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Avian leukosis virus usurps the cellular SERBP1 protein to enhance its transcription and promote productive infections in avian cells

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ABSTRACT Avian leukosis virus subgroup K (**ALV-K**) is composed of newly emerging isolates, which cluster separately from the well-characterized subgroups A, B, C, D, E, and J in sequence analysis, and exhibits a specific host range and a unique pattern of superinfection interference. Avian leukosis virus subgroup K replicate more slowly in avian cells than other ALV strains, leading to escaped detection during ALV eradication, but the underlying mechanism are largely unknown. In our previous study, we have reported that JS11C1 and most of other suspected ALV-K strains possessed unique mutations in the U3 region. Here, we selected 5 mutations in some important transcriptional regulation elements to explore the possible factor contributing for the lower activity of LTR, including CA-TG mutation in the CAAT box, 21 nt deletion in the CAAT box, A-G and A-T mutations in the CArG boxes, 11 nt insertion in the PRE boxes, and C-T mutation in the TATA box. On the basis of infectious clone of JS11C1, we demonstrated that the 11 nt fragment in the PRE boxes was associated with the transcription activity of LTR, the enhancer ability of U3, and the replication capacity of the virus. Notably, we determined the differential U3-protein interaction profile of ALVs and found that the 11 nt fragment specifically binds to cellular SERPINE1 mRNA binding protein 1 (**SERBP1**) to increase the LTR activity and enhance virus replication. Collectively, these findings reveal that a 11 nt fragment in the U3 gene contributed to its binding ability to the cellular SERBP1 to enhance its transcription and the infectious virus productions in avian cells. This study highlighted the vital role of host factor in retrovirus replication and thus provides a new perspective to elucidate the interaction between retrovirus and its host and a molecular basis to develop efficient strategies against retroviruses.

Key words: avian leukosis virus, U3, mutation, SERBP1, interaction

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INTRODUCTION

Avian leukosis virus (**ALV**) has spread all over the world and causes enormous economic losses in the international poultry industry. Avian leukosis virus has been divided into 11 different subgroups from A to K based on receptor interference patterns, host range, and neutralization by antibodies (Payne et al., 1991; Cui et al., 2014; Payne and Nair, 2012). Among them, ALV subgroup K (**ALV-K**) is composed of newly emerging isolates, which cluster separately from the well-characterized subgroups A, B, C, D, E, J in sequence analysis (Cui et al., 2014), exhibit a specific host range and possess a unique pattern of superinfection interference (Přikryl et al., 2019). In recent years, more ALV-K infection cases have been reported in many countries, and most of the viruses were isolated from local healthy flocks (Dong et al., 2015; Freick et al., 2022). Usually, ALV-K isolates replicate more slowly and are less pathogenic than some other ALV strains (Li et al., 2016; Zhao et al., 2018). However, ALV-K causes subclinical infections leading to growth retardation and immune organ atrophy that may cause immunosuppression, which is often neglected until clinical signs are developed (Cui et al., 2014).

Avian leukosis virus, a member of retroviruses, contains the overall structure of a typical slow-transforming

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replication-competent ALV: 5′-LTR-leader-gag/pol-env-LTR-3′. LTR is located at both ends of the retrovirus genome and plays vital roles in viral replication, pathogenesis and tumorigenesis. It contains powerful transcription regulatory elements that might differ among viruses and determine the host and viral gene expression. Several groups have characterized ALV-K as a low pathogenic ALV mainly due to its LTR, which displayed high similarity with the endogenous counterpart sequence (Li et al., 2016; Li et al., 2022). It was supposed that the viruses with endogenous ALV LTRs may be responsible for lower viral transcription and pathogenicity. Promoter activity of ALV-K derived LTR was significantly lower than that of other exogenous subgroups based on luciferase expression activity (Li et al., 2022). Substitution of ALV-J derived LTR enhanced the replication of ALV-K on avian cell (Li et al., 2022). Therefore, these ALV-K isolates replicate more slowly, results in a low level of viral gene transcription and p27 antigen expression, indicating that they can easily escape detection (Shao et al., 2017; Li et al., 2021). Although it was reported relatively late, ALV-K may have existed in local chickens for a long time and probably as a persistent infection, causing interference to ALV control and eradication.

U3 gene enclosed within LTR is 224-233 nt in length and involves in retrovirus transcription and activation of host proto-oncogenes (Hayward et al., 1981). Mutations in U3 region exert a crucial effect on virus virulence (Tomioka et al., 2004). A 19 nt insertion in the U3 region with an additional CCAAT/enhancer element increased the enhancer activity of the U3 region and the replication capacity of the ALV (Gao et al., 2015). The U3 region contains a variety of cis-acting elements that can interact with transcription factors and other regulatory proteins of host cells, and then drive RNA polymerase II to transcribe the pro-virus and control the transcription rate. Therefore, the profile of U3-interacted host factors might change due to the mutations, especially mutant fragment containing cis-acting elements. Indeed, the DNA-protein interaction profile of endogenous ALV LTRs appears to be different from previously described exogenous virus enhancer binding proteins because of lacking some U3 enhancer domains (Zachow and Conklin, 1992). However, few host factors interacted with U3 gene were reported currently and the related mechanisms utilized by retroviruses to coordinate virus replication are largely unknown (Ruddell, 1995; Ruddell et al., 1988). In this study, we determined the differential U3-protein interaction profile of ALVs and showed that an 11-nt fragment deletion in the U3 gene resulted in loss of the ability to recruit the cellular SERPINE1 mRNA binding protein 1 (**SERBP1**) to enhance its transcription and the infectious virus productions in avian cells. These findings provide a new perspective to elucidate the interaction between retrovirus and its host and might shed light on ALV infection and tumorigenesis.

