

**Comparative analysis of the 5' UTR-mediated translational
regulation of X/P mRNA among avian orthobornaviruses
using reverse genetics**

Meng-Chi Wu

Abstract

Bornaviruses are non-segmented, negative-sense RNA viruses causing neurological disease in mammalian and avian hosts. Because they uniquely replicate in the nucleus and establish persistent infections, precise control of viral protein production is critical. Viral replication depends on polymerase activity, which is regulated by two proteins: the X protein, a suppressor, and the phosphoprotein (P), a cofactor. Notably, both proteins are encoded by a single X/P mRNA. In mammalian bornaviruses such as Borna disease virus 1 (BoDV-1, clade-1), the 5' untranslated region (5' UTR) of this mRNA contains regulatory elements that control the translational balance between X and P, thereby supporting efficient replication. In avian bornaviruses (ABVs), which divide into two groups, clade-2 and clade-3, their X/P mRNAs differ markedly in 5' UTR length. Clade-2 viruses possess long 5' UTRs containing stem-loop elements, whereas clade-3 viruses harbor short 5' UTRs lacking such elements. Despite this divergence, the functional consequences for translational regulation and viral replication remain unclear.

To address this, I investigated the role of the 5' UTR in X/P mRNA in regulating X and P expression and viral replication in clade-2 and clade-3 ABVs. I established reverse genetics systems for clade-2 viruses, including parrot bornavirus 5 (PaBV-5) and aquatic bird bornavirus 1 (ABBV-1), and exchanged the X/P 5' UTRs between clade-2 viruses and the clade-3 PaBV-4. Replacing the native 5' UTR in clade-2 viruses with a clade-3 sequence impaired viral growth, whereas introducing a clade-2 5' UTR into clade-3 PaBV-4 did not affect replication. Mechanistically, clade-2 chimeric viruses carrying a short clade-3 5' UTR showed disrupted X and P expressions and nuclear retention of viral proteins, which were associated with delayed viral spread.

Together, these findings reveal that while clade-2 viruses strictly require a long 5'

UTR to control X and P translation, clade-3 viruses replicate independently of such regulatory elements. This study highlights the evolutionary divergence in translational control among bornaviruses.

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Abbreviation

293T	Human Embryonic Kidney 293T
ABBV-1	Aquatic bird bornavirus 1
ABV	Avian bornavirus
bp	Base pair
BoDV-1	Borna disease virus 1
CnBV-1	Canary bornavirus 1
cDNA	Complementary DNA
DAPI	4', 6-Diamidino-2-phenylindole
dpc	Days post-coculture
DDX21	DEAD-box RNA helicase 21
DMEM	Dulbecco's Modified Eagle Medium
GOI	Gene-of-interest
G	Glycoprotein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gRNA	Genomic RNA
hpt	Hours post-transfection
IFA	Immunofluorescence assay
IFIT	Interferon-induced protein with tetratricopeptide repeats
kb	Kilobase
L	Large polymerase protein
Le	Leader
M	Matrix protein
mRNA	Messenger RNA

MuBV-1	Munia bornavirus 1
N	Nucleoprotein
ns	Not significant
nt	Nucleotides
ORF	Open reading frame
P	Phosphoprotein
PaBV	Parrot bornavirus
qPCR	Quantitative polymerase chain reaction
QM7	Quail muscle clone 7
QM7-T	QM7 cells expressing the simian vacuolating virus 40 T antigen
QT6	Quail fibroblast-like cells
QT6-Blast	Blasticidin-resistant QT6 cells
QT6-T	QT6 cells expressing the simian vacuolating virus 40 T antigen
RT	Reverse transcription
Rz	Hammerhead ribozyme
Tr	Trailer
UTR	Untranslated region
VSBV-1	Variegated squirrel bornavirus 1
vRNP	Viral ribonucleoprotein
WT	Wild-type
X	Accessory protein
μg	Microgram
μm	Micrometer

Chapter 1

Literature Review and Introduction

1.1 Virological characteristics of orthobornaviruses

Orthobornaviruses are a group of viruses classified in the genus *Orthobornavirus*, family *Bornaviridae*, order *Mononegavirales* [1-3]. They are enveloped viruses with a roughly spherical morphology, with approximately 80-100 nm in diameter [4, 5] (**Fig. 1A**). Their genome is a nonsegmented, single-stranded, negative-sense RNA of about 9 kilobase (kb), encoding at least six open reading frames (ORF) in the 3' to 5' order: nucleoprotein (N), accessory protein (X), phosphoprotein (P), matrix protein (M), glycoprotein (G), and large polymerase protein (L) [6-8] (**Fig. 1B**). In addition, the genome termini contain sequences essential for regulating transcription and replication, namely the 3' leader (Le) and the 5' trailer (Tr).

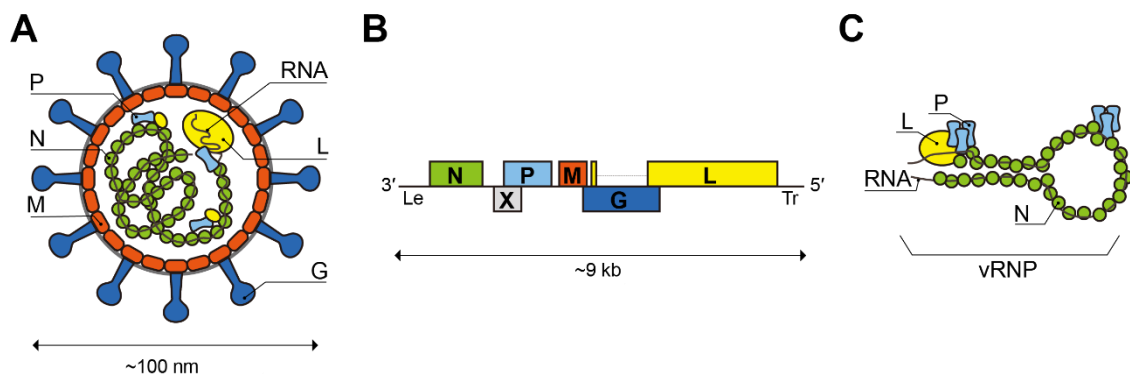


Figure 1. Morphological and genomic features of orthobornaviruses.

(A) Schematic illustration of the orthobornavirus virion. (B) Schematic diagram of the orthobornavirus genome organization. (C) Schematic illustration of the viral ribonucleoprotein (vRNP) complex.

As Borna disease virus 1 (BoDV-1) is the prototypic member of the genus *Orthobornavirus*, current understanding of orthobornavirus biology is primarily derived from studies of BoDV-1. The viral ribonucleoprotein (vRNP), composed of N, P, and L proteins together with the genomic and antigenomic RNAs, is responsible for transcription and replication (**Fig. 1C**) [9-11]. The M and G proteins mediate viral

assembly and entry [12-15]. X is a nonstructural protein that negatively regulates the polymerase activity of BoDV-1 through its interaction with P [16-19]. Distinguished from most RNA viruses, orthobornaviruses transcribe and replicate in the nucleus [20]. Another key characteristic is their ability to establish persistent infection without causing overt cytopathic effects [20-24]. Moreover, viral spread is predominantly cell-associated, occurring via cell division or direct cell-to-cell contact, with only very small amounts of virions being released from infected cells into the supernatant [25, 26]. During mitosis, vRNPs tether to host chromosomes and are distributed to daughter cells, thereby maintaining persistent infection even in the dividing cells [20, 25].

1.2 Classification and the host range of orthobornaviruses

Orthobornaviruses have been documented to infect a wide range of vertebrate species, including mammals and avians [27-29]. Currently, the genus contains at least 25 viruses belonging to 9 different viral species [1-3]. Phylogenetically, orthobornaviruses can be classified into four clades [30, 31] (**Fig. 2**). Clade-1 comprises mammalian bornaviruses such as BoDV-1 and variegated squirrel bornavirus 1 (VSBV-1). Clade-2 and clade-3 include avian-infecting viruses: clade-2 comprises non-psittacine viruses such as aquatic bird bornaviruses (ABBV) and canary bornaviruses (CnBVs), and also includes parrot bornavirus 5 (PaBV-5); clade-3 comprises most psittacine viruses such as PaBV-2 and PaBV-4, which are highly pathogenic in parrots [32, 33]. Clade-4 includes reptile-derived viruses, such as Loveridge's garter snake virus 1 (LGSV-1).

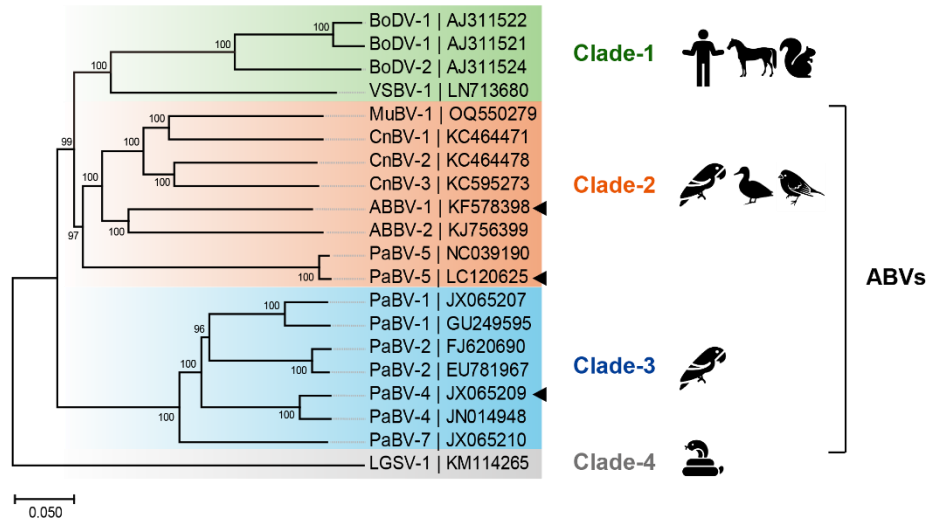


Figure 2. Phylogenetic classification and host range of orthobornaviruses.

Phylogenetic tree based on whole-genome nucleotide sequences excluding 5' and 3' terminal regions. Representative viruses from each clade are shown, with their accession numbers. The tree was constructed using the neighbor-joining method with the Jukes-Cantor model and 10,000 bootstrap replicates in MEGA12. Viruses analyzed in this study are marked with arrowheads. Major host species for each clade are illustrated on the right. Clade-2 and -3 viruses constitute the avian bornaviruses (ABVs). This figure was adapted with modifications from Reference 31 (Fig. 1). Note that PaBV-3, -6, and -8 were excluded due to the unavailability of complete genome sequences.

1.3 Genetic diversity and pathogenicity of avian bornaviruses

For several decades, BoDV-1 was believed to be the sole member in the family *Bornaviridae* (now reclassified within the genus *Orthobornavirus*). This paradigm shifted in 2008 when bornavirus-like sequences were detected in psittacines suffering from proventricular dilatation disease (PDD) [34, 35]. Since then, at least 15 genetically distinct viruses have been identified across diverse avian hosts, all of which cluster phylogenetically within *Orthobornavirus* clade-2 or clade-3 (**Fig. 2**) [29]. In this thesis, the term “avian bornaviruses” (ABVs) is used as a collective designation for these avian orthobornaviruses, although the unified species name “avian bornavirus” was formally

abandoned to reflect the distinctness of these viral species [1-3].

Among ABVs, parrot bornaviruses (PaBVs; PaBV-1 to -8) are the most clinically important viruses, as they are strongly associated with PDD in companion parrots [29, 36, 37]. PaBVs infection is typically progressive and often fatal, presenting with neurological signs (ataxia, seizures, behavioral changes) and/or gastrointestinal dysfunction (weight loss, undigested seeds, proventricular dilation) [29, 38, 39]. Experimental infections have confirmed PaBV-2 and PaBV-4 as causative agents of PDD in cockatiels [32, 33, 40-42], whereas the pathogenicity of other PaBVs remains unclear. Notably, PaBV-5 has also been associated with atypical signs such as feather-picking [43]. Epidemiological studies show that clade-3 PaBV-4, followed by PaBV-2, is the predominant virus in PDD-affected parrots worldwide [44-46]. In contrast, clade-2 viruses such as PaBV-5 have been reported only sporadically, mainly in East Asia, Turkey, and the United States [30, 31, 43, 46-48]. Although clade-2 PaBV-5 and clade-3 PaBV-4 appear to differ in their geographic distribution and pathogenic outcomes, the molecular mechanisms underlying these differences remain unclear. This limited understanding of the virological properties of PaBVs has hampered the development of effective antivirals and vaccines against PaBVs [49-53].

In addition to psittacine ABVs, non-psittacine viruses occupy clade-2 and exhibit variable pathogenicity across hosts [29]. For example, canary bornaviruses (CnBV-1 to -3) circulate widely in captive canaries but generally cause subclinical or mild disease [54, 55]. In contrast, aquatic bird bornavirus 1 (ABBV-1) has been linked to neurological disorders across a broad host range, including waterfowl, chickens, and turkeys [56-59].

