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Short communication

Bovine leukemia virus G4 enhances virus production

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ABSTRACT

The nonstructural *G4* gene of bovine leukemia virus (BLV) has been thought to function in virus replication. However, the discovery of the *AS1* gene on the antisense strand of the *G4* gene has affected this interpretation. In this study, we investigated the function of G4 in virus production independent of the *AS1* gene using a reverse genetic approach, and briefly examined the association of the G4 protein with Tax, which is also a nonstructural protein that promotes virus replication. First, we constructed a mutant molecular clone of BLV with a nonsense mutation in *G4* that had a minimal effect on the *AS1* gene. Comparison of the wild-type and mutant molecular clones indicated that the nonsense mutation resulted in a reduction of virus in the culture supernatant and accumulation of viral RNA (vRNA) in cells. Moreover, G4 and Tax expression in cells was shown to synergistically enhance virus production. Therefore, we suggest that G4 enhances virus production through abrogation of vRNA accumulation.

Bovine leukemia virus (BLV), which belongs to the family *Retroviridae* genus *Deltaretrovirus*, causes the development of a malignant lymphoma/leukemia in cattle, known as enzootic bovine leukosis (EBL). Although only a small percentage of BLV-infected cattle develop EBL, BLV infection also results in negative effects on the livestock industry by reducing lifetime milk production, reproductive efficiency and lifespan (Brenner et al., 1989; Nekouei et al., 2016; Schwartz and Levy, 1994). BLV randomly integrates into the genomic DNA of peripheral blood cells as a provirus and propagates in infected cattle (Gillet et al., 2007; Murakami et al., 2011). Although virus propagation in BLV-infected cattle is considered to be closely related to the pathogenesis of BLV infection and its transmissibility to other cattle (Jimba et al., 2010; Juliarena et al., 2016; Somura et al., 2014), the propagation mechanism of BLV is not fully understood.

One of the important genes responsible for virus propagation and replication is *G4*, which is encoded on the sense strand of the BLV genome and produces a small nonstructural protein (105 amino acids, molecular weight 11.6 kDa) (Florins et al., 2006; Murakami et al., 2016; Willems et al., 1994). However, a recent transcriptomic study of BLV-infected cells showed that a putative gene, *AS1*, is encoded on the antisense strand of the *G4* gene in the proviral genome (Durkin et al., 2016). Therefore, previous studies did not examine the function of G4 independent of that of AS1 protein. Moreover, it is uncertain whether G4 is related to the nonstructural protein Tax, which plays a critical role in virus replication through activation of viral transcription (Aida et al., 2013; Derse, 1987). In this study, we investigated the role of G4 in virus production using a reverse genetic approach with minimal influence on the *AS1* gene, and briefly examined the importance in virus production of the G4 protein combined with the viral transcriptional activator Tax.

A BLV molecular clone and plasmid were prepared to examine the association of *G4* with virus production. The molecular clone pBLV-AN903, which was previously constructed from a provirus derived from a cow that developed EBL (Murakami et al., 2016), was used in this study. The genome of this BLV molecular clone was designed to impair G4 function with the least possible effect on AS1. Because amino acids 48–51 of G4 (RLPL; Fig. 1) seem to be important for virus production (Murakami et al., 2016), a nonsense mutation upstream of the 48th codon of the *G4* gene will impair G4 protein function. However, the induction of such a nonsense mutation also results in a missense mutation in the *AS1* gene. To ensure that this missense mutation had the least possible influence on AS1, the cytosines at nucleotide positions 7122 and 7123 were replaced with adenine and guanine, respectively (Fig. 1). These changes induced nonsense and missense mutations at the 42nd codon of the *G4* gene and at the 31st codon of the *AS1* gene, respectively. The missense mutation of the *AS1* gene caused an amino acid substitution from glycine to alanine at the 31st residue of the AS1