MATERIALS AND METHODS

Cells and Plasmid

HEK293T and DF-1 cell lines (from ATCC, kept in our lab) were cultivated in Dulbecco's modified Eagle's medium (DMEM; Gibco, California, USA) containing 10% fetal bovine serum (FBS; Gibco, California, USA). Both cell lines used in this study were maintained at 37° C in 5% CO_2. The ALV-K infectious clone was constructed by inserting the proviral DNA of ALV-K isolate JS11C1 into pBluescript II KS(+) backbone. The pGL3-LTR vectors were constructed by cloning the LTR region into the linearized pGL3-basic vector (Promega, Promega, Madison, Wisconsin, USA) at the Kpn I site using the ClonExpress II one-step cloning kit. The pGL3-U3-promoter vectors were constructed by cloning the U3 region into the linearized pGL3-promoter vector (Promega, Promega, Madison, Wisconsin, USA) at the Kpn I site upstream of the SV40 promoter using the ClonExpress II one-step cloning kit. Site-directed mutagenesis and overlap PCR strategies were used to generate mutant LTR and U3 genes. The pRL-TK plasmid (Promega, Madison, Wisconsin, USA) was used as a control plasmid that expresses the Renilla luciferase reporter gene. The CDS of gallus SERBP1 from chicken cells was cloned into vectors pcDNA3.1 with fusion expression of HIS tag and named pcDNA3.1-SERBP1-HIS.

Reverse Genetics

On the basis of the infectious clone of JS11C1 in our previous study (Cui et al., 2014), the viral genome was cleaved into 3 fragments and inserted into the pBlueScript II KS in order using homologous recombination. Overlapping PCR was used to introduce the mutant sites in the U3 gene of LTR at both ends of the genome. To generate the parental and mutant viruses, the plasmid was transfected into DF1 cells with Lipofectamine 3000 (Invitrogen, California, USA) and supplemented with Opti-MEM (Gibco, California, USA). At 5 h post transfection, the medium was replaced with DMEM with 2% FBS. At 7 d post transfection, the supernatant was collected and blindly inoculated into fresh DF-1 cells followed by serial passages. The rescued viruses were all identified by ELISA, IFA, PCR, and sequencing as previously described (Cui et al., 2014).

Viral Growth Kinetics

DF1 cells were seeded in a 6-well plate at 8×10^5 cells per well and inoculated with different rescued ALV viruses at an MOI of 0.01. At 2 h post infection (**p.i.**), the cells were washed with PBS and fresh DMEM with 2% FBS was added to maintain the cells. Two hundred microliters of the infected cell cultures were collected at various time points, and an equal amount of fresh medium was added to each well. The viral titers of the collected supernatants were determined in DF-1 cells

using $TCID_{50}$ and calculated by the Reed-Muench method. The final viral growth curves were constructed with GraphPad Prism 5 software.

Luciferase Reporter Gene Assay

HEK293T / DF-1 cells ($\sim 1 \times 10^5$) were seeded in 24-well dishes and transfected with pGL3-LTR or pGL3-U3-promoter the following day using Lipofectamine 3000 (Invitrogen, California, USA). pRL-TK was cotransfected into the cells in combination with each transfection to normalize the transfection efficiency. Reporter luciferase activity was measured at 48 h post-transfection using the Dual-Luciferase Reporter Assay System (Promega, Madison, Wisconsin, USA) and a Tecan Innite M1000 luminometer (Tecan, Maennedorf, Switzerland) according to the manufacturer's instructions. Data are representative of 3 independent experiments, performed in triplicate.

SPR-MS Analysis

The U3 binding proteins were collected by surface plasmon resonance (**SPR**) performed on a BIAcore 2000 instrument (GE Healthcare, Marlborough, USA) at 25° C as previously described (Lupu et al., 2021). Briefly, biotin oligonucleotides (oligo) 5′-biotin-AATG-TAGCCTTACACAATAGC and 5′-GTTTAT-TAGGTCAAGCCAGGC (25 nmoles) were synthesized by commercial company (Sangon Biotech Co., Ltd., Shanghai, China). The U3 nucleic acid DNA fragments were prepared using the biotinylated oligonucleotides and captured using a streptavidin-coated sensor chip. Only the 5′ ends in the upper strands of U3 gene were biotinylated. DF1 cells collected were lysed with RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris, pH 7.5), and the lysates were centrifuged at 10,000 g for 20 min to obtain the target protein complex. Binding of DF1 proteins to the chip was performed at a flow rate of 20 μL/min in a solution containing 0.01 M HEPES-KOH, pH 7.4, 0.1 M NaCl, 3.4 mM EDTA, 8.4 mM MgCl2, 0.005% (v/v) Surfactant P20. The dissociated U3 binding proteins were finally subjected to MS for identification.

Microscale Thermophoresis Assay

MST measurements were performed to test the direct interaction of SERBP1 with U3 genes using a NanoTemper Monolith NT.115 instrument (NanoTemper Technologies GmbH). SERBP1 protein was expressed in HEK293T cells by transfecting pcDNA3.1-SERBP1-HIS plasmid into the cells. Cells are lysed at 24h to collect protein for Western blotting using anti-HIS monoclonal antibody (Sigma, 1:6000) or labeled with RED-tris-NTA 2nd generation dye using the Monolith NT.115 Protein Labelling Kit for MST analysis. The U3 nucleic acid DNA fragments were amplified and purified using the oligonucleotides without biotin labeling. DNA-protein affinity interaction measurements were performed at 24°C using 40% LED power and 60% microscale thermophoresis power. All experiments were repeated twice for each measurement. Data were analyzed using NanoTemper analysis software.

RNA Interference and CRISPR-Cas9

Small interfering RNA (**siRNA**) was used for transient knockdown of SERBP1. SERBP1 siRNA and control siRNA were purchased from the TSINGKE Biological technology (Qingdao, China) and transfected using Lipofectamine RNAi MAX following the recommendations of the manufacturer. The final concentration of the siRNA was 50 nmol/L and the cells were collected after 24h and 48 h for transfection efficiency determined by RT-qPCR. SERBP1 siRNA sequence1 (**SE1**): 5′-GGAGAAUUCUCUGUUGAUA-3′. SERBP1 siRNA sequence1 (**SE2**): 5′-GAAUGGAAAGCUAUUCAAA-3′. Data are representative of 3 independent experiments, performed in triplicate.