1.4 Transcription of orthobornaviruses

Orthobornaviruses transcribe their viral mRNAs via the stop-start mechanism, directed by conserved transcription start (S) and termination/polyadenylation (T) signals [60]. This mechanism, followed by the subsequent processing of primary transcripts, yields at least five major capped and polyadenylated mRNAs, including those encoding N, X/P, M, G, and L [8, 22, 24] (**Fig. 3**).

A monocistronic mRNA encodes the N protein, whereas a bicistronic mRNA encodes the overlapping X and P proteins. The downstream M, G, and L proteins are translated from a set of transcripts initiated at the third transcription start site (S3). Transcripts terminated at T3 produce the M and G proteins, whereas read-through transcripts that bypass T3 and terminate at T4 produce the L protein. Notably, expressions of G and L require subsequent processing by the host splicing machinery [8, 9, 61-63].

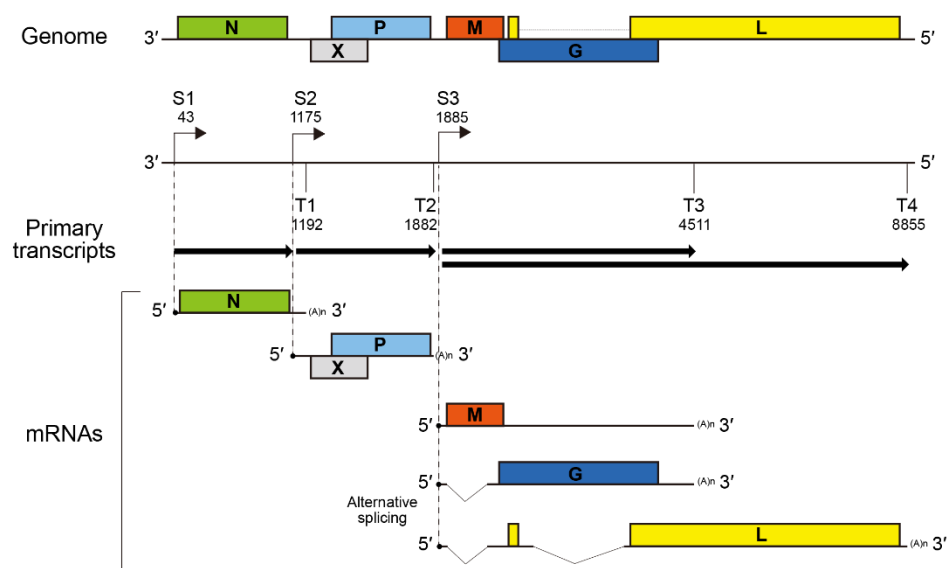


Figure 3. Subgenomic transcript map of orthobornaviruses.

Capped and polyadenylated mRNAs are transcribed from the orthobornavirus genome. The nucleotide positions shown for the transcription start (S) and stop (T) sites correspond to the BoDV-1 genome (GenBank accession number, NC001607). This figure was adapted with modifications from Reference 1 (Fig. 2).

1.5 Translational control of X/P mRNA and clade-specific features in its 5' UTR

Bicistronic X/P mRNA encodes two proteins with opposing effects on polymerase activity: P, an essential polymerase cofactor, and X, which negatively regulates polymerase activity through its interaction with P [16-19]. In BoDV-1 (clade-1), the expression ratio of X and P is tightly controlled in mammalian cells by regulatory elements within the 5' untranslated region (5' UTR), including a secondary stem-loop structure and an upstream ORF (uORF) [64] (**Fig. 4**). Generally, the 5' UTR is defined as the sequence extending from the transcription start site to the start codon of the X ORF. In cases where a uORF is present, the uORF sequence initiates within the 5' UTR and may overlap with the X start codon or extend into the main coding region. Such uORFs can interrupt the translation of the main ORF, so they are called 'out-of-frame overlapping uORFs' [65, 66]. In the early phase of BoDV-1 infection, a predicted stem-loop in the 5' UTR recruits the host DEAD-box RNA helicase 21 (DDX21) and stabilizes its secondary structure. This interaction inhibits termination-coupled ribosomal reinitiation at the X start codon, which overlaps with the uORF stop codon. Consequently, translation of P is facilitated, while suppressing translation of X (**Fig. 4A**).

As infection progresses, accumulating P alters DDX21 phosphorylation status, weakening its binding to the 5' UTR and thereby allowing X translation to increase [64] (**Fig. 4B**). This accumulation of X subsequently drives the export of P from the nucleus to the cytoplasm [64] (**Fig. 4A, B**). While relocalization of P in response to increasing X expression appears to be conserved among orthobornaviruses [67], the molecular mechanisms by which ABVs control the translational balance between X and P in avian cells remain largely unexplored.

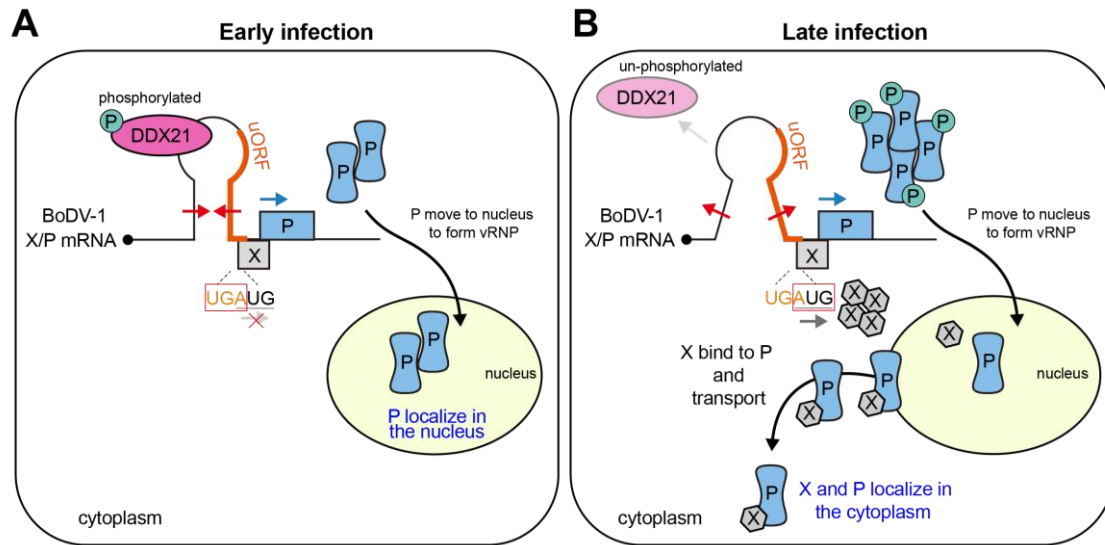


Figure 4. The model of the autogenous regulation of BoDV-1 X/P mRNA translation.

(A) During early infection, phosphorylated DDX21 binds the 5' UTR of the X/P mRNA, hindering recognition of the X start codon and reducing termination-coupled reinitiation after scanning through the stop codon of upstream ORF (uORF). At this stage, P localizes mainly to the nucleus. (B) As P accumulates, it interferes with DDX21 phosphorylation, leading to its detachment from the 5' UTR and allowing more efficient ribosomal reinitiation at the X AUG codon. Increased X leads to relocalization of P to the cytoplasm. This figure was adapted with modifications from Reference 64 (Fig. 10).

Despite similar genome organization among orthobornaviruses, the 5' UTRs of the X/P mRNA display distinct differences in length and structure between clades [43, 67, 68] (**Fig. 5A-D**). Similar to clade-1, clade-2 ABVs possess long, structured 5' UTRs (>50 nucleotides; nt) containing predicted stem-loops and uORFs [64] (**Fig. 5A, B**). By contrast, clade-3 ABVs feature significantly shorter, unstructured 5' UTRs (<30 nt) that lack these regulatory motifs (**Fig. 5A, B**). Quantitatively, the 5' UTRs in clade-1 and clade-2 viruses can reach nearly 90 nt, approximately three times the length of those in clade-3 viruses (**Fig. 5C**). Furthermore, the putative uORFs within these sequences are predicted to encode peptides ranging from 8 to 24 amino acids (**Fig. 5D**). This structural divergence suggests that the translational control of X and P likely differs between clades.

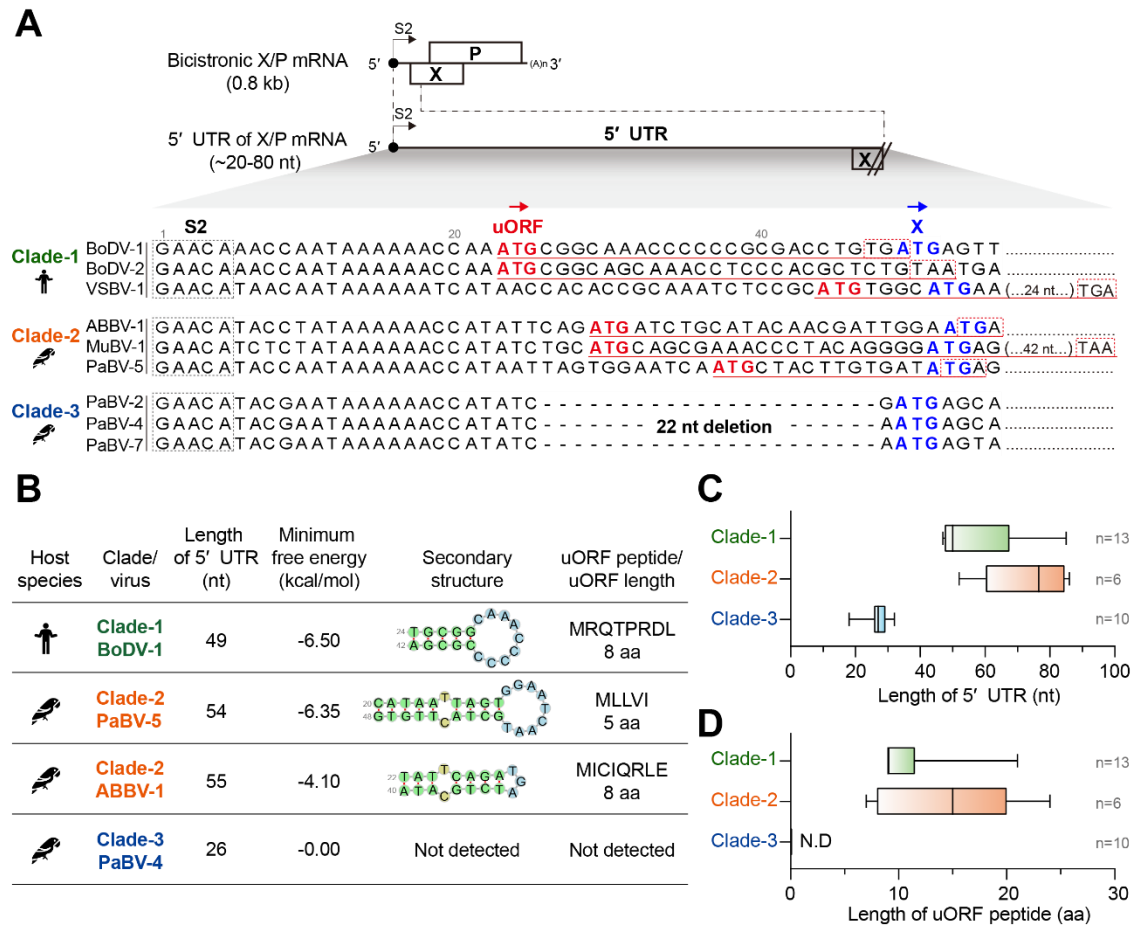


Figure 5. The clade-specific features of the 5' UTR of the X/P mRNA.

(A) Schematic of the 5' UTR of the X/P mRNA. Alignments of representative clades show S2 (black dashed box), putative uORFs (start codons in bold red, coding regions underlined, stop codons boxed), and X start codons (bold blue). (B) Summary of 5' UTR features for BoDV-1 (clade-1), PaBV-5 and ABVV-1 (clade-2), and PaBV-4 (clade-3), including UTR length, predicted structures, minimum free energy (MFE), and putative uORF peptides. (C) Boxplot comparison of 5' UTR length across clades (sample sizes shown in right). (D) Boxplot comparison of uORF peptide length across clades (sample sizes shown in right).

1.6 Reverse genetics in molecular virology

While forward genetics screens for the genetic basis of a specific phenotype, reverse genetics elucidates gene function by introducing defined mutations and characterizing the resulting phenotypic changes [69]. Facilitated by advances in sequencing and molecular cloning, reverse genetics has evolved into a cornerstone of modern virology, allowing the

de novo generation of infectious viruses entirely from cloned cDNA [70].

