells were used as indicator host cells. Two days after the transfection of HeLa cells with pBLV-AF967 and pBLV-FLK, the culture supernatants of the HeLa cells were added to CC81 cells and then

Fig. 1. Analysis of provirus pvAF967 in cow AF967. (a) Proviral loads of BLV-infected cows in 2014 and 2015. The solid and open bars show the proviral loads in the BLV-infected cattle in 2014 and 2015, respectively. The experiments were performed in triplicate. The bar graph with an error bar indicates the mean value with se. Single and double asterisks indicate the statistical significance (P<0.05 and P<0.01, respectively). (b) Site of deletion mutation found in the pvAF967 genome. The top panel shows a map of the BLV proviral genome. On the genome map, the right-handed and left-handed arrows indicate the genes encoded on the sense and antisense strands, respectively. The deletion site identified in the pvAF967 genome is indicated by a cross mark. The deletion mutation is located on the overlapping G4 and AS1 genes. The bottom panel shows DNA and protein sequences around the deletion site of pvFLK and pvAF967 (2014 and 2015).
Table 1. BLV molecular clones used in this study

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<tr>
<th>Name</th>
<th>Origin</th>
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<td>Molecular clones constructed from proviral genome sequences</td>
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<tr>
<td>pBLV-FLK</td>
<td>pvFLK (provirus in FLK-BLV cell line)</td>
<td>The provirus genome sequences were amplified and cloned into pSMART LC Amp (Lucigen). Primers for PCR amplification of the proviral genome, BLV-FF, BLV-FR, BLV-LF and BLV-LR, were used (see Table S2). The GenBank accession numbers for the pBLV-FLK, pvAF019, pvAF967 and pvAN903 sequence of BL proviral full genomes are LC164083, LC164084, LC164085 and LC164086</td>
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<tr>
<td>pBLV-AF967</td>
<td>pvAF967 (provirus in an aleukaemic cow AF967)</td>
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<td>pBLV-AF019</td>
<td>pvAF019 (provirus in an aleukaemic cow AF019)</td>
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<tr>
<td>pBLV-AN903</td>
<td>pvAN903 (provirus in an EBL-developing cow AN903)</td>
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<td>Genetically modified clones</td>
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<td>pBLV-AF967insRLPL</td>
<td>pBLV-AF967</td>
<td>Molecular clone induced with the addition of 12 nt (i.e. 5’-CGGCTGCCTGCT-3’) was constructed by inverse PCR method. Primers, RLPL-F and insRLPL-R, were used for PCR amplification of the molecular clone (see Table S2)</td>
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<td>pBLV-FLKARLPL</td>
<td>pBLV-FLK</td>
<td>Molecular clones induced with the deletion of the 12 nt (i.e. 5’-CGGCTGCCTGCT-3’) were constructed by inverse PCR method. Primers RLPL-F and ΔRLPL-R were used (see Table S2)</td>
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<td>pBLV-AF019ARLPL</td>
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<td>pBLV-AN903ARLPL</td>
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cultured. The supernatants of cells transfected with both clones caused syncytia formation by CC81 cells. However, the supernatant of pBLV-AF967-transfected cells caused the formation of significantly fewer syncytia than that of the pBLV-FLK-transfected cells. These data for infectious virus production are similar to those for viral RNA replication described above. Together, the data suggest that pBLV-AF967 transfection of the HeLa cells produced infectious viruses less efficiently than transfection with pBLV-FLK (Fig. 2c).

Although viral properties in vitro might not be completely reflected in the in vitro assay system, it was speculated that the virus production in vitro was related to the viral propagation in vivo. Indeed, the virus production occurred for 2 weeks after the initial BLV infection (Kono et al., 1983; Pyeon & Splitter, 1999) and it would be important for viral propagation in vivo. Since the cow AF967 showed the static proviral load, it was thought that the pvAF967 strain did not efficiently propagate in vivo. Examining the account of low virus production in pvAF967 was considered to be important for relationship between the genetic variation and BLV pathogenesis in vivo. Therefore, we next examined whether the deletion mutation in the G4 gene caused low virus production in vitro.