CRISPR-Cas9 mediated ablation of the SERBP1 was achieved with CRISPR-Cas9 RNP (provided by Haixing Bioscience, Suzhou, China) containing expression cassettes for hSpCas9 and chimeric guide RNA. To target exon 1∼exon 10 of the SERBP1, 2 guide RNA sequence of CTGCGCGTTTATTCGCGTGG AGG and CGGA-GAGGAAGGCCCGACGA GGG were selected through the http://crispr.mit.edu website. Plasmid containing the guide RNA sequence was electrotransfected into DF1 cells using Neon transfection system according to the manufacturer's instructions (ThermoFisher Scientific, California, USA). After 2 d, single colonies were transferred into 96-well plates and determined the presence of insertions or deletions in SERBP1 targeted clones using PCR amplification with primers flanking exon and then sequenced by Sanger sequencing (GENEWIZ, Suzhou, China). Clones with mutations in both alleles were selected as gene (−/−) cells and used for downstream studies.

RT-qPCR Assay

Total RNA was isolated using the SPARKeasy Cell RNA Kit (Sparkjade, Jinan, China) according to the manufacturer's protocol. RNA was reversing transcribed using HiScript II Reverse Transcriptase with gDNA wiper (Vazyme, Nanjing, China) and the resulting cDNA was used for RT-qPCR. ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) was used for RT-qPCR. RT-qPCR was performed using LightCycler 96 (Roche, Basel, Switzerland). The following primers were used: SERBP1 forward primer, 5′−ACAGGAGACCTGAGAGACGA−3′; SERBP1 reverse primer, 5′−TCGGTCCAGGATGG GTTTATC−3′; β−actin forward primer, 5′−ATGA AGCCCAGAGCAAAAGA−3′; and β−actin reverse primer, 5′−GGGGTGTTGAAGGTCTCAAA−3′.

Samples were run in duplicate in 96-well plates. Relative gene expression data were analyzed using the $2^{-\Delta\Delta Cq}$ method.

Statistical Analysis

Statistical analysis and figure creation were performed using GraphPad Prism 5.0 (La Jolla, CA). Comparisons between 2 groups were conducted using the unpaired t test. Comparisons among 3 or more groups were conducted using contrasts following 1-way or 2-way ANOVA with statistical significance. Results are expressed as means ± SEM. Values of $P < 0.05$ were considered statistically significant.

RESULTS

The 11 nt Fragment Insertion in the U3 Gene Enhanced the Transcription Activity of LTR and the Enhancer Ability of U3

In our previous study, we have reported that JS11C1 and most of other suspected ALV-K strains possessed unique mutations in the U3 region (Cui et al., 2014). Here, we selected 5 mutations in some important transcriptional regulation elements to explore the possible factor contributing for the lower activity of LTR, including CA-TG mutation in the CAAT box (**EB1**), 21 nt deletion (5′-ATGTAACGATGAGTTAGCAAC-3′) in the CAAT box (**EB2**), A-G and A-T mutations in the CArG boxes (**CB**), 11 nt insertion (5′-GTGGTATGATC-3′) in the PRE boxes (**PB**), and C-T mutation in the TATA box (**TB**). Luciferase reporter gene assay was performed to study the transcription activity of LTR and the enhancer ability of U3. JS11C1 parental LTR and mutant LTRs were cloned into pGL3-basic vector upstream of luciferase gene respectively as shown in Figure 1A. The mutant LTR with 11 nt insertion in the PRE boxes showed the highest promoter activity, which is significantly higher compared with parental LTR. JS11C1 parental U3 gene and mutant U3 genes was clone into pGL3-promoter vector upstream of SV40 promoter and luciferase gene as shown in Figure 1B. The mutant U3 with 11 nt insertion in the PRE boxes showed the highest enhancer activity, which is significantly higher compared with parental U3. Here, we concluded that the 11 nt fragment insertion in ALV strains enhanced the transcription activity of LTR and the enhancer ability of U3.

The 11 nt Fragment Insertion in the U3 Gene Enhanced the Replication of ALV

On the basis of the infectious clone of JS11C1 in our previous study (Cui et al. 2014), we further constructed the infectious clone of PB mutant strain of JS11C1 and rescued the mutant virus via reverse genetics method (Figure 2A). Rescued parental and mutant viruses were