Generating viruses from genetic material dates to 1957, when T2 phage DNA was shown to be infectious [70, 71]. For DNA and positive-sense RNA viruses, reverse genetics is straightforward because transfection of full-length cDNA or RNA initiates replication [70]. By contrast, negative-sense RNA viruses resisted this approach because naked genomic RNA is noninfectious and must be assembled into a vRNP complex. This barrier was overcome in 1994 by rescuing recombinant rabies virus through co-transfection of plasmids encoding proteins needed for vRNP formation [72]. This strategy enabled the subsequent rescue of recombinant BoDV-1 (rBoDV-1) in 2005 [73] (**Fig. 6A**).

Today, reverse genetics serves as an indispensable platform for dissecting viral replication dynamics and pathogenesis. Beyond basic research, it supports translational applications, including the development of vaccines, antiviral therapeutics, and viral vectors for gene therapy [74-76].

1.7 Reverse genetics for avian bornaviruses

To date, reverse genetics systems for ABVs have been established only for the clade-3 PaBV-4 [62], adapted from the BoDV-1 platform [76] (**Fig. 6A**). Briefly, the full-length viral antigenomic cDNA is flanked by a 5' hammerhead ribozyme (HamRz; Rz) and a 3' hepatitis delta virus ribozyme (HdvRz; δ). This cassette is transcribed from an RNA polymerase II-driven CAG promoter and terminated by a polyadenylation signal (**Fig. 6B**). To rescue recombinant PaBV-4 (rPaBV-4), this antigenomic plasmid is cotransfected with helper plasmids encoding N, P, L, M, and G into QT6 cells expressing the SV40 T antigen (QT6-T) (**Fig. 6A, C**). The transfected cells are then cocultured with blasticidin-resistant QT6 cells (QT6-Blast) and serially passaged for viral propagation. Rescue is

confirmed by detecting viral antigen-positive cells, even at low infection levels (**Fig. 6A**). Besides, inserting green fluorescent protein (GFP) or another gene-of-interest (GOI) between the P and M genes produces a reporter virus [14, 62] (**Fig. 6B**).

However, despite the availability of complete genome sequences for several clade-2 ABVs, no reverse genetics systems have been established, which limits direct comparisons of replication and pathogenesis between clade-2 and clade-3 viruses.

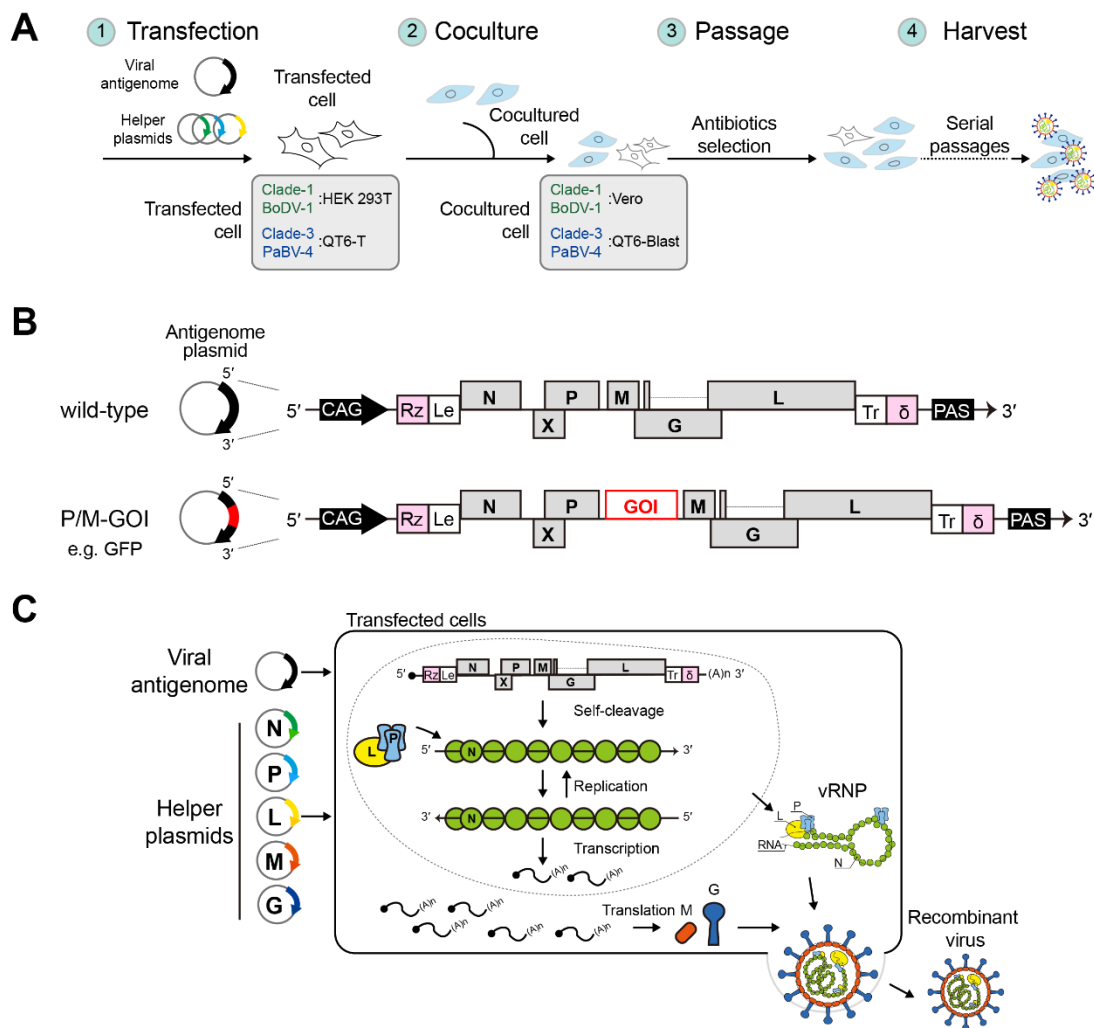


Figure 6. The reverse genetics systems for mammalian and avian bornaviruses.

(A) Schematic workflow of reverse genetics for BoDV-1 and PaBV-4. (B) Configuration of the bornavirus antigenome plasmid. Rz, 5' hammerhead ribozyme; δ , 3' hepatitis delta virus ribozyme; CAG, RNA polymerase II-driven CAG promoter; PAS, polyadenylation signal; GFP, green fluorescent protein; GOI, gene-of-interest. (C) Illustration of the generating recombinant bornavirus after plasmid transfection.

1.8 Research rationale and objectives of the study

Although translational regulation of the bicistronic X/P mRNA has been characterized in BoDV-1 (clade-1) [64], how ABVs regulate the translation of X and P remains largely unknown. In BoDV-1, structural elements within the 5' UTR tightly control the relative expression of X and P, a balance essential for maintaining persistent infection. However, ABVs show remarkable clade-specific differences in their 5' UTR of X/P mRNA: clade-2 viruses possess long, structured 5' UTRs, whereas clade-3 viruses contain short, unstructured 5' UTRs [43, 67, 68] (**Fig. 5**). The impact of these divergent architectures on translational control and viral replication remains unclear.

In this doctoral study, I aimed to clarify the molecular basis of clade-specific translational regulation of X and P proteins and its impact on viral replication between clade-2 and clade-3 orthobornaviruses. I first assessed the translational control using transient expression plasmids. Next, to investigate the functional significance of the 5' UTR of X/P mRNA during infection, I established reverse genetics systems for clade-2 ABVs. Using clade-2 and clade-3 reverse genetics platforms, I generated chimeric viruses by exchanging the 5' UTR of X/P mRNA between clades and compared their growth kinetics with those of the corresponding wild-type viruses. To further explore the mechanisms underlying 5' UTR exchange, I analyzed viral protein expression, RNA synthesis, and subcellular localization. Collectively, this study provides insights into clade-specific translational regulatory strategies among orthobornaviruses.

Chapter 2

Results

2.1 5' UTR-dependent translation in PaBV-4 and PaBV-5 X/P mRNA

To assess the impact of clade-specific 5' UTRs on X/P mRNA translation, I placed the clade-3 PaBV-4 or clade-2 PaBV-5 X and P coding regions downstream of native or heterologous 5' UTRs (**Fig. 7A and 8A**).

Following transfection into QM7 cells expressing the SV40 T antigen (QM7-T) cells, PaBV-4 constructs yielded comparable X and P protein levels regardless of whether the wild-type PaBV-4 or PaBV-5 5' UTR was used, resulting in consistent X-to-P ratios at 18 hours post-transfection (hpt) (**Fig. 7B**). RT-qPCR confirmed comparable mRNA levels across conditions (**Fig. 7C**). These findings indicate that the translational control of the clade-3 PaBV-4 X/P mRNA is insensitive to these specific 5' UTR variations.

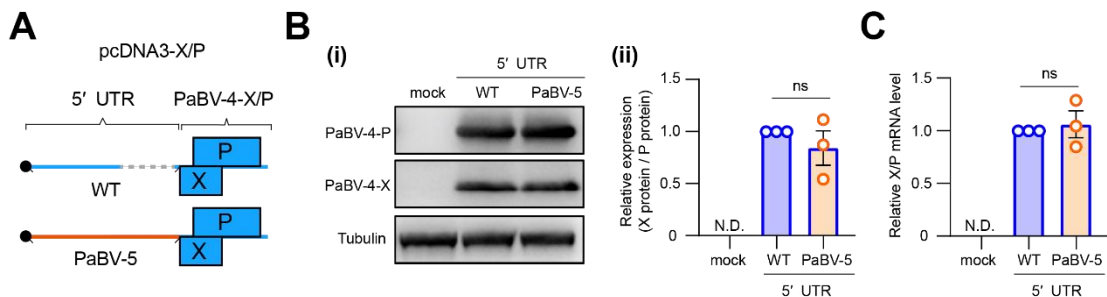


Figure 7. Translation of PaBV-4 X/P in a plasmid-based assay.

(A) Schematic of PaBV-4 X/P constructs with either the wild-type (WT) PaBV-4 or PaBV-5 5' UTR. (B) QM7-T cells were transfected with 0.6 μ g of each PaBV-4 X/P plasmid. (i) X and P protein expression at 18 hpt detected by Western blotting. (ii) X-to-P protein ratios quantified from band intensities using ImageJ. (C) X/P mRNA levels at 18 hpt measured by RT-qPCR from total RNA. Data represent the means $\pm$ standard errors of the means ($\pm$ SEM) from three independent experiments. Statistical significance was determined by an unpaired *t*-test (B, C); ns, not significant.

In contrast, PaBV-5 X/P constructs carrying the PaBV-4 5' UTR exhibited significantly reduced X protein levels, while P expression remained comparable to the wild-type PaBV-5 construct (**Fig. 8B**). Consequently, the X-to-P ratio decreased

approximately two-fold at 18 hpt (**Fig. 8B**). RT-qPCR showed comparable mRNA levels between the wild-type- and PaBV-4-5' UTR plasmids (**Fig. 8C**), indicating that this change in the X-to-P ratio arose from translational rather than transcriptional regulation. These results indicate that PaBV-5 X translation is critically dependent on the integrity of its native 5' UTR.

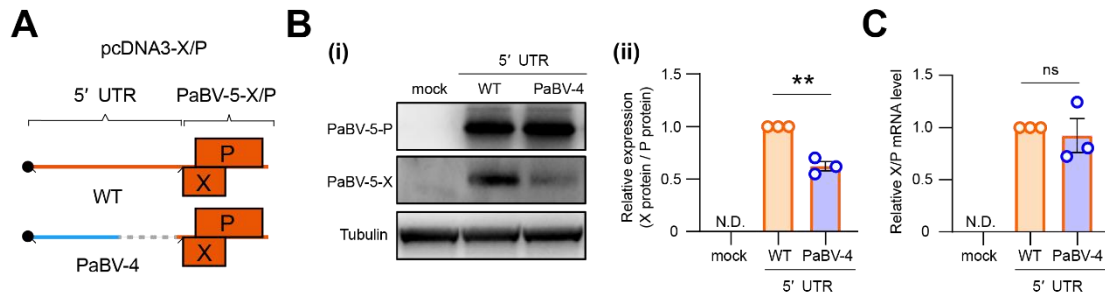


Figure 8. Translation of PaBV-5 X/P in a plasmid-based assay.

(A) Schematic of PaBV-5 X/P constructs with either the wild-type (WT) PaBV-5 5' UTR or PaBV-4 5' UTR. (B) QM7-T cells were transfected with 0.6 μ g of each PaBV-5 X/P plasmid. (i) X and P protein expression at 18 hpt detected by Western blotting. (ii) X-to-P protein ratios quantified from band intensities using ImageJ. (C) X/P mRNA levels at 18 hpt measured by RT-qPCR from total RNA. Data represent the means $\pm$ SEM from three independent experiments. Statistical significance was determined by an unpaired *t*-test (B, C); ns, not significant; ** $p < 0.01$.