Examination of the effect of the deletion mutation in the G4 gene on virus production using an in vitro assay system

Experiments to complement the deletion mutation in the G4 gene were performed. First, an in vitro complementation experiment was performed in HeLa cells expressing full-length G4 protein. HeLa cells stably expressing a hemagglutinin (HA) tag or an HA-tagged G4 protein (HA/HeLa and G4-HA/HeLa, respectively) were prepared (Fig. 3a). The HA/HeLa and G4-HA/HeLa cells were transfected with pBLV-FLK or pBLV-AF967, and the production of infectious virus in their supernatants 2 days posttransfection was examined using the syncytium assay. The transfaction of pBLV-AF967 into G4-HA/HeLa cells produced significantly higher titre of virus than transfection into HA/HeLa cells, while the transfection of pBLV-FLK to HA/HeLa and G4-HA/HeLa cells resulted in similar virus titre (Fig. 3a).

Next, another complementation experiment was performed in HeLa cells using a genetically modified clone, pBLV-AF967insRLPL, in which the 12 nt (5’-CGGCTGCCTGCT-3’) of the G4 deletion mutation site were induced into pBLV-AF967 (Table 1). Then, pBLV-AF967 and pBLV-AF967insRLPL were transfected into HeLa cells and the production of infectious viruses 2 days post-transfection was measured with the syncytium assay. The titre of viruses produced by pBLV-AF967insRLPL was significantly higher than that produced by pBLV-AF967 (Fig. 3b). We also examined the influence of the deletion mutation in the G4 gene using clones of pBLV-FLK and two other wild-type proviruses that infected with cow AF019 and AN903 (pvAF019 and pvAN903, respectively). The cow AF019 was kept on the same farm as the cow AF967, in which the proviral load was quickly increased (Fig. 1a), while the cow AN903, which developed EBL, was kept on a different farm. pBLV-AF019 and pBLV-AN903 were constructed from the pvAF019 and pvAN903, respectively (Table 1). The degree of DNA sequence identity of pBLV-AF019 and pBLV-AN903 to that of pBLV-FLK was 97.47 % and 99.24 %, respectively. Neither clone harboured any deletion mutations of the G4 gene. The same deletion mutation of the G4 gene seen in pBLV-
AF967 was induced to pBLV-FLK, pBLV-AF019 and pBLV-AN903, producing clones designated as pBLV-FLK\textsubscript{DRLPL}, pBLV-AF019\textsubscript{DRLPL} and pBLV-AN903\textsubscript{DRLPL}, respectively (Table 1). The same assay as described above was performed and showed that pBLV-FLK\textsubscript{DRLPL}, pBLV-AF019\textsubscript{DRLPL} and pBLV-AN903\textsubscript{DRLPL} produced significantly less virus than their parental clones pBLV-FLK, pBLV-AF019 and pBLV-AN903, respectively (Fig. 3c). Therefore, the deletion of 12 nt in the \textit{G4} gene of these clones resulted in low virus production in the \textit{in vitro} assay system.

**Effect of the deletion mutation on viral transcriptional activity of Tax**

Tax protein is a viral transcriptional activator via interaction with the 5'-LTR of the BLV proviral genome, and is considered to be essential for viral replication and production (Aida \textit{et al.}, 2013). We examined the correlation between presence of the deletion mutation in the \textit{G4} gene seen in pvAF967 virus production and the viral transcriptional activity of Tax. First, pBapo-LTR-Luc together with the parental clones (pBLV-FLK, pBLV-AF967, pBLV-AF019 and pBLV-AN903) or the clones containing the deletion or insertion mutation (pBLV-FLK\textsubscript{DRLPL}, pBLV-AF967\textsubscript{insRLPL}, pBLV-AF019\textsubscript{DRLPL} and pBLV-AN903\textsubscript{DRLPL}) were transfected into HeLa cells, and the viral transcriptional activity was measured using a luciferase assay. No significant difference was observed in the Tax activities of the parental clones and the clones containing the deletion or insertion mutation (Fig. 4), which did not correspond with differences between them in virus production (Fig. 3b, c). In addition, transfection with pBLV-FLK resulted in significantly higher Tax activity than pBLV-FLK transfection (Student’s \textit{t}-test, \textit{P}<0.01), which was completely the opposite result to that for virus production in the \textit{in vitro} assay system. Therefore, the deletion mutation in the \textit{G4} gene
was not associated with effects on viral replication mediated by Tax activity.