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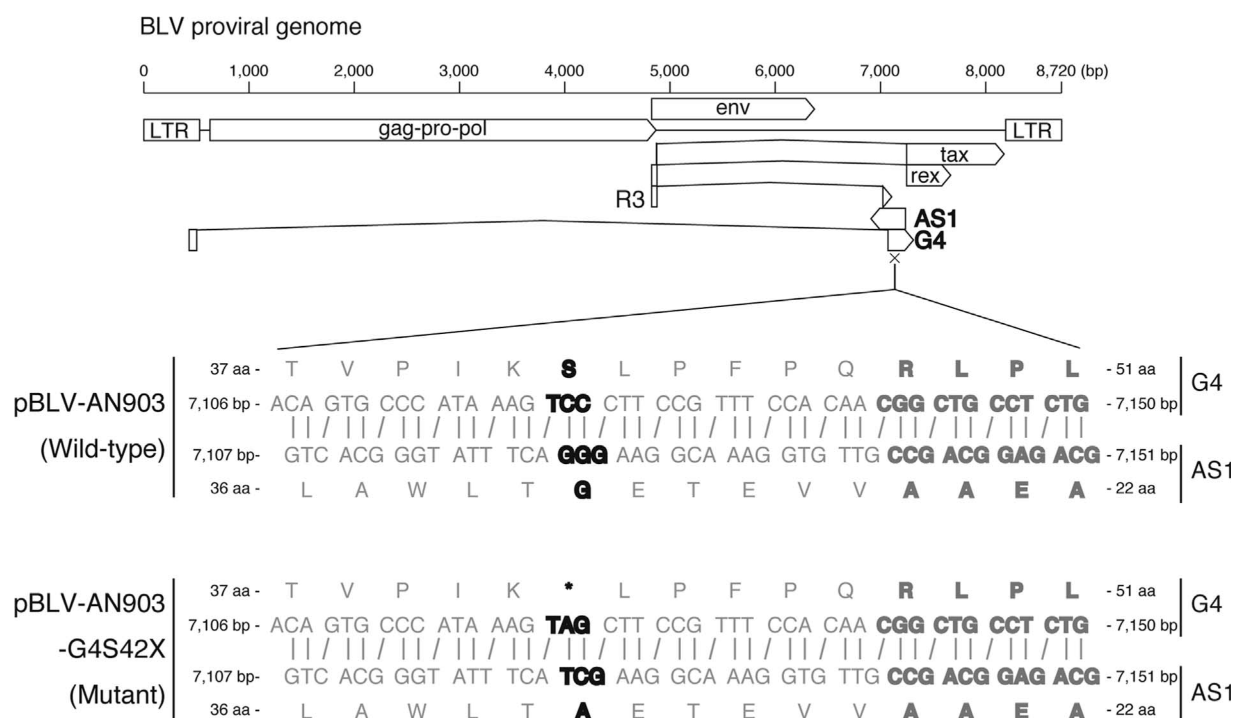

Fig. 1. Mutation induced in the *G4* gene of BLV molecular clone pBLV-AN903. The site of the induced mutation in BLV molecular clone pBLV-AN903. The top panel shows the map of the BLV proviral genome. On the genome map, the right- and the left-facing arrows indicate the genes encoded on the sense and antisense strands, respectively. The induced mutation site overlaps the *G4* and *AS1* genes. The bottom panel shows the DNA and protein sequences around the mutation site of the molecular clone. The sequences important for virus production are shown in bold gray characters. The induced mutation codon is shown in bold black characters. The asterisk indicates the stop codon. The wild-type molecular clone pBLV-AN903 and its mutant molecular clone pBLV-AN903-G4S42X are shown in the upper and lower columns, respectively.

protein. Because glycine and alanine are structurally similar and apolar amino acids, this missense mutation of the *AS1* gene was considered to have minimal effect on the function of the AS1 protein. This mutation was induced in pBLV-AN903 using an In-Fusion HD Cloning Kit (Takara Bio, Shiga, Japan) to construct a mutant molecular clone, pBLV-AN903-G4S42X (Fig. 1). As a control for the transfection efficiency of the clones when conducting experiments on virus replication, we also prepared pTK-Luc, in which the *Renilla luciferase* gene of pRL-TK (Toyo Ink, Tokyo, Japan) was replaced with the *firefly luciferase* gene. Virus replication in culture supernatant and the level of viral RNA (vRNA) in cells were normalized using luciferase activity to correct for the influence of transfection efficiency.