2.2 Development of reverse genetics systems for clade-2

ABVs

To examine the role of the 5' UTR of X/P mRNA in viral replication, I established reverse genetics systems for two clade-2 viruses, PaBV-5 and ABBV-1. PaBV-5 represents psittacine viruses, whereas ABBV-1 represents non-psittacine (waterfowl) viruses. By adapting protocols developed for clade-3 PaBV-4 and clade-1 BoDV-1 [62, 76], I successfully rescued recombinant wild-type PaBV-5 (WT-rPaBV-5) and ABBV-1 (WT-rABBV-1) (**Fig. 9A-C**).

Briefly, 293T cells were cotransfected with the full-length viral genomic plasmid and five helper plasmids (encoding N, P, L, M, and G) [76] and then cocultured with QT6-Blast cells [62] (**Fig. 9A**). The cocultures were passaged every 3 to 4 days, and viral propagation was monitored by immunofluorescence assay (IFA) for over one month. By 36 days post-coculture (dpc), both wild-type rPaBV-5 and rABBV-1 had spread throughout the culture (**Fig. 9B, C**).

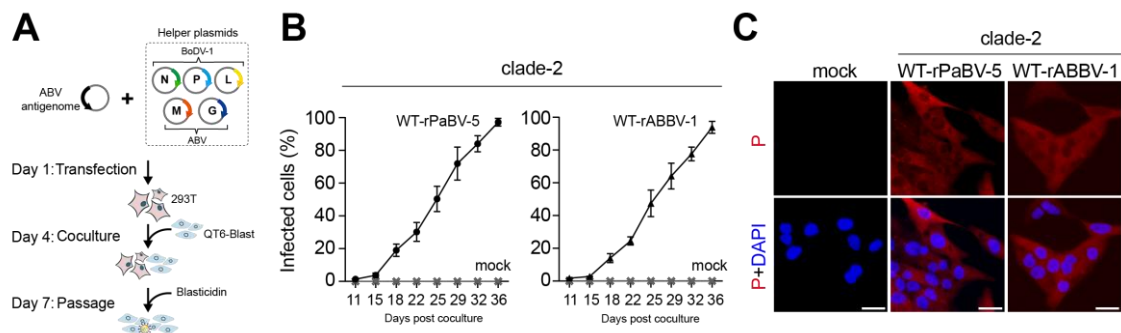


Figure 9. Establishment of reverse genetics systems for clade-2 ABVs.

(A) Schematic overview of the workflow for rescuing clade-2 ABVs. (B) Spread of wild-type rPaBV-5 (WT-rPaBV-5; left) and rABBV-1 (WT-rABBV-1) infection in cocultured QT6-Blast cells at the indicated days post-coculture (dpc). Cells were passaged every 3 or 4 days, and the proportion of infected cells was determined by IFA. Data represent the mean $\pm$ SEM from three independent experiments. (C) Representative IFA images at 36 dpc showing infection with WT-rPaBV-5 and WT-rABBV-1. Anti-BoDV-1 P antibodies were used. Scale bar, 25 μ m.

2.3 Reciprocal exchange of the 5' UTR of the X/P mRNA impairs replication of PaBV-5 but not PaBV-4

To assess the effect of the X/P mRNA 5' UTR on viral replication, I generated chimeric recombinant viruses by reciprocally exchanging the native 5' UTRs between clade-3 PaBV-4 and clade-2 PaBV-5. For the clade-3 virus, both wild-type PaBV-4 (WT-rPaBV-4) and the chimeric virus carrying the PaBV-5 5' UTR (chimeric-rPaBV-4) were

successfully rescued (**Fig. 10A, B**).

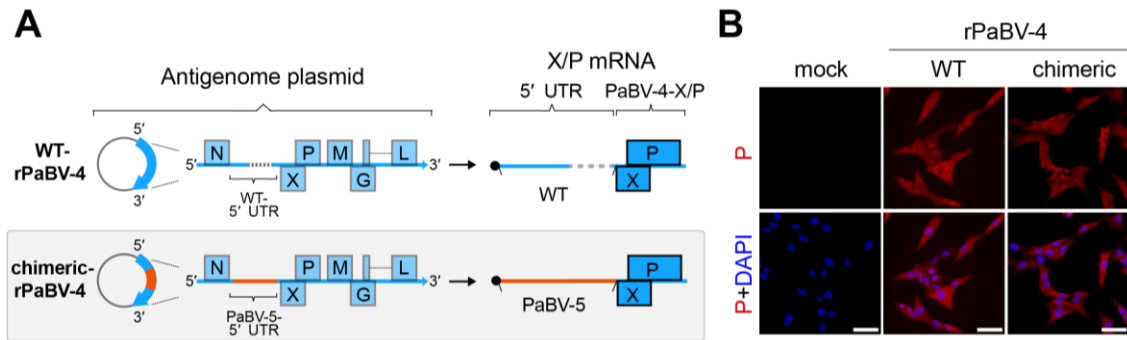


Figure 10. Rescue of wild-type- and chimeric-rPaBV-4.

(A) Schematic diagrams of antigenome plasmids for wild-type (WT)-rPaBV-4 and chimeric-rPaBV-4. The corresponding X/P mRNAs with the indicated 5' UTRs are shown on the right. (B) Rescue of WT- and chimeric-rPaBV-4 in QT6 cells, confirmed by IFA. Representative images at 36 dpc are shown. P protein was detected with an anti-BoDV-1 P antibody (red), and nuclei were counterstained with DAPI (blue). Scale bar, 50 μ m.

To evaluate viral growth kinetics, uninfected and infected QT6 cells were cocultured at the ratio of 95 to 5 and passaged twice per week (**Fig. 11A**). Over around one month, wild-type-rPaBV-4 and chimeric-rPaBV-4 exhibited indistinguishable growth curves (**Fig. 11B**) and yielded similar viral titers (**Fig. 11C**). These findings indicate that PaBV-4 replication is largely independent of the 5' UTR origin in the X/P mRNA.

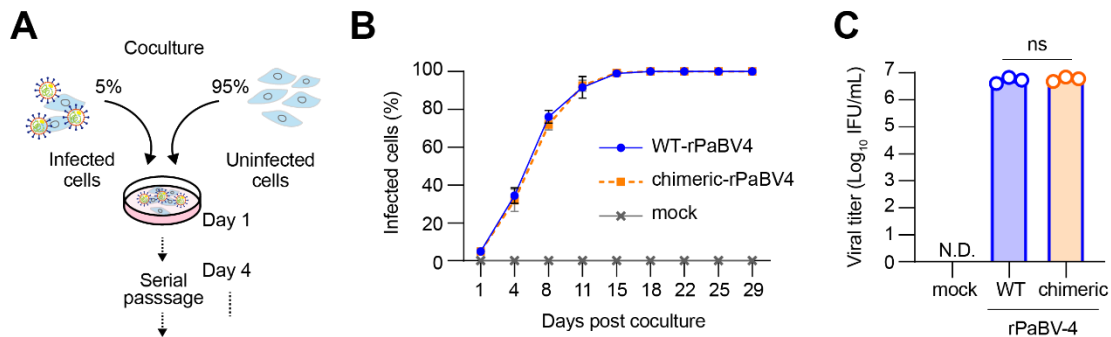


Figure 11. Growth kinetics and titers of wild-type- and chimeric-rPaBV-4.

(A) Schematic of the growth kinetics assay. Infected cells were mixed with uninfected cells at a 5:95 ratio. Cocultures were serially passaged every 3 or 4 days. (B) Growth kinetics of wild-type (WT)-rPaBV-4 and chimeric-rPaBV-4 carrying PaBV-5 5' UTR in QT6 cells. The infected ratio was determined by IFA when

cells were passaged. (C) Infectious titers of rPaBV-4s. Viruses rescued at 29 dpc were titrated on QT6 cells. Data represent the means $\pm$ SEM from three independent experiments. Statistical significance was determined by two-way ANOVA with Tukey's multiple-comparison test (B), and unpaired *t*-test (C); ns, not significant.

Next, I examined clade-2 PaBV-5. Both wild-type-rPaBV-5 and the chimeric virus carrying the PaBV-4 5' UTR (chimeric-rPaBV-5) were successfully rescued (**Fig. 12A, B**). In contrast to clade-3 viruses, the clade-2 chimera with PaBV-4 5' UTR exhibited a severe replication defect. When uninfected and infected QT6 cells were cocultured at the ratio of 95 to 5 and passaged twice per week, chimeric-rPaBV-5 showed markedly delayed growth kinetics compared to the wild-type-rPaBV-5 (**Fig. 13A**). Furthermore, even after reaching 100% infection, viral titer recovered from chimeric-rPaBV-5 were approximately 100-fold lower than that of wild-type-rPaBV-5 (**Fig. 13B**). These findings indicate that PaBV-5 replication is critically dependent on the integrity of its native long 5' UTR in the X/P mRNA.

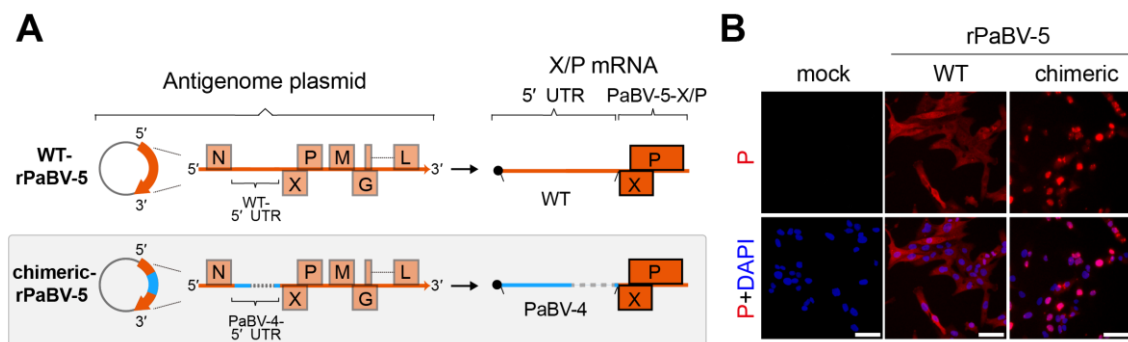


Figure 12. Rescue of wild-type- and chimeric-rPaBV-5.

(A) Schematic diagrams of antigenome plasmids for wild-type (WT)-rPaBV-5 and chimeric-rPaBV-5. The corresponding X/P mRNAs with the indicated 5' UTRs are shown on the right. (B) Rescue of WT- and chimeric-rPaBV-5 in QT6 cells, confirmed by IFA. Representative images at 36 dpc are shown. P protein was detected with an anti-BoDV-1 P antibody (red), and nuclei were counterstained with DAPI (blue). Scale bar, 50 μ m.

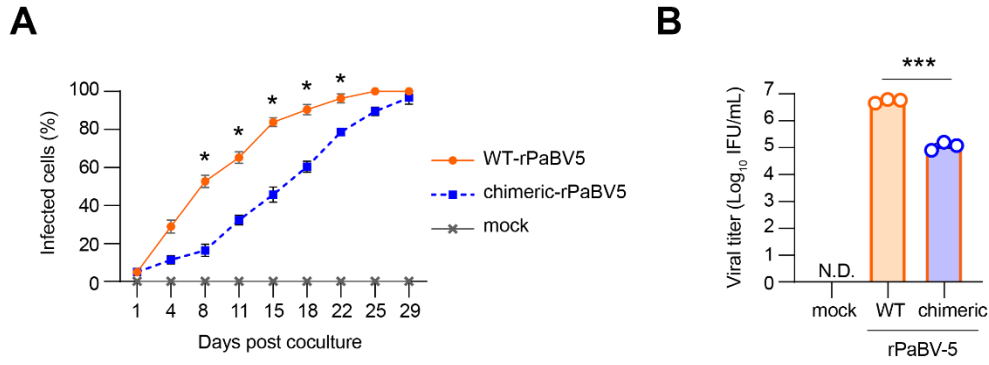


Figure 13. Growth kinetics and titers of wild-type- and chimeric-rPaBV-5.

(A) Growth kinetics of wild-type (WT)-rPaBV-5 and chimeric-rPaBV-5 carrying PaBV-4 5' UTR in QT6 cells. Infected and uninfected QT6 cells were cocultured at a 5:95 ratio, passaged every 3 or 4 days, and the infected ratio was determined by IFA. (B) Infectious titers of rPaBV-5s. Viruses rescued at 29 dpc were titrated on QT6 cells. Data represent the means $\pm$ SEM from three independent experiments. Statistical significance was determined by two-way ANOVA with Tukey's multiple-comparison test (A), and unpaired *t*-test (B); ns, not significant; * $p < 0.05$; *** $p < 0.001$.