DISCUSSION

In the present study, we identified and studied a unique BLV strain pvAF967 that showed a static proviral load. In addition, pBLV-AF019, which showed high levels of virus production, was isolated from a cow with a rapidly elevated proviral load. A genome comparison showed a deletion mutation of 12 nt in the G4 gene of the pvAF967 genome. Moreover, an in vitro assay system using molecular clones showed that this deletion mutation led to low virus production, which did not seem to be caused by a change in Tax activity. Assuming that the virus production in vitro is related to the proviral load in vivo, virus production by a small population of BLV-infected cells during latent BLV infection would be reduced for AF967. Therefore, pvAF967 would not propagate efficiently in vivo because of inefficient virus production.

The G4 protein in BLV and the p13 protein in HTLV-1 are analogous; both localize to mitochondria and bind to farnesyl pyrophosphate synthetase (Lefèbvre et al., 2002), and it has been speculated that they have the same evolutionary function. However, the G4 and p13 proteins have different biological effects on the virus and its hosts. First, based on this study and others, the G4 protein appears to be an enhancer of BLV replication and production (Florins et al., 2000).
2006, 2007), while the p13 protein is a suppressor of HTLV-1 viral replication (Andresen et al., 2011; Bai & Nicot, 2012). Second, the expression of G4 protein with Harvey rat sarcoma viral oncoprotein in the cells possibly leads to transformation in nude mice (Kerkhofs et al., 1998), while the p13 protein can inhibit tumour cell proliferation (Silic-Benussi et al., 2004; D’Agostino et al., 2005; Hiraragi et al., 2005). Thus, although BLV and HTLV-1 are both members of the genus Deltaretrovirus, they may have different evolutionary traits in their host animals. The relationship of BLV with cattle is considered to be antagonistic evolution. Cattle have a defence system against BLV infection: e.g. expression of interferon \( \gamma \) reduces the proviral load (Murakami et al., 2004). Genetic variations in the bovine leukocyte antigen have been shown to be associated with not only resistance to the development of BLV-induced leukemogenesis in an experimental setting (Nagaoka et al., 1999; Aida, 2001) and to persistent lymphocytosis (Xu et al., 1993; Starkenburg et al., 1997; Juliarena et al., 2008) but also to the elevation of proviral load (Miyasaka et al., 2013). In contrast, the BLV may have acquired mechanisms to evade and escape the host defence system through antagonistic evolution with the host, similar to those developed by other retroviruses such as human immunodeficiency virus type 1 (Arien et al., 2007). BLV strains that have lower viral replication and proviral load like the pvAF967 strain is less likely to stimulate the immune system than ordinary BLV strains. This suggests that some BLV strains may have evolved to be symbiotic with cattle.

In the present study, the BLV proviral strain pvAF967, which showed a static proviral load, was identified, and the relationship between its spontaneous deletion mutation and low viral replication was studied. Such deletion mutation may be an evolutionary trait of BLV. We believe that the accumulation of knowledge about BLV variants and properties of virus related to BLV pathogenesis will be useful for future development of BLV control strategies such as new diagnostic systems and attenuated virus vaccines.

**METHODS**

Isolation of serum and genomic DNA from blood. Blood samples were collected from cattle (Holstein Friesian) that were kept on dairy farms in Japan. Serum was prepared from the blood by centrifugation. The genomic DNA was extracted from the whole blood using Wizard Genomic DNA Purification kit (Promega), according to the manufacturer’s instructions.