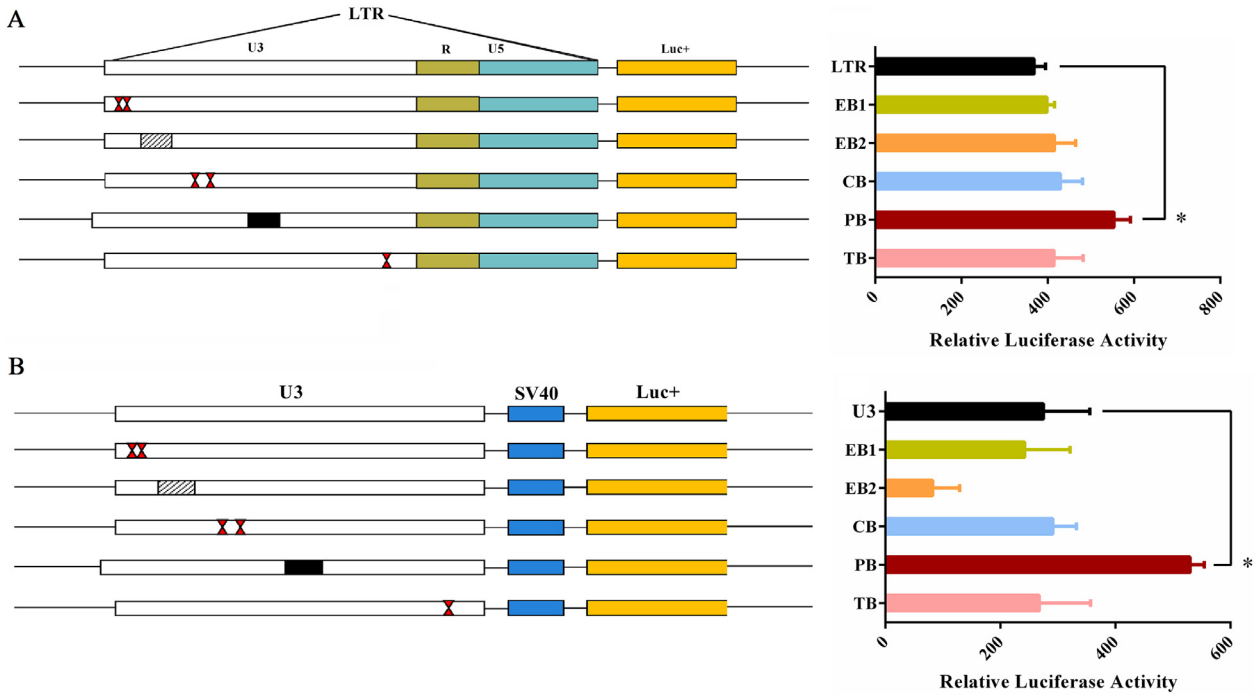

Figure 1. The 11 nt fragment insertion in the U3 gene enhanced the transcription activity of LTR (A) and the enhancer ability of U3 (B). Locations of the mutant sites in U3 gene were indicated in left diagram. EB1 indicates CA-TG mutation in the CAAT box. EB2 indicates 21 nt deletion (5′-ATGTAACGATGAGTTAGCAAC-3′) in the CAAT box, CB indicates A-G and A-T mutations in the CArG boxes, PB indicates 11 nt insertion (5′-GTGGTATGATC-3′) in the PRE boxes. TB indicates C-T mutation in the TATA box. JS11C1 parental LTR and mutant LTRs was clone into pGL3-basic vector upstream of luciferase gene. JS11C1 parental U3 gene and mutant U3 genes was clone into pGL3-promoter vector upstream of SV40 promoter and luciferase gene. Luciferase activity reporter plasmid pGL3-LTR or pGL3-U3-promoter were cotransfected with reference plasmid pRL-TK. Reporter luciferase activity was measured at 48 h post-transfection. Data are representative of 3 independent experiments. Error bars represent ± SEM for triplicate experiments.

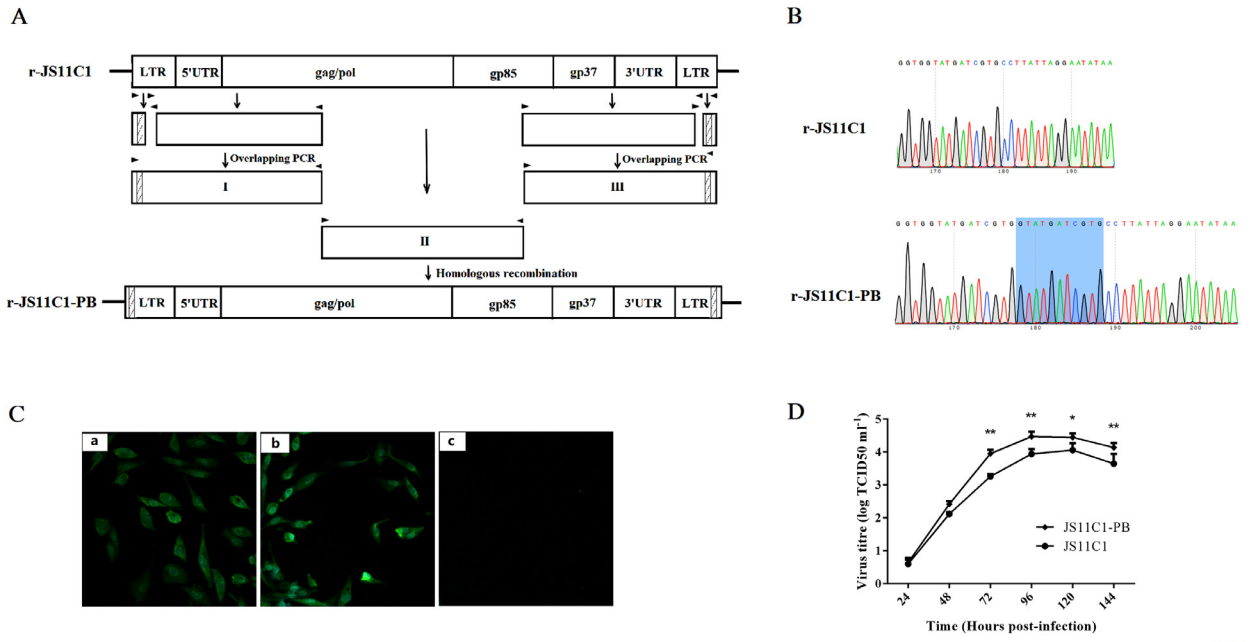

Figure 2. The 11 nt fragment insertion in the U3 gene enhanced the replication of ALV. (A) Strategy for construction of the mutant ALV with the 11bp fragment deletion in the U3 gene. The viral genome was cleaved into 3 fragments and inserted into the pBlueScript II KS in order using homologous recombination. Overlapping PCR was used to introduce the mutant sites in the U3 gene of LTR in fragment I and fragment III. The plasmid was transfected into DF1 cells to generate the parental and mutant viruses. At 7 d post transfection, the supernatant was collected and blindly inoculated into fresh DF-1 cells followed by serial passages. U3 genes of the viruses were amplified for sequence verification of the mutant site (B). (C) IFA verification for the rescued viruses. Parental (a) or mutant viruses (b) were inoculated into DF-1 cells at an MOI of 0.01 respectively, and the mock-infected cells (c) were used as control. The cells were fixed at 48 h post-infection and incubated with anti-gp85 polyclonal antibody as the primary antibody and FITC conjugated goat anti-rat antibody (Sigma) as secondary antibody. (D) Growth curves of parental and mutant viruses. The viruses were inoculated into DF-1 cells at an MOI of 0.01, and the virus-containing supernatants were collected at the indicated time points for virus titration. Error bars represent ± SEM for triplicate experiments.

confirmed by sequencing of U3 gene (Figure 2B) and IFA assay using anti-gp85 polyclonal antibody (Figure 2C), respectively. Replication capacity of parental and mutant viruses was compared on DF1 cells. The PB mutant virus replicated faster and showed significantly higher virus titers at 72, 96, 120, and 144 h p.i than the JS11C1 parental virus (Figure 2D). Therefore, the 11 nt fragment insertion in the U3 gene enhanced the replication of ALV in avian cells.