2.4 The long 5' UTR of X/P mRNA is required to maintain the translational balance between X and P proteins in PaBV-5

To examine why 5' UTR swapping impaired replication in clade-2 PaBV-5 but not in clade-3 PaBV-4, I compared viral gene expression between cultures infected with the wild-type virus and those infected with the heterologous 5' UTR chimera. Firstly, I compared protein and RNA levels in rPaBV-4 cultures. Reflecting the replication kinetics (**Fig. 11B**), both wild-type virus and the chimeric virus carrying PaBV-5 5' UTR exhibited comparable expression levels of N, X, and P proteins from Western blot analysis (**Fig. 14A**). Their X-to-P protein ratios remained statistically indistinguishable (**Fig. 14B**). Consistently, RT-qPCR confirmed that N mRNA, X/P mRNA, and genomic RNA levels were virtually identical between the two infections (**Fig. 15A-C**). These data indicated

that PaBV-4 gene expression is largely unaffected by the origin of the 5' UTR.

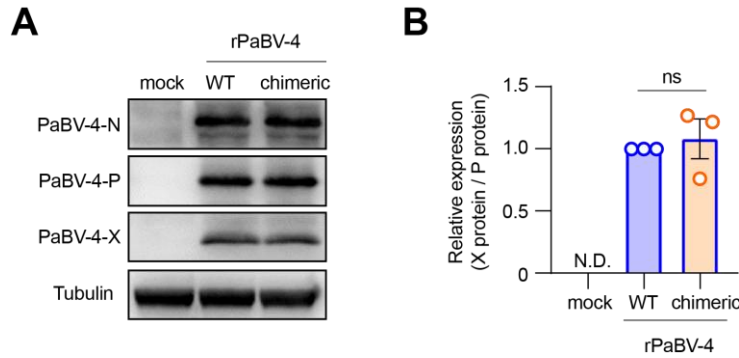


Figure 14. Viral protein expression in wild-type- and chimeric-rPaBV-4-infected cells.

(A) N, X, and P protein expression in wild-type (WT)-rPaBV-4 and chimeric-rPaBV-4 carrying PaBV-5 5' UTR in QT6 cells was detected by Western blotting. (B) X-to-P protein ratios quantified from band intensities using ImageJ. Data represent the means $\pm$ SEM of three independent experiments. Statistical significance was determined by an unpaired *t*-test (B); ns, not significant.

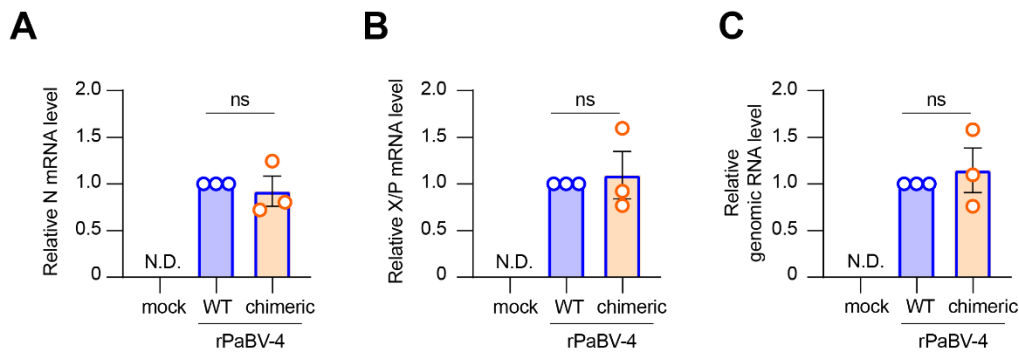


Figure 15. Viral RNA levels in wild-type- and chimeric-rPaBV-4-infected cells.

Relative levels of (A) N mRNA, (B) X/P mRNA, and (C) genomic RNA in WT-rPaBV-4- and chimeric-rPaBV-4- (with PaBV-5 5' UTR) infected QT6 cells were quantified by RT-qPCR. Data represent the means $\pm$ SEM of three independent experiments. Statistical significance was determined by an unpaired *t*-test (A-C); ns, not significant.

On the other hand, analysis of clade-2 rPaBV-5 infections revealed a distinct phenotype. Western blotting demonstrated that while N protein levels remained comparable between infections, the chimeric virus carrying PaBV-4 5' UTR displayed a marked reduction in X protein, accompanied by increased P protein levels relative to the

wild-type rPaBV-5 infection (**Fig. 16A**). Consequently, the X-to-P protein ratio was significantly reduced in cells infected with the chimeric virus (**Fig. 16B**).

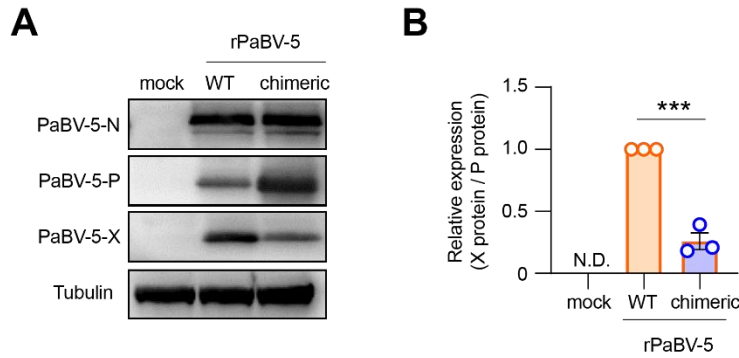


Figure 16. Viral protein expression in wild-type- and chimeric-rPaBV-5-infected cells.

(A) N, X, and P protein expression in wild-type (WT)-rPaBV-5 and chimeric-rPaBV-5 carrying PaBV-4 5' UTR in QT6 cells was detected by Western blotting. (B) X-to-P protein ratios quantified from band intensities using ImageJ. Data represent the means $\pm$ SEM of three independent experiments. Statistical significance was determined by an unpaired *t*-test (B); ns, not significant; *** *p* < 0.001.

Surprisingly, despite the lower infectious titers observed (**Fig. 13B**), RT-qPCR revealed a dramatic upregulation of viral RNA in chimeric-rPaBV-5-infected cells (**Fig. 17A-C**). N mRNA (**Fig. 17A**) and X/P mRNA (**Fig. 17B**) levels were elevated by approximately 18.7-fold and 21.0-fold, respectively. Moreover, genomic RNA levels also increased roughly 6.8-fold compared to the wild-type rPaBV-5 infection (**Fig. 17C**). These results indicate that the native PaBV-5 5' UTR is critical not only for maintaining the proper translational balance between X and P proteins (**Fig. 16**) but also for regulating viral transcription and genome replication to homeostatic levels.

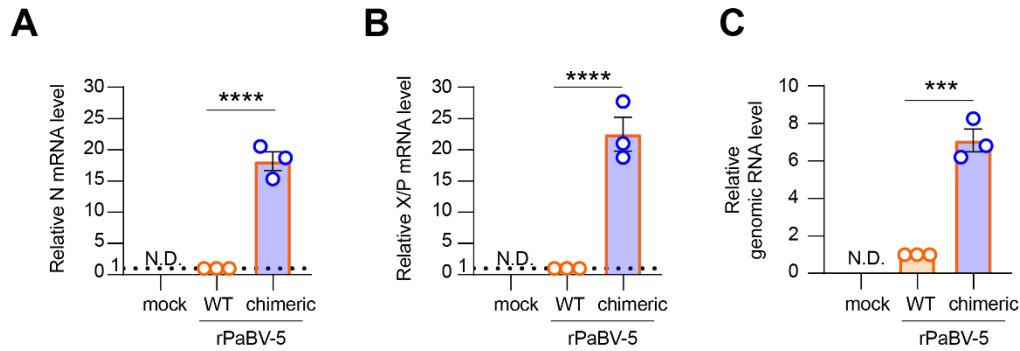


Figure 17. Viral RNA levels in wild-type- and chimeric-rPaBV-5-infected cells.

Relative levels of (A) N mRNA, (B) X/P mRNA, and (C) genomic RNA in in WT-rPaBV-5- and chimeric-rPaBV-5- (with PaBV-4 5' UTR) infected QT6 cells were quantified by RT-qPCR. Data represent the means $\pm$ SEM of three independent experiments. Statistical significance was determined by an unpaired *t*-test (A-C); *** $p < 0.001$; **** $p < 0.0001$.

2.5 Exchange of 5' UTR altered the subcellular localization of viral proteins in PaBV-5

Previous studies on BoDV-1 have shown that X protein regulates nucleocytoplasmic trafficking of P, facilitating P shift from the nucleus to the cytoplasm as infection progresses [64]. Given the disrupted X-to-P ratio in cells infected with chimeric rPaBV-5 carrying the PaBV-4 5' UTR (**Fig. 16B**), I examined viral protein subcellular localization in detail.

In both wild-type rPaBV-4-infected cells and rPaBV-4 chimeras carrying the PaBV-5 5' UTR, the N and P proteins showed comparable subcellular distributions, localizing to both the nucleus and cytoplasm (**Fig. 18A-B**). Specifically, N signals were more prominent in the nucleus (**Fig. 18A**), whereas P signals were stronger in the cytoplasm (**Fig. 18B**).

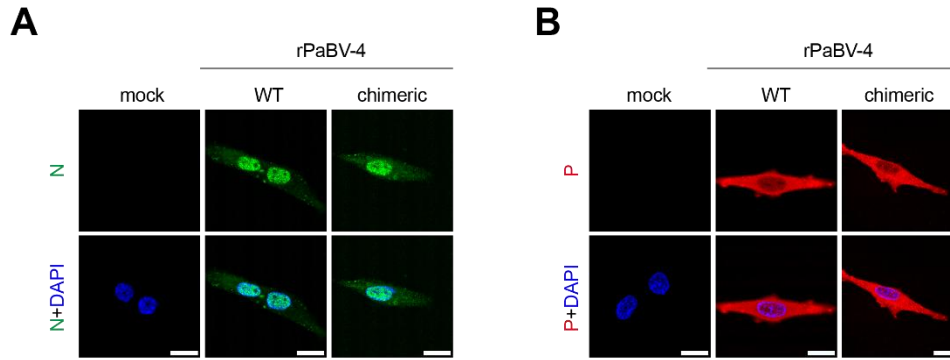


Figure 18. Subcellular localization of N and P proteins in rPaBV-4-infected cells.

Subcellular localization of (A) N and (B) P was analyzed in QT6 cells infected with WT-rPaBV-4 and chimeric-rPaBV-4 (with PaBV-5 5' UTR) by IFA and confocal microscopy. Primary antibodies are indicated to the left of each panel; nuclei were counterstained with DAPI (blue). Scale bar, 20 μm.

The subcellular distribution of N and P in wild-type-rPaBV-5-infected cells paralleled the pattern observed in rPaBV-4 infections (**Fig. 19A-B**). In sharp contrast, the chimeric virus carrying short PaBV-4 5' UTR showed exclusive nuclear retention, where both N and P were strictly confined to the nucleus with no detectable cytoplasmic signal (**Fig. 19A-B**). These observations indicate that the native 5' UTR of the PaBV-5 X/P mRNA was critical for proper subcellular trafficking of viral proteins, which appears to be required for efficient replication and viral spread (**Fig. 13A**).

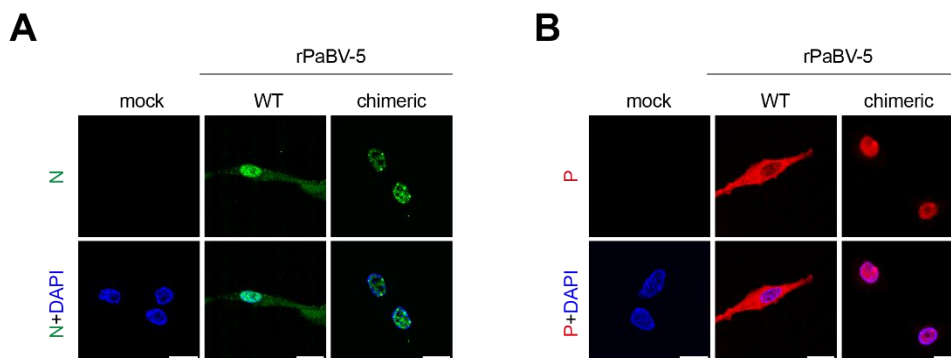


Figure 19. Subcellular localization of N and P proteins in rPaBV-5-infected cells.

Subcellular localization of (A) N and (B) P was analyzed in QT6 cells infected with WT-rPaBV-5 and chimeric-rPaBV-5 (with PaBV-4 5' UTR) by IFA and confocal microscopy. Primary antibodies are indicated to the left of each panel; nuclei were counterstained with DAPI (blue). Scale bar, 20 μm.

2.6 Inter-clade but not intra-clade exchange of the 5' UTR of the X/P mRNA impairs replication of clade-2 ABVs

To determine whether the dependence on a long 5' UTR of X/P mRNA observed in PaBV-5 is conserved among clade-2 ABVs, I extended the analysis to ABBV-1. In addition to wild-type rABBV-1 (WT-rABBV-1) (**Fig. 9B, C**), two chimeric recombinant viruses were generated (**Fig. 20**). An inter-clade chimera was created by replacing the native ABBV-1 5' UTR with the clade-3 PaBV-4 5' UTR (PaBV-4-5' UTR-rABBV-1), like **Fig. 12A**. An intra-clade chimera was generated by replacing the ABBV-1 5' UTR with the clade-2 PaBV-5 5' UTR (PaBV-5-5' UTR-rABBV-1) to test functional compatibility of regulatory elements (stem-loop and uORF) within clade-2 5' UTRs, despite sequence divergence (**Fig. 5A**).