AGID test. The gel used for the AGID test consisted of 50 mM Tris-HCl (pH 8.6), 0.8% Noble agar and 8.5% NaCl. One central well with six surrounding wells was made in the gel. The BLV antigen and the positive reference serum were placed into the central well and into two symmetrical outer wells. The serum samples were placed into the remaining four wells. After incubation in a moisture chamber at room temperature for 48 h, the precipitation lines indicating the presence of the BLV-specific antibodies were examined by eye. Antibody titres were expressed as the reciprocal of the serum highest dilution that showed a positive reaction, according to previous study (Kono et al., 1981).

Construction of molecular clones and plasmids. The BLV proviral genome was amplified by PCR using PrimeSTAR GXL DNA polymerase (Takara Bio) and cloned into pSMART LC Amp (Lucigen).
The BLV LTR sequence, G4 gene sequence and G4 gene sequence fused with an HA tag sequence were amplified by PCR from the genomic DNA of FLK-BLV. Firefly luciferase gene was amplified by PCR using pCMV-Luc plasmid vector (Promega) as template DNA. The amplified PCR products of LTRs together with those of the firefly luciferase gene, pCMV-Luc plasmid vector (Promega) as template DNA. The amplified PCR products of LTRs together with those of the firefly luciferase gene, the firefly luciferase gene alone, the HA tag alone and HA-tagged G4 were cloned into pBApo-EF1 α–Neo (Takara Bio) to construct pBApo-LTR-Luc, pBApo-EF1α-Luc, pBApo-EF1α-HA and pBApo-EF1α-G4-HA, respectively (Table S1).

The clones and plasmids were validated by sequencing. All the primers used in this study are listed in Table S2.

Cell lines and culture conditions. The FLK-BLV cell line, which is persistently infected with BLV (Van Der Maaten & Miller, 1975), was maintained in Eagle’s minimum essential medium (EMEM; Nissui Pharmaceutical) supplemented with 5% FBS (DS Pharma Biomedical). The HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Nissui Pharmaceutical) supplemented with 10% FBS. FBS and 2.95% tryptose phosphate broth solution was added to EMEM at 5% and 10% concentration, respectively, and the medium was used for the maintenance of CC81 cell line.

Light microscopy. The molecular clones were transfected into CC81 cells using FuGENE HD (Promega). Two days post-transfection, the CC81 cells were washed with PBS. The cells were fixed with absolute methanol for 1 min and then stained with Giemsa stain (Sigma-Aldrich) for 15 min. After washing with water, the cells were observed under a light microscope (IX83, Olympus).

Fluorescence microscopy. CC81 cells were seeded on a 24-well plate (5×10⁴ cells well⁻¹). After 24 h, the molecular clones were transfected into the CC81 cells using FuGENE HD (Promega). Two days post-transfection, the CC81 cells were fixed with 3.6% formaldehyde in PBS for 10 min at room temperature and then permeabilized with PBS containing 0.2% Triton X-100 for 5 min on ice. After washing with PBS, the cells were incubated with PBS containing 5% skimmed milk for 30 min at room temperature. The cells were incubated with anti-p24 mAb (VMRD) in PBS containing 2.5% skimmed milk for 1 h at room temperature. The cells were washed three times with PBS and then incubated with Alexa 488-conjugated anti-mouse IgG polyclonal antibodies (Abcam) in PBS containing 2.5% skimmed milk for 45 min. After washing with PBS three times, the cells were observed under a fluorescence microscope (IX83, Olympus).