The 11 nt Fragment Binds to SERBP1 to Increase Virus Replication in Avian Cells

Differential binding host proteins between U3 with/without the 11 nt fragment deletion were identified by employing surface plasmon resonance (**SPR**) spectroscopy using BIAcore technology. Collectively, there were 70 host proteins bind to both parental and mutant U3 genes (Figure 3A). Specially, there were 109 host proteins bind to mutant U3 gene with the SERBP1 possessed the highest score among the nuclear proteins (Figure 3B). We therefore performed MST measurements to confirm the biospecific interactions of SERBP1 protein with different U3 genes. SERBP1 protein was expressed in high level at 24h after transfecting pcDNA3.1-SERBP1-HIS plasmid into HEK293 cells as verified by anti-HIS monoclonal antibody (Figures 4A and 4B). SERBP1 specifically binds to mutant U3 with the Signal to Noise value of more than 5, whereas there was no interaction signal between SERBP1 and parental U3 (Figure 4C). The same results were observed between the 2 repeats, indicating consistency and reliability between the MST experiments, which confirmed the interaction between U3 gene and SERBP1 identified in SPR assays.

To test the role of SERBP1 in ALV replication, 2 SERBP1 siRNA (Figure 5A) were firstly synthesized to knock down the expression of SERBP1 in DF1 cells. The expression level of SERBP1 was 66.4% and 57.5% lower than the control group at 24h and 48h after transfecting siRNA (Figure 5B). Then, we tested the role of SERBP1 on the transcription activity of LTR, the enhancer ability of U3 and the replication of ALV at different points. Knockdown of SERBP1 significantly reduced the transcription activity of LTR and the enhancer ability of U3 with the 11 nt fragment (Figures 5F and 5G), whereas there were no obvious influence on the parental LTR and U3 activities (Figures 5C and 5D). The result of virus replication dynamics is similar. Interfering with the expression of SERBP1 inhibits the replication of PB mutant virus (Figure 5H), whereas there was no influence on the parental JS11C1 virus (Figure 5E).

Ablation of the SERBP1 was further achieved with the CRISPR-Cas9 knockout system to obtain the SERBP1 knockout (ΔSE) DF1 cells. Primers flanking exon 1 or exon 10 could amplified an 806 nt or 697 nt

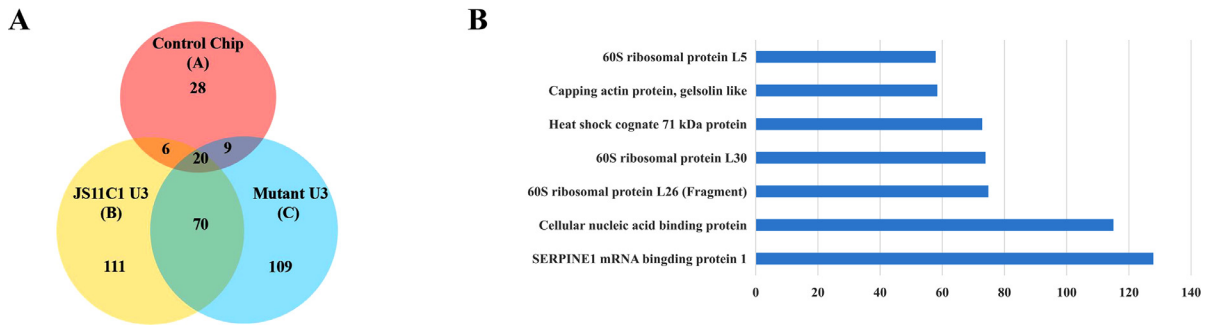

Figure 3. The 11 nt fragment deletion leads to differential profile of U3 binding proteins. (A) Summary of the U3 binding proteins identified by SPR-MS. (B) Nuclear proteins that specially bind by U3 gene with the 11 nt fragment deletion (Score > 50).

fragment in wild type (**WT**) DF1 cells, while they were negative in the ΔSE DF1 cells. Meanwhile, only the 728 nt length fragment was amplified in the ΔSE DF1 cells using the primers located at the upstream of exon 1 and downstream of exon 10 respectively, which verified the gene (−/−) cells were achieved (Figure 6A). The ablation of a 16,164 nt fragment in the genome was confirmed further by Sanger sequencing (Figure 6B). PB mutant virus exhibited a dramatic reduce of gp85 protein transcription and virus replication in DF1ΔSE cells over time (Figures 6E and 6F), whereas there were no obvious influence on those of parental JS11C1 virus (Figures 6C and 6D). Viral protein transcription and replication of PB mutant virus is restored (Figures 7B and 7C) when SERBP1 knockout cells are reconstituted with avian SERBP1 (Figure 7A). Hence, we concluded that ALV usurps the cellular SERBP1 protein by the 11 nt fragment in the