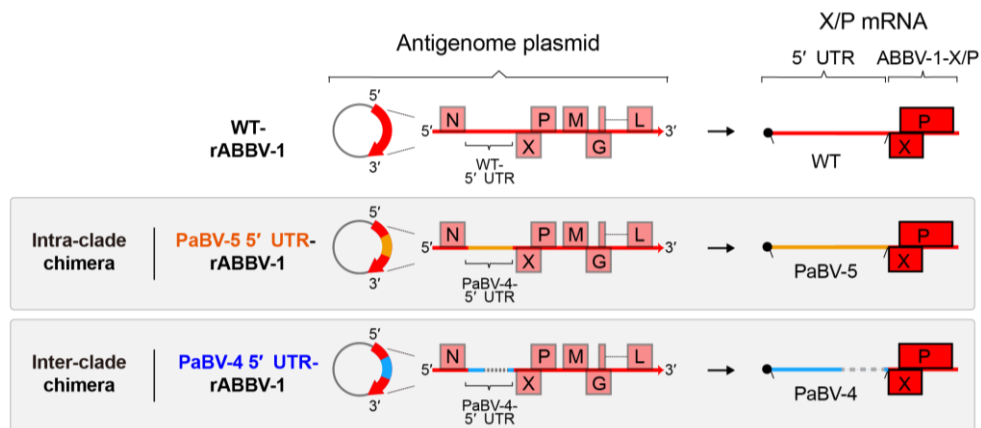


Figure 20. Schematic of rABBV-1 antigenome constructs.

Schematic diagrams of antigenome plasmids for rABBV-1 carrying either the wild-type (WT)-, PaBV-5-derived (intra-clade), or PaBV-4-derived 5' UTR (inter-clade) of the X/P mRNA. The corresponding X/P mRNAs with the indicated 5' UTRs are shown on the right.

To compare growth kinetics, infected and uninfected QT6 cells were cocultured at a 5:95 ratio. Consistent with PaBV-5 kinetics (**Fig. 13A**), the inter-clade chimera carrying the short PaBV-4 5' UTR (PaBV-4-5' UTR-rABBV-1) exhibited markedly delayed growth

(**Fig. 21A**). When infection reached ~50% of cells, viral titers of the inter-clade chimera were approximately 100-fold lower than those of wild-type rABBV-1 (**Fig. 21B**). By contrast, the intra-clade chimera (PaBV-5-5' UTR-rABBV-1) showed growth kinetics and viral titers indistinguishable from wild-type rABBV-1 (**Fig. 21A, B**), demonstrating that intra-clade exchange of the long 5' UTR does not impair replication. These results indicate that the requirement for a long 5' UTR is a shared feature of clade-2 viruses and is associated with conserved regulatory elements such as stem-loop structures and a uORF rather than with the exact nucleotide sequence.

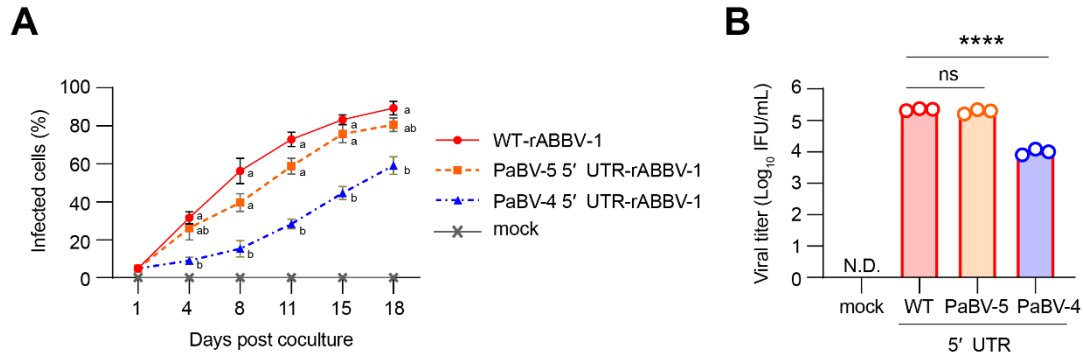


Figure 21. Growth kinetics and titers of wild-type- and chimeric-rABBV-1s.

(**A**) Growth kinetics of rABBV-1s in QT6 cells, including WT-, PaBV-5-5' UTR-, and PaBV-4-5' UTR-rABBV-1. Infected and uninfected QT6 cells were cocultured at a 5:95 ratio, passaged every 3 or 4 days, and the infected ratio was determined by IFA. (**B**) Infectious titers of rABBV-1s. Viruses were rescued when cultures reached 50% infection and titrated on QT6 cells. Data represent the means $\pm$ SEM of three independent experiments. Statistical significance was determined by two-way (A) and one-way ANOVA (B) and Tukey's multiple-comparison test (A, B); ns, not significant; **** $p < 0.0001$. In panel A, different letters (a, b) next to the bars indicate statistically significant differences at $p < 0.05$.

To elucidate the molecular basis of the observed differences in replication, I analyzed viral protein expression in rABBV-1-infected QT6 cells. Consistent with the growth kinetics, cells infected with the intra-clade chimera (PaBV-5-5' UTR-rABBV-1) exhibited N, X, and P protein levels comparable to wild-type-rABBV-1 infection (**Fig. 22A**). In contrast, the inter-clade chimera (PaBV-4-5' UTR-rABBV-1) displayed a dysregulated

expression pattern characterized by reduced X and increased P protein levels (**Fig. 22A**). Consequently, the X-to-P ratio was significantly reduced in cells infected with the inter-clade chimera compared to those infected with wild-type or intra-clade viruses (**Fig. 22B**).

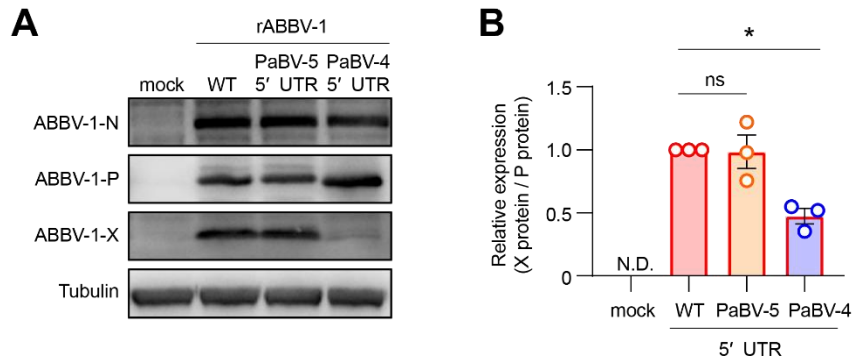


Figure 22. Viral protein expression in wild-type- and chimeric-rABBV-1-infected cells.

(A) N, X, and P protein expression in WT-, PaBV-5-5' UTR-, and PaBV-4-5' UTR-rABBV-1-infected QT6 cells was detected by Western blotting. (B) X-to-P protein ratios quantified from band intensities using ImageJ. Data represent the means $\pm$ SEM of three independent experiments. Statistical significance was determined by one-way ANOVA and Tukey's multiple-comparison test (B); ns, not significant; * $p < 0.05$.

RT-qPCR showed that intra-clade 5' UTR exchange did not alter viral RNA levels. The levels of N mRNA, X/P mRNA, and genomic RNA in cells infected with the intra-clade chimera (PaBV-5-5' UTR-rABBV-1) were comparable to those of the wild-type virus (**Fig. 23A-C**). In contrast, the inter-clade chimera (PaBV-4-5' UTR-rABBV-1) exhibited a dramatic upregulation of viral RNA. Specifically, compared to wild-type rABBV-1, N mRNA and X/P mRNA levels increased approximately 10.7-fold and 15.1-fold, respectively (**Fig. 23A, B**), while genomic RNA levels were elevated roughly 2.8-fold (**Fig. 23C**). These findings suggest that a clade-2-specific long 5' UTR is essential for maintaining the proper translational balance between X and P proteins and for regulating viral transcription and genome replication at appropriate levels.

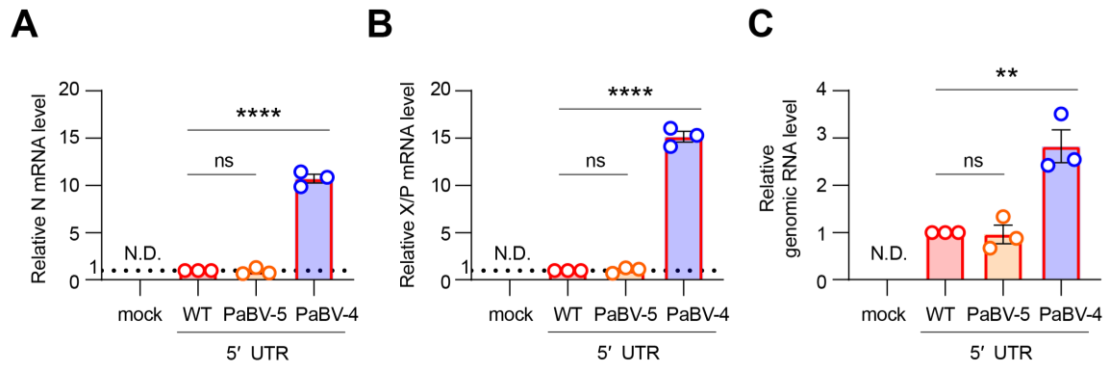


Figure 23. Viral RNA levels in wild-type- and chimeric-rABBV-1-infected cells.

Relative levels of (A) N mRNA, (B) X/P mRNA, and (C) genomic RNA in WT-, PaBV-5-5' UTR-, and PaBV-4-5' UTR-rABBV-1-infected QT6 cells were quantified by RT-qPCR. Data represent the means $\pm$ SEM of three independent experiments. Statistical significance was determined by one-way ANOVA and Tukey's multiple-comparison test (A-C); ns, not significant; ** $p < 0.01$; **** $p < 0.0001$.

Finally, I examined the subcellular localization of viral proteins in rABBV-1-infected cells. In cells infected with wild-type rABBV-1 and the intra-clade chimera (PaBV-5-5' UTR-rABBV-1), N and P proteins showed similar localization (**Fig. 24A, B**). In contrast, the inter-clade chimera (PaBV-4-5' UTR-rABBV-1) exhibited strict nuclear retention of both N and P proteins (**Fig. 24A, B**), mirroring the phenotype observed in chimeric-rPaBV-5 carrying the PaBV-4 5' UTR (**Fig. 19A, B**).

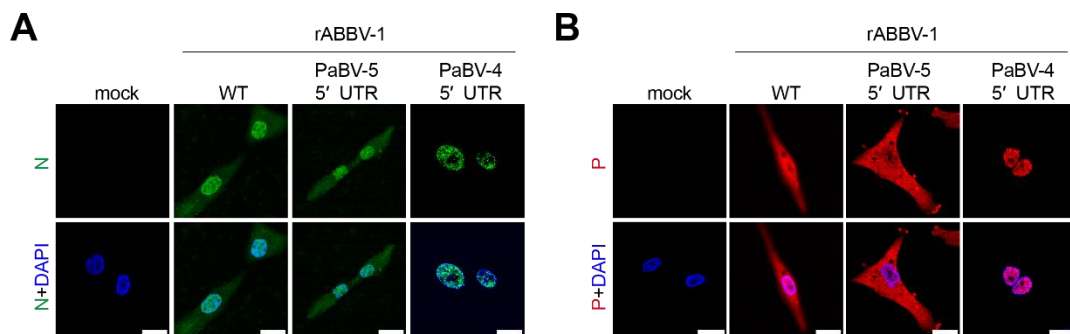


Figure 24. Subcellular localization of N and P proteins in rABBV-1-infected cells.

Subcellular localization of (A) N and (B) P was analyzed in QT6 cells infected with WT-, PaBV-5-5' UTR-, and PaBV-4-5' UTR-rABBV-1 by IFA and confocal microscopy. Primary antibodies are indicated to the left of each panel; nuclei were counterstained with DAPI (blue). Scale bar, 20 μ m.

Taken together, these findings demonstrate that clade-2 viruses require a clade-2-specific long 5' UTR containing regulatory elements to support efficient replication. This requirement reflects its role in maintaining the translational balance between X and P proteins and enabling proper nucleocytoplasmic trafficking.