To determine whether virus production was affected by the mutation in pBLV-AN903, we examined virus production using a syncytium assay or quantitative polymerase chain reaction (qPCR). First, pBLV-AN903 or pBLV-AN903-G4S42X together with pTK-Luc were transfected into 293T or HeLa cells. At 24 h posttransfection, the cells were washed twice with Dulbecco's modified Eagle's minimal essential medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan), and the medium was replaced with fresh growth medium (10% fetal bovine serum, 90% DMEM). At 48 h posttransfection, the supernatants of the transfected cells were centrifuged to remove cell debris and were used for a syncytium assay and qPCR as described previously (Murakami et al., 2016). The syncytium assay was performed as follows. Briefly, CC81 cells were cultured with the serially-diluted supernatants in the presence of polybrene (Sigma-Aldrich, St. Louis, MO, USA) at 4 μg/ml in growth media (5% fetal bovine serum and 10% of 2.95% tryptose phosphate broth added to Eagle's minimal essential medium) until confluent. Supernatants from both 293T and HeLa cells transfected with pBLV-AN903 and those transfected with pBLV-AN903-G4S42X formed syncytia in the CC81 cell line. For qPCR, cell debris was removed from the supernatants of the transfected cells, which were then used for vRNA extraction using a High Pure Viral Nucleic Acid Kit (Roche, Penzberg, Germany), according to the manufacturer's instructions. cDNA was synthesized from the vRNA using ReverTra Ace qPCR RT Master Mix with gDNA remover (Toyobo, Osaka, Japan), according to the manufacturer's instructions. The qPCR was performed using primers and a probe against the *polymerase* gene using the cDNA as a template. All statistical analyses in this study were performed using the statistical software R, version 3.3.3 (R Core Team, 2017).

First, we measured the virus titers produced from pBLV-AN903-G4S42X and pBLV-AN903-transfected 293T and HeLa cells. The viruses released from the 293T cells caused the formation of larger and more distinct syncytia than those released from the HeLa cells used in our previous study (Murakami et al., 2016) (Supplementary Fig. S1A, B). Regarding virus production, the supernatants from the pBLV-AN903-G4S42X-transfected 293T and HeLa cells formed significantly fewer than those from pBLV-AN903-transfected 293T and HeLa cells (Fig. 2A, Supplementary Fig. S1C). Therefore, 293T and HeLa cells transfected with pBLV-AN903 and pBLV-AN903-G4S42X showed similar tends for virus production, which demonstrated that the mutation in pBLV-AN903-G4S42X resulted in low virus production in both cell types. In addition, these results showed that the syncytium assay was improved by using 293T cells. Therefore, further studies used 293T cells for analysis of virus replication.

To determine whether the *G4* gene was responsible for virus production, the virus production by the molecular clones was further examined in cells expressing G4 and AS1 proteins. First, the *G4* and *AS1* genes fused with a hemagglutinin (HA) tag sequence at their 3′ end were cloned into the pCAGGS expression vector (Niwa et al., 1991) using cDNA synthesized from fetal ovine kidney cells persistently infected with BLV (FLK-BLV) (Van Der Maaten and Miller, 1975), and pCAGGS-G4-HA and pCAGGS-AS1-HA were prepared. The G4 and AS1 proteins of pBLV-AN903 showed 99.05% and 98.85% identity, respectively, to that derived from FLK-BLV.

Using these plasmids together with the molecular clones, a complementation experiment was performed. The molecular clones and pTK-Luc together with pCAGGS-G4-HA or with empty vector pCAGGS

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