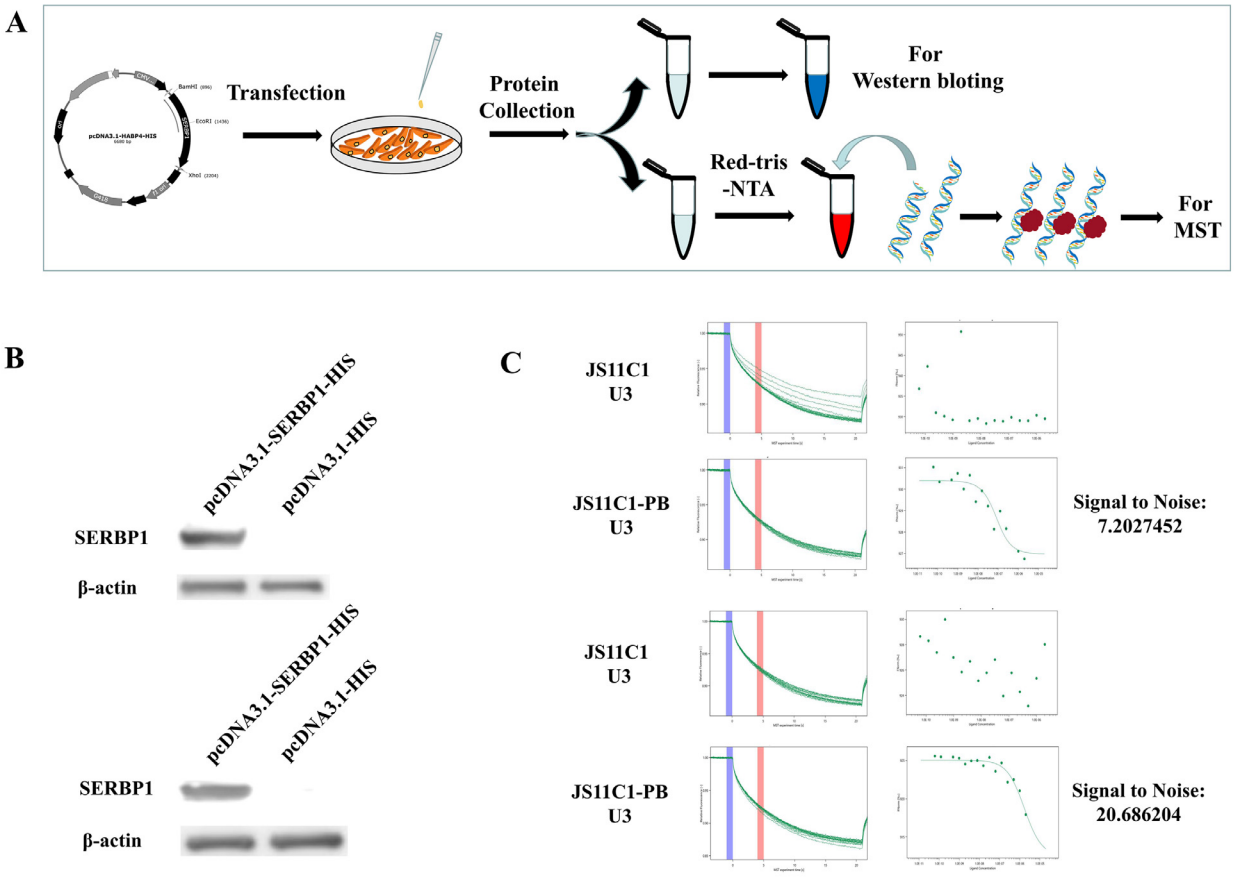

Figure 4. SERBP1 specially binds to the 11 nt fragment in the U3 gene. (A) Flowchart for validation the interaction of U3-SERBP1. SERBP1 protein was fusion expressed with his-tag in HEK293T cells by transfecting pcDNA3.1-SERBP1-HIS plasmid into HEK293 cells. Cells are lysed at 24h to collect protein for Western blotting using anti-HIS monoclonal antibody (B) or subjected MST analysis (C). In the MST assays, over-expressed SERBP1-His fusion protein was labeled with RED-tris-NTA 2nd generation dye. The U3 nucleic acid DNA fragments were amplified, purified and serially diluted at a 2-fold dilution. Interaction of SERBP1 with U3 was determined with the cutoff of the Signal to Noise value of more than 5. All experiments were repeated twice times for each measurement.