Chapter 3

Discussions

3.1 The significance of this study

In this study, I established novel reverse genetics systems for clade-2 ABVs, enabling the first functional investigation into how clade-specific 5' UTR architectures regulate bicistronic X/P mRNA translation and viral replication. The results revealed a striking functional divergence between clade-2 and clade-3 ABVs. In the clade-2 PaBV-5 and ABBV-1, replacement of the native 5' UTR with that of the clade-3 PaBV-4 reduced X expression while increasing P expression and markedly attenuated viral growth. By contrast, the PaBV-4 tolerated the introduction of the PaBV-5 5' UTR without compromising translational balance between X and P or impairing the viral replication. Collectively, these findings indicate that the long clade-2-specific 5' UTR, which contains a putative stem-loop structure and a uORF, is essential for clade-2 ABVs to maintain the appropriate stoichiometry between X and P proteins required for efficient viral replication.

3.2 Predicted mechanism of 5' UTR-mediated translational control in clade-2 viruses

This study demonstrated that the long 5' UTR of the X/P mRNA in clade-2 ABVs is crucial for maintaining the translational balance between X and P, as evidenced in both plasmid-based assays (**Fig. 8**) and recombinant viruses (**Fig. 16 and 22**). These results parallel findings for clade-1 BoDV-1, where the stem-loop structure and uORF are critical for regulating X expression [64] (**Fig. 4**). Given the structural similarities in the 5' UTR of X/P mRNA between clade-1 and clade-2 viruses (**Fig. 5**), it is plausible that clade-2 viruses utilize a similar regulatory mechanism.

Several lines of evidence provide support for this shared "structure-dependent"

model. First, the predicted secondary structures in the clade-1 and clade-2 5' UTRs exhibit thermodynamic stability, with minimum free energy values ranging from around -4 to -6 kcal/mol (**Fig. 5**). This suggests the formation of stable stem-loop structures capable of impeding ribosomal progression. Second, although the uORF peptide sequences are poorly conserved across clades, studies in BoDV-1 indicated that translational regulation relies on the positioning of start/stop codons relative to the secondary structure rather than on the encoded peptide itself [64]. Our results from the intra-clade 5' UTR exchange in rABBV-1 (**Fig. 22**) further substantiated this concept, as translational regulation was maintained despite sequence differences of the 5' UTR between PaBV-5 and ABBV-1. Finally, the amino acid sequence of DDX21, a key RNA-binding protein that interacts with the BoDV-1 stem-loop, is highly conserved between human and avian hosts [77]. It is therefore plausible that the clade-2 stem-loop similarly recruits avian DDX21, which works in concert with the uORF to regulate X expression, thereby regulating the proper translational balance between X and P.

Applying the BoDV-1-like model for translational regulation of X and P in clade-2 viruses can explain how replacing the clade-2 5' UTR with the unstructured clade-3 UTR disrupts the X and P expressions. Loss of critical regulatory elements, such as the stem-loop and uORF, likely permits unrestricted ribosomal scanning, thereby biasing translation toward the downstream P ORF. Accordingly, chimeric clade-2 viruses carrying the PaBV-4 5' UTR showed P upregulation (**Fig. 16**). In contrast, P levels remained unchanged in transient plasmid-based assays at 18 h post-transfection (**Fig. 8**). This discrepancy likely reflects the cumulative effect of dysregulated translation. Continuous upregulation of P during prolonged infection leads to pronounced accumulation, which is hardly detected in short-term plasmid assays.

To validate this hypothesized model of translational control of X and P in clade-2

ABVs, the reverse genetics systems established here, combined with protein-RNA interaction assays, will be instrumental in determining whether the clade-2 stem-loop recruits avian DDX21 to mediate this control and mapping the specific binding sites on the viral RNA.

3.3 Divergent host sensing pathways and the predicted 5' UTR-independent evasion of clade-3 viruses

In BoDV-1, disruption of the 5' UTR regulatory elements, including uORF and stem-loop, in X/P mRNA enhanced X translation in mammalian cells [64], whereas in PaBV-5, replacing the long clade-2 5' UTR with the short PaBV-4-derived 5' UTR reduced X expression in avian cells (**Fig. 8 and 16**). A plausible explanation for these opposing phenotypes lies in fundamental differences in how mammalian and avian hosts sense and regulate viral mRNAs. Host cells employ sophisticated surveillance pathways that monitor 5' UTR length [78] and secondary structure [79] to selectively repress viral transcripts, thereby maintaining cellular homeostasis. For example, while mammalian cells utilize interferon-induced proteins with tetratricopeptide repeats (IFIT) 2 and 3 to detect and restrict RNAs with short or unstructured 5' UTRs, avian species lack orthologs for IFIT2 and IFIT3, and their compensatory mechanisms remain uncharacterized [78, 80, 81]. Such divergence in sensing pathways could explain how a similar disruption in 5' UTR architecture could promote X translation in mammalian cells but suppress it in avian cells. Although further experiments are needed to clarify how host cells sense the structure or length of the 5' UTR, our findings suggest that the functional role of the 5' UTR differs between the mammalian and avian hosts.

By contrast, substituting the PaBV-4 5' UTR of X/P mRNA with the PaBV-5-derived 5' UTR did not disturb the translational balance between X and P and replication (**Fig. 7, 11, and 14**). Together with the sensing mechanism proposed above, this finding suggests that the PaBV-4 X/P mRNA may evolve an RNA structure that evades host-sensing pathways and enables efficient translation despite its short 5' UTR. Importantly, this regulatory architecture likely extends beyond the 5' UTR into the X and P coding regions [67]. Consistent with this hypothesis, attempts to rescue an rPaBV-4 mutant in which the secondary structure extending from the 5' UTR into the X and P coding regions was disrupted were unsuccessful (data not shown). These results suggest that translational regulation of PaBV-4 X/P mRNA may depend on the overall mRNA architecture rather than on the 5' UTR alone. Unraveling how PaBV-4 evades host sensing through its specific X/P mRNA architecture will provide key insights into the unique translational strategies of clade-3 orthobornaviruses in avian hosts.

3.4 Decoupling between viral transcription and translation

Chimeric rPaBV-5 and rABBV-1 carrying the PaBV-4 5' UTR showed substantially increased viral mRNA and genomic RNA levels (**Fig. 17 and 23**), despite their attenuated viral spread and yields (**Fig. 13 and 21**). In BoDV-1, X acts as a negative regulator of polymerase activity through interacting with P [16-19, 82-85]. By analogy, lower X expression in the chimeric viruses was likely to relieve this inhibition, thereby enhancing genomic RNA synthesis and increasing the template pool for transcription. However, this increase in transcript abundance did not lead to a proportional increase in viral protein levels in chimeric virus-infected cells (**Fig. 16 and 22**), indicating a functional decoupling between viral transcription and translation. Similar uncoupling phenomena have been

reported in vesicular stomatitis virus infection, where they are attributed to the saturation of the available host ribosome pool under conditions that otherwise preserve cellular homeostasis [86]. Similarly, studies on persistent dengue virus infection have linked transcription-translation decoupling to a decline in infectious viral yields [87]. Although the precise mechanisms warrant further investigation, these findings suggest that the excessive relief of X-mediated polymerase inhibition creates a discordance where RNA accumulation is uncoupled from protein synthesis, thereby paradoxically limiting productive viral replication.

3.5 Insufficient X expression and nuclear retention of viral proteins

The imbalance between X and P expression observed in the clade-2 chimeric viruses carrying the short PaBV-4 5' UTR (**Fig. 16 and 22**) was mirrored by a distinct alteration in the intracellular localization of viral proteins. Chimeric rPaBV-5 and rABBV-1 carrying the PaBV-4 5' UTR showed that both N and P were primarily restricted to the nucleus (**Fig. 19 and 24**), whereas wild-type viruses expressed these proteins mainly in the cytoplasm (**Fig. 19 and 24**). BoDV-1 P is exported to the cytoplasm when co-expressed with X, and PaBV-5 X similarly promoted cytoplasmic relocalization of P, analogous to BoDV-1 X [67, 82]. Since P acts as a central scaffold within the vRNP complex (including N, P, and L) [10, 20, 88-91], excess P in the absence of sufficient X is therefore expected to retain vRNPs in the nucleus and delay viral spread. Thus, the nuclear confinement of N and P in clade-2 chimeric viruses likely reflected inadequate X expression (**Fig. 16 and 22**). Without sufficient X to facilitate the nuclear export of P,

vRNPs remained trapped in the nucleus, thereby blocking efficient viral propagation (**Fig. 13 and 21**). Elucidating the specific host factors that modulate this X-dependent export machinery in avian cells will be crucial for fully understanding how orthobornaviruses maintain the delicate balance between nuclear replication and cytoplasmic spread during persistent infection.

3.6 Predicted evolutionary scenario: ancestral simplicity to adaptive complexity

The striking difference in 5' UTR requirements for X/P mRNA translation between clade-2 and clade-3 ABVs raises an intriguing evolutionary question: did this complex architecture represent an evolutionary gain in clade-2, or a reductive loss in clade-3? It is currently unclear whether the requirement of a long 5' UTR for efficient replication in clade-2 viruses has been selected for or has arisen by chance. A phylogenetic study revealed that reptile bornaviruses in clade-4 branch off at a basal position, indicating that the lineage leading to clade-3 PaBV-4 diverged before the separation of clade-2 PaBV-5 and clade-1 BoDV-1 [92]. This topology and the short 5' UTRs observed in clade-3 and clade-4 X/P mRNAs [67] suggest a scenario in which the complex regulatory sequences, such as stem-loops and uORFs, in the 5' UTRs of X/P mRNA of clade-1 and clade-2 were absent in the ancestor and were subsequently acquired after divergence from the clade-3 lineage (**Fig. 25**). Although such structural complexity can incur a fitness cost by impeding ribosomal scanning [93], these additional nucleotides were likely retained because they may confer adaptive advantages. For example, they may enable finer regulation of X and P expression via the interactions with host factors [64], which may

help explain the broader host range of clade-1 and clade-2 viruses, as they can infect both mammalian and avian cells in culture [94]. Conversely, clade-3 viruses retain a more ancestral, streamlined 5' UTR architecture (**Fig. 25**). By avoiding these structural burdens, they prioritize translational efficiency [95] but appear to sacrifice this adaptive potential, potentially contributing to a host range largely confined to avian species [94]. It will therefore be important to experimentally examine the hypothesized advantages and costs that these alternative 5' UTR architectures impose on viral fitness.

Nonetheless, this study reveals a remarkable nuance regarding 5' UTR dependence in X/P mRNA for efficient replication between clade-2 and clade-3 ABVs, facilitated by newly established clade-2 reverse genetics systems. Comparative analysis suggests that ABVs have adopted two contrasting strategies for translating their X/P mRNAs: clade-2 viruses favor regulatory complexity, and clade-3 viruses preserve a more ancestral simplicity (**Fig. 25**). Further elucidation of the underlying molecular mechanisms may identify critical host-virus interactions that constrain viral replication and clarify the evolutionary logic linking 5' UTR design to host adaptation.

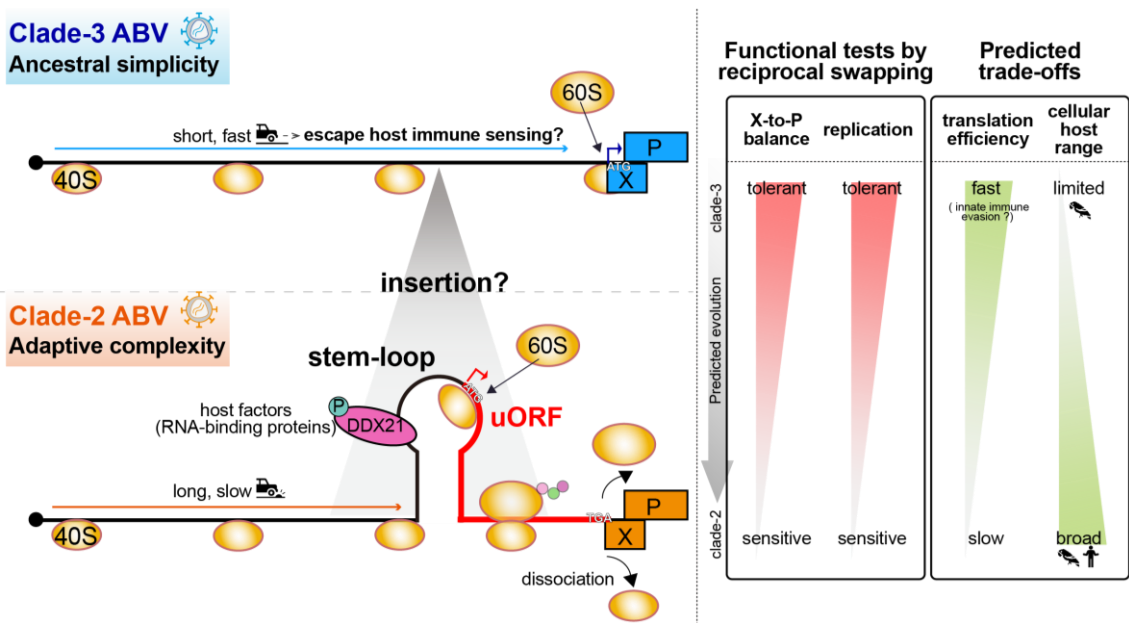


Figure 25. Proposed models for the evolutionary divergence of X/P mRNA translational control strategies.