Extraction of viral RNA and synthesis of cDNA. Viral RNA was extracted from the culture medium of the HeLa cells transfected with molecular clone, using QIAamp Viral RNA Mini Kit (Qiagen), according to the manufacturer’s instructions. cDNA was synthesized from the viral RNA, using ReverTra Ace qPCR RT Master Mix with gDNA remover (ToyoBo), according to the manufacturer’s instructions.

qPCR. The qPCR to quantify viral and proviral BLV genome was performed for all samples using the 7500 Real-Time PCR system (Applied Biosystems). The primers 5'-GGACAAAATGGACTGCTCAACACG-3' and 5'-CTCCCATCTGTCTTTAAGATTTG-3' and the probe 5'-FAM-C TTCGCCAGACTCGGCGCTTCTCTAM-3' were used to measure the proviral load in BLV culture and viral copy number in the supernatant of clone-transfected HeLa cells. The primers and probes used in this study were designed using the polymerase gene region, which is highly conserved. A standard curve was generated using pBLV-FLK as the template and the viral copy number was calculated.

Syncytium assay. The clones together with pBApo-EF1α-Luc were transfected into HeLa cells using FuGENE HD (Promega). The pBApo-EF1α-Luc vector was used to measure transfection efficiency. Twenty-four hours post-transfection, the cells were washed twice with PBS and the growth medium was replaced. Forty-eight hours post-transfection, the luciferase and the syncytium assays were performed using the cells and the cell supernatants. First, the luciferase activity of the cells was measured to determine transfection efficiency using the luciferase assay system (Promega), according to the instruction manual. Next, the syncytium assay was performed. The CC81 cells had been cultured for 24 h. After debris was removed by centrifugation, the cell supernatants of the HeLa cells transfected with each clone were serially diluted. The CC81 cells were cultured with the serially diluted supernatants supplemented with polybrene at a final concentration 4 µg mL⁻¹. The CC81 cells were cultered until confluent and syncytia were visualized by Giemsa stain and light microscopy. Cells containing more than four nuclei were defined as syncyti. To validate the syncytia induced by BLV infection, it was confirmed that the number of the syncytia was dependent on the serial dilution of the supernatant. The syncytium-forming units were normalized by transfection efficiency. The plasmid vectors used for the experiment are listed in Table S1.

Establishment of stably expression cells. The plasmids pBApo-HA and pBApo-G4-HA were constructed as described in Table S1 and transfected into HeLa cells. The cells were cultured in DMEM containing 10% FBS and 800 µg mL⁻¹ G418 sulfate. The growth medium containing G418 sulfate was replaced every 3 days, and the cells were cultured for 3 weeks.

Immunoblotting. Cells were collected, washed with PBS, dissolved in 1× Laemmli sample buffer and heat denatured. The proteins were separated in a 20% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon, Millipore). The blotted membrane was treated with Tris-buffered saline containing 0.05% Tween 20 (TBST) containing 5% skimmed milk at room temperature for 1 h. After washing with TBST three times, the membrane was incubated with anti-HA mAb (MBL) or anti-β-actin polyclonal antibody (MBL) diluted with TBST containing 3% skimmed milk at 4°C for 16–18 h. After washing with TBST three times with TBST, the membrane was incubated with HRP-conjugated anti-mouse IgG and HRP-conjugated anti-rabbit IgG at room temperature for 1 h, to detect anti-HA antibody and anti-β-actin antibody, respectively. Each antibody was diluted with TBST containing 3% skimmed milk. After washing three times with TBST, detection was performed using Clarity Western ECL Substrate (Bio-Rad Laboratories) according to manufacturer’s instructions. Signals were detected using an LAS 4000 mini (GE Healthcare).

Measurement of viral transcriptional activity. Each clone together with pBApo-LTR-Luc and pTK-RL (Toyo Ink) was transfected into HeLa cells. The pBApo-LTR-Luc and pTK-RL were used to measure Tax viral transcriptional activity and transfection efficiency, respectively. Forty-eight hours post-transfection, the firefly and Renilla luciferase activity in cells was measured using the Pikka Gene Dual Assay Kit (Toyo Ink), according to instruction manual. The viral transcriptional activity and transfection efficiency were assessed by firefly and Renilla luciferase activity, respectively. The firefly luciferase activity was normalized to the Renilla luciferase activity. The plasmid vectors used for the measurement of viral transcriptional activity are listed in Table S1.

Statistical analysis. Statistical analysis was performed with paired or unpaired Student’s t-test using statistical software R version 3.2.0.
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