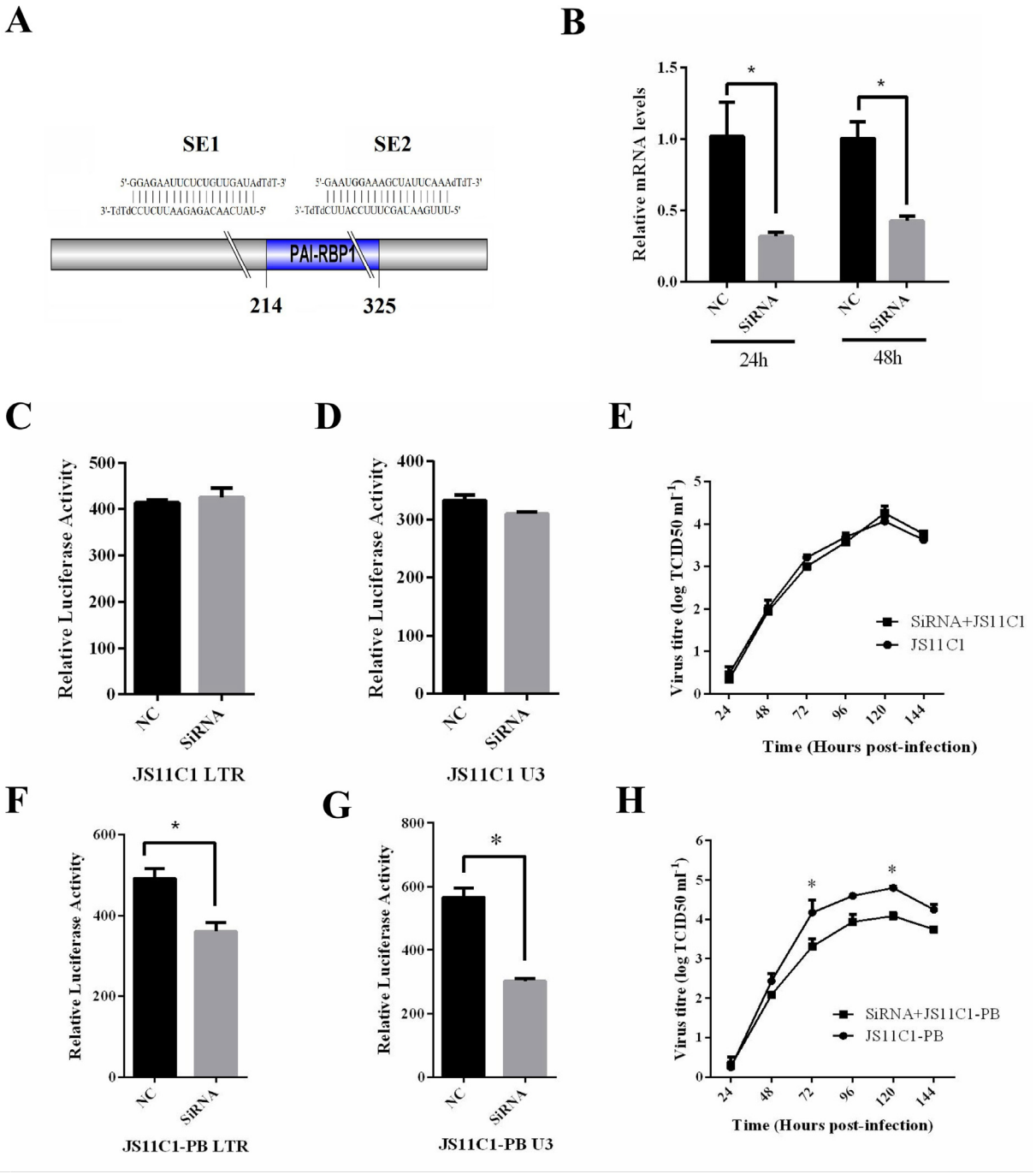

Figure 5. Knockdown of SERBP1 reduced the LTR activity and inhibited ALV replication in DF1 cells. (A) Location and sequence of SERBP1 siRNA. (B) siRNA-mediated knockdown of SERBP1 in DF-1 cells was performed by transfecting the SERBP1 siRNA into DF1 cells at the final concentration of 50 nmol/L and the cells were collected after 24h and 48 h for transfection efficiency determined by real-time PCR. NC, negative control. Error bars represent ± SEM for triplicate experiments. DF-1 cells were then transfected with JS11C1 parental LTR-mediated reporter (C), U3-mediated enhancer reporters (D), mutant LTR-mediated reporter (F), or mutant U3-mediated enhancer reporters (G). Cells were harvested and lysed for assessment of luciferase activity at 48h post transfection. Replication of JS11C1 parental virus (E) and mutant virus (H) in control and siRNA-mediated knockdown of SERBP1 in DF-1 cells. The viruses were inoculated into DF-1 cells at an MOI of 0.01, and the virus-containing supernatants were collected at the indicated time points for virus titration. Error bars represent ± SEM for triplicate experiments.

U3 gene to enhance its transcription and promote productive infections in avian cells.

DISCUSSION

Retrovirus LTR contains powerful transcription regulatory elements that might differ among viruses and determine the host and viral gene expression (Ruddell, 1995), although the molecular basis of this process is unclear. In this study, we found that the 11 nt fragment in the U3 gene of LTR specifically binds to SERBP1 protein to enhance ALV replication in avian cells via increasing the transcription activity of LTR and the enhancer ability of U3, representing a novel mechanism employed by U3 gene to modulate the virus replication.

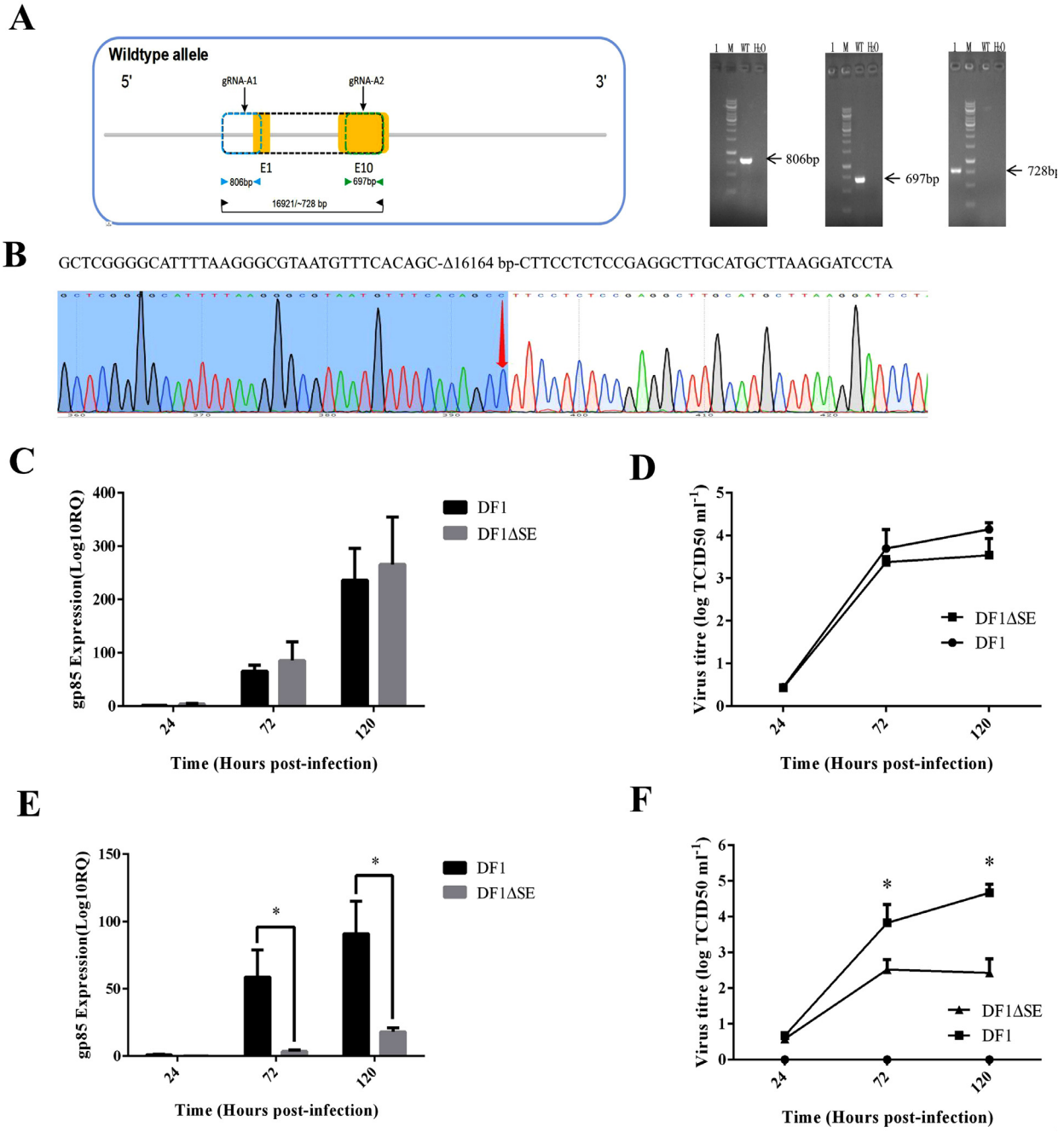

Figure 6. Knockout of SERBP1 dramatically reduced the gp85 protein transcription and virus replication in DF1 cells. CRISPR-Cas9 mediated ablation of the SERBP1 gene was achieved with CRISPR-Cas9 RNP containing expression cassettes for hSpCas9 and chimeric guide RNA (gRNA-A1 and gRNA-A2). Single colonies were transferred into 96-well plates and determined the presence of insertions or deletions in SERBP1 targeted clones using PCR (A) and then sequenced by Sanger sequencing (B). Clones with mutations in both alleles were selected as gene (−/−) cells and used for downstream studies. Gp85 expression and replication of JS11C1 parental virus (C, D) and mutant virus (E, F) were tested on the SERBP1-knockout (ΔSE) and wild type (WT) DF1 cells respectively. Error bars represent ± SEM for triplicate experiments.