(Left) Proposed mechanisms of X/P mRNA translational control. Clade-3 ABVs utilize a short 5' UTR for efficient ribosomal scanning and potential evasion of host sensing. Conversely, Clade-2 ABVs are predicted to evolve complex 5' UTRs from ancestral clade-3 viruses. A stem-loop and uORF together interact with host factors (e.g., DDX21) to regulate the proper balance of X and P expression, a strategy analogous to that of clade-1 BoDV-1. **(Right)** Functional roles and evolutionary trade-offs. Red arrows summarize the findings from this study: clade-2 ABVs require their native 5' UTR to maintain the proper X-to-P ratio for efficient replication, whereas clade-3 ABVs tolerate heterologous 5' UTRs without compromising viral growth. Green arrows show the predicted evolutionary trade-offs: clade-3 prioritizes translational efficiency but remains restricted to avian hosts, whereas clade-2 sacrifices efficiency to gain the regulatory plasticity required to infect both avian and mammalian hosts.

Chapter 4

Materials and Methods

4.1 Cell culture

Quail fibroblast cell lines QT6 (ATCC CRL-1708), QM7 (ATCC CRL-1962), and human embryonic kidney 293T cells (293T) (ATCC CRL-3216), were maintained in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, USA; #11965118) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, USA; #10270-106) and 1% penicillin/streptomycin/amphotericin B solution (Fujifilm Wako, Japan; #161-23181). All cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

A QM7 subline expressing simian virus 40 large T antigen (QM7-T) was generated using the PiggyBac Transposon Vector System (System Biosciences, USA; #PB210PA-1-SBI) following previously established protocols [62]. Similarly, a blasticidin-resistant derivative of QT6 cells (QT6-Blast) was established via lentiviral transduction of a construct encoding the blasticidin-resistance gene [62]. Antibiotic selection and clonal expansion procedures were performed, and the resulting stable cell lines were routinely cultured under the same conditions as parental cells.

4.2 Plasmid construction and transfection

Viral genomic and subgenomic sequences were retrieved from the GenBank database: PaBV-4 (accession number JX065209), PaBV-5 (accession number LC120625), and ABBV-1 (accession number KF578398). cDNA fragments corresponding to the X/P bicistronic mRNA of PaBV-4 and PaBV-5 were cloned into the pcDNA3 vector as previously described [64]. To minimize non-viral nucleotides, residual vector sequences between the transcriptional start site and the viral 5' UTR were excised

during construct validation.

For transient expression assays, QM7-T cells cultured overnight in 12-well plates were transfected with 0.6 µg of each plasmid using Lipofectamine 3000® (Invitrogen, USA; #L3000001) according to the manufacturer's instructions. Cells were harvested 18 hr post-transfection for protein and mRNA analysis.

Full-length cDNAs of viral antigenomic RNA for clade-2 (PaBV-5 and ABBV-1) and clade-3 (PaBV-4) were cloned into pCAG-HRSV3 [96]. Each construct was flanked by a hammerhead ribozyme (HamRz; Rz) at the 5' terminus and a hepatitis delta virus ribozyme (HdvRz; δ) at the 3' terminus to ensure precise transcript ends [14, 76]. In addition, five helper plasmids encoding the N, P, L, M, and G proteins for each virus and BoDV-1 (accession number PX246123) were generated in the pCAGGS vector as described previously [62, 76]. Site-directed mutagenesis using a PCR-based protocol was employed to introduce specific nucleotide substitutions or deletions into selected plasmids.

4.3 Reverse genetics for ABVs and cell-free virus preparation

The reverse genetics procedure for ABVs was performed following and adapting previously established protocols [62, 76]. Human 293T cells were seeded in six-well plates one day prior to transfection and co-transfected with 2 µg of the full-length antigenomic cDNA plasmid together with five helper plasmids encoding the N, P, L, M, and G proteins. The standard plasmid ratio per well was 0.5 µg pCAGGS-N, 0.025 µg pCAGGS-P, 0.25 µg pCAGGS-L, 0.03 µg pCAGGS-M, and 0.01 µg pCAGGS-G.

Transfection was performed using the TransIT-293 transfection reagent (Mirus Bio, USA; #MIR2700) according to the manufacturer's recommendations.

At three days post-transfection, the transfected 293T cells were overlaid with blasticidin-resistant QT6 cells to initiate coculture. The mixed cells were passaged every three to four days to facilitate virus spread. Residual 293T cells were eliminated by treating the culture medium with 8 $\mu\text{g ml}^{-1}$ blasticidin (InvivoGen, USA; #ant-bl-1) for approximately one week.

To prepare cell-free viral stocks, cocultured cells were harvested by trypsinization, pelleted by centrifugation, and resuspended in Opti-MEM (Invitrogen, USA; #31985070). The cell suspension was disrupted by sonication using a Bioruptor II (Sonic Bio, Japan) and clarified by centrifugation at $1,200 \times g$ for 25 min at 4 °C. The supernatant was collected as virus-containing material and stored at -80 °C until use.

4.4 Immunofluorescence assay (IFA)

For immunofluorescence staining, cells were washed once with phosphate-buffered saline (PBS) to remove residual culture medium and fixed in 4%-paraformaldehyde phosphate buffer solution (Nacalai Tesque, Japan; #09154-85) for 15 min at room temperature. Paraformaldehyde-fixed cells were permeabilized and blocked in PBS containing 0.5% Triton X-100 (Wako, Japan; #169-21105) and 5% bovine serum albumin (BSA; Sigma-Aldrich, USA; #A3059) for 15 min.

The cells were then incubated with rabbit polyclonal antibodies against BoDV-1 N (HB01; 1:4,000 dilution) or BoDV-1 P (25F/10; 1:3,000 dilution) followed by Alexa Fluor[®] 488- or 555-conjugated anti-rabbit IgG secondary antibodies (1:1,000 dilution; Thermo Fisher Scientific, USA; #A-11034, #A-21429). Nuclei were counterstained with

300 nM 4',6-diamidino-2-phenylindole (DAPI; Merck, Germany; #28718-90-3). Fluorescence signals were visualized using an ECLIPSE TE2000-U fluorescence microscope or an ECLIPSE Ti confocal laser-scanning microscope (Nikon, Japan). Image acquisition parameters were kept constant within each experiment to ensure comparability.

4.5 Growth kinetics assay

QT6 cells persistently infected with recombinant ABVs were cocultured with uninfected QT6 cells at a ratio of 5:95 to initiate low-multiplicity spread. The cocultures were maintained under standard growth conditions and passaged every three to four days. At each passage, the proportion of infected cells was quantified by IFA using rabbit polyclonal antibodies against BoDV-1 P (25F/10; 1:3,000 dilution) followed by Alexa Fluor® 555-conjugated anti-rabbit IgG secondary antibodies (1:1,000 dilution; Thermo Fisher Scientific, USA; #A-21429). The infection ratio at each time point was used to monitor viral propagation across serial passages.

4.6 Titration assay

Ten-fold serial dilutions of cell-free virus suspensions were prepared in Opti-MEM (Invitrogen, USA; #31985070), and 50 µl of each dilution was added to monolayers of QT6 cells seeded in a 96-well plate the previous day. After incubation at 37 °C for 1 hr to allow viral adsorption, the inoculum was replaced with fresh growth medium, and cultures were maintained for three days. Infected cells were detected by IFA. The number of positive wells at each dilution was recorded, and viral titers were calculated according to the previously published method [97]. Results were expressed as infectious focus units

(IFU/mL).

4.7 Reverse transcription quantitative PCR (RT-qPCR)

Total RNA was isolated from cultured cells using the NucleoSpin RNA Plus (Macherey-Nagel, Germany; #740984) according to the manufacturer's instructions. For reverse transcription, 1 µg of total RNA was used as template in a 20-µl reaction with the Verso cDNA synthesis kit (Thermo Fisher Scientific, USA; #AB1453). cDNA was synthesized either with oligo-dT primers to quantify viral messenger RNAs or with virus-specific primers to detect genomic RNA.

Quantitative PCR (qPCR) was carried out using the Luna Universal qPCR Master Mix (New England Biolabs, USA; #M3003) on a CFX Connect real-time PCR system (Bio-Rad, USA). qPCR was performed under the following thermal cycling conditions: an initial denaturation at 95 °C for 10 min, followed by 35 cycles of 95 °C for 5 s and 60 °C for 30 s. Fluorescence signals were recorded at the end of each annealing/extension step. A final dissociation stage was carried out at 95 °C for 10 s, during which melting curves were generated by continuous fluorescence acquisition at 0.5 °C increments from 65 °C to 95 °C. Primer sequences used in this study are listed in **Table 1**. Relative RNA expression levels were calculated by the comparative Ct ($\Delta\Delta C_t$) method, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as the internal normalization control.

Target gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
PaBV-4-N	CACGATTTAGACGGCGAGAA	GCGAATCCGGTTACACCTATTA
PaBV-4-P [†]	CTAACTGTGCCCCTCGAGAA	TTAAGCCCCTCTGCCTCGAT
PaBV-4-gRNA-specific RT [†]	GTTGCGGTAACAACCAACGCAAC	-
PaBV-5-N	CCGCCTGGAAAGGAGTTTAT	AACTGCAGGGATGGTTATGG
PaBV-5-P	GCACTGACTCAACCTGTGGA	AGTCCCTCATTCTCAATCAT
PaBV-5-gRNA-specific RT	GTTGCGTTAACAACAAACCAGCGACC	-
ABBV-1-N	GTTACGCGCAGATGACTACTT	GTACGCGACAGCTGGAATAA
ABBV-1-P	GCTCTGACACAACCAGTCGAAC	AAACCTTCAGTTCGACCATGC
ABBV-1-gRNA-specific RT	GTTGCGGTAACAACAAACCAACCTCC	-
Quail GAPDH [†]	GCAACCGTGTGTGGACTTG	GGGAACAGAACTGGCCTCTC

[†] Primer sequences from Komorizono et al [62].

Table 1. Sequences of primers used for RT-qPCR

4.8 Western blotting

QM7-T cells transfected with expression plasmids or rABV-infected cells were lysed directly in 2× sample buffer (Nacalai Tesque, Japan; #30566-22) and boiled for 10 min at 95 °C to denature proteins. The lysates were separated by SDS-PAGE using 10-20% gradient ePAGEL gels (ATTO Corporation, Japan; #E-R1020L) and electrotransferred onto Trans-Blot Turbo Mini nitrocellulose membranes (Bio-Rad, USA; #1704156).

Membranes were blocked with Tris-buffered saline (TBS; TaKaRa Bio Inc., Japan; #T9141) containing 0.1% Tween-20 and 5% (w/v) skim milk, followed by incubation with primary antibodies: anti-BoDV-1 N (rabbit polyclonal HB01, 1:1,000), anti-BoDV-1 P (rabbit polyclonal HB03, 1:1,000), anti-BoDV-1 X (rabbit polyclonal H1766, 1:100), or anti- α -tubulin (Sigma-Aldrich, USA; #T5168, 1:10,000). After washing, membranes were probed with horseradish peroxidase (HRP)-conjugated secondary antibodies, anti-rabbit IgG or anti-mouse IgG (Jackson ImmunoResearch, USA; #715-035-150), at a

1:10,000 dilution. Chemiluminescent detection was performed using Clarity Western ECL substrate (Bio-Rad, USA; #170-5060), and protein bands were visualized with a Fusion Solo S imaging system (Vilber, France). Exposure conditions were standardized across samples to allow quantitative comparison. Band intensities were quantified using ImageJ software (NIH, USA), and the X-to-P protein ratio was calculated from normalized signal values.

4.9 RNA structure prediction

The secondary structure of the full-length 5' UTR of the X/P mRNA was predicted using RNAfold implemented in the ViennaRNA Package 2.0. Minimum free energy (MFE) folding was applied to estimate the most thermodynamically stable conformation.

4.10 Statistical analysis

Statistical analyses were performed using GraphPad Prism software (version 10). The statistics tests applied to each dataset are described in the corresponding figure legends. Data are presented as the mean $\pm$ standard error of the mean (SEM) unless otherwise indicated, and a *p* value of < 0.05 was considered statistically significant.

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Appendice

The work, which is being presented in this thesis entitled “**Comparative analysis of the 5’ UTR-mediated translational regulation of X/P mRNA among avian orthobornaviruses using reverse genetics**”, is mainly based on the published article:

“**Distinct 5’ UTR requirements for translation of the bicistronic X/P mRNA among avian orthobornaviruses**”

Meng-Chi Wu, Takehiro Kanda, Ryo Komorizono, Madoka Sakai, Alexander Leacy, Leonardo Susta, Akiko Makino, Keizo Tomonaga

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