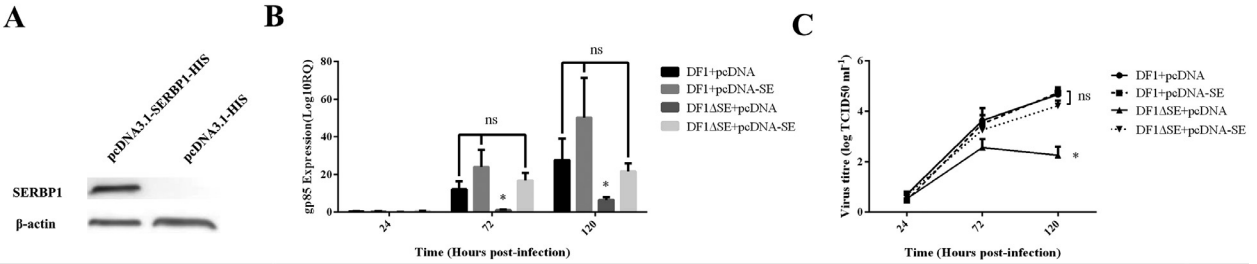

Figure 7. Expression completation of SERBP1 restored the gp85 expression and replication of S11C1 mutant virus in ΔSE DF1 cells. Expression completation of SERBP1 was achieved by transfecting pcDNA3.1-SERBP1-HIS plasmid into the DF1ΔSE cells and verified by Western blotting (A). Gp85 expression (B) and replication (C) of JS11C1 mutant virus were tested respectively.

ALV-K strains were shown previously that replicated more slowly than some other ALV strains and the endogenous-like ALV LTRs may be responsible for lower viral transcription (Li et al., 2016; Zhao et al., 2018; Li et al., 2022). U3 portion of the viral LTR contains a strong enhancer, which is required for high-level expression from the viral promoter and also able to augment transcription from various heterologous promoters. During evolution, retroviruses showed extremely great genetic diversity due to the high error rate of their polymerases and a high recombination rate. Indeed, our previous study showed that most of the supposed ALV-K isolates possess coincident mutations and deletions in the U3 gene (Cui et al., 2014). Therefore, we firstly tested the effect of these unique mutations and deletions on LTR activities. We observed that the 11 nt fragment deletion decreased the transcription activity of LTR and the enhancer ability of U3, while other mutations have no significant effect. The U3-associated enhancer is also an important contributor to cellular transformation, since it is responsible for the increased transcription of cellular oncogenes adjacent to sites of provirus integration. Interestingly, a unique 11 nt deletion was also observed in the U3 gene of partial ALV-J strains associated with hemangioma (Shi et al., 2011). Online service analysis in previous studies showed that the 11 nt deletion in ALV resulted in the loss of the AIB REP1 box transcription factor, implying that the 11 nt deletion is associated with the LTR activities (Liu et al., 2021). The AIB REP1 box is a regulatory factor of the human gene Apo-AI, which is associated with hepatocellular carcinoma with portal vein tumor thrombus in human (Xu et al., 2011). It further suggested that the 11 nt deletion might also play a vital role in oncogenesis of ALVs.

In fact, multiple cis-acting elements in the U3 gene of retroviruses can utilize host polymerase and translation system to regulate transcription initiation. An understanding of host cellular components that mediate the activity of U3 gene is important for elucidating the mechanism for efficient retroviral transcription and retrovirus-mediated tumorigenesis (Tang et al., 2022). SERBP1, the focus of the present study, was found to specifically interact with U3 gene that possessed the 11 nt fragment insertion and contributed virus replication in host cell. SERBP1 (synonyms: CGI-55, CHD3IP, HABP4L, PAI-RBP1, SERPINE 1) is an mRNA-binding protein. With today's knowledge of SERBP1, it is suggested that the protein is associated with a plethora of important cellular processes separate from its action on mRNA (Colleti et al., 2019; Brugier et al., 2022). It has been well established that SERBP1 interacts with a number of transcriptional regulators, reducing its transcriptional activity by blocking its DNA binding (Nery et al., 2006). The U3 region contains a variety of cis-acting elements that can interact with transcription factors and other regulatory proteins of host cells to control the transcription rate. For example, the avian p53 inhibits ALV replication through binding to the U3 region of the promoter (Duan et al., 2022). Combined our study, the SERBP1 might enhance the LTR activity via binding to U3 region directly or alleviate the inhibitory activity of transcriptional regulators like p53 on LTR activity via forming protein complex with them to coordinate its transcriptional activity. In another aspect, chromatin conformation of genomic regions targeted by retroviral integration can change the gene expression patterns, which is also an important factor for retroviral replication and viral pathogenesis (Collora and Ho, 2023). SERBP1 interact with the chromatin remodeling protein, which regulates gene transcription (Lemos et al., 2003). It is high possible that the U3-SERBP1 interaction may change the chromatin remodeling and further affect ALV replication. In particular, SERBP1 was implicated in tumorigenicity via regulating mitotic cell cycle and apoptosis as well as the aforementioned gene transcription/translation regulation and RNA recognition (Colleti et al., 2019). Chromatin remodeling is also important for the maintenance of genomic stability and plays important roles in tumor growth (Sadikovic et al., 2008). Meanwhile, SERBP1 was reported to interacted with cellular oncogenes including c-fos (Colleti et al., 2019). Proto-oncogene c-fos is frequently involved in ALV-induced tumors, as the proviral integration of slow-transforming ALVs in the host genome could activate cellular proto-oncogenes through enhancer or promoter insertions to develop tumors (Collart et al., 1990). Therefore, although the exact mechanisms are still unknown, considering the function of U3 gene and SERBP1 protein, the interaction complex may also involve in in ALV induced tumors as well as viral reproduction.

Collectively, our study established the SERBP1 as a novel cellular factor required for efficient ALV infection for the first time. Furthermore, we demonstrated that the 11 nt fragment in the U3 gene specifically binds to SERBP1 to enhance virus replication in avian cells via increasing the LTR activity. The subtle pattern of U3-SERBP1 interaction may provide a novel prospective to underlying ALV infection and tumorigenesis.

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DISCLOSURES

The authors declare no conflicts of interest.

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