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IDENTIFICATION OF CONSERVED THREE-WAY JUNCTION IN THE GENOME OF THE BOVINE FOAMY VIRUS

Balak O. K. [1], Limanskaya O. Yu. [2]

[1] Kharkiv National Medical University, Kharkiv, Ukraine
[2] National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine', Kharkiv, Ukraine, e-mail: olgaliman@ukr.net

Summary. Three-way junctions (3WJs) belong to unusual structures in DNA and RNA. 3WJs are non-canonical structures like G-quadruplexes, triplexes (H-DNA), cruciform, hairpin structures, A-DNA, and Z-DNA that differ from the classic double-stranded B-DNA. 3WJs play an important role in many biological processes and may be associated with some human diseases. This study aimed to search for putative 3WJ structures in the mRNA of bovine foamy virus (BFV). Bioinformatic analysis was used to analyze conserved RNA structural motifs of intramolecular 3WJ in BFV mRNA. The Vfold2D software was used to search for structural motifs in the 3WJ RNA. Multiple sequence alignment was conducted using MEGA software. For the confirmation of secondary structures and the determination of the thermodynamic parameters of 3WJs, Mfold software from the UNAFold web server was utilized. Based on multiple alignments of 37 BFV isolates with the complete genome, we found 6 putative 3WJ structures in the BFV mRNA, which are stabilized by 20–26 complementary nucleotides pairs (ntp) and localized in the *gag*, *env*, *bel2* genes, as well as in the 5'LTR. However, only two 3WJ structures in *gag* and *env* genes from the abovementioned six ones, designed by the Mfold software, coincide with 3WJ structures determined by the Vfold2D software. Five 3WJ structures from 6 identified ones are not conserved. Conserved 3WJ structure with a length of 73 nt for a set of 37 BFV isolates with complete genome is localized between 5'-LTR and 5'-end of *gag* gene and partially covers 5'-end of *gag* gene. This intramolecular secondary structure is formed by three duplexes and stabilized by 20 complementary ntp with a free energy of −19.8 kcal/mol. Our analysis of SNPs in the paper (Bao et al., 2020), which arose after serial passages of BFV Riems-infected MDBK cells has shown that the determined 3WJ structure is retained, indicating the importance of this alternative structure for BFV functioning

Keywords: bovine foamy virus, three-way junction, 3WJ, structural motif

Introduction. Hairpins, cruciform structures, internal loops, bulges, G-quadruplexes, and multi-helix junctions are alternative elements of the secondary structure of nucleic acid molecules. They are formed from complementary strand fragments, contain varying numbers of duplexes, have a branched structure, and play significant roles in various biological processes (Kardrmas, Ravin, and Leontis, 1995). Some biophysical methods and algorithms are used for modeling and prediction of these non-canonical structures to characterize multi-helix junctions, to determine their kinetic and thermodynamic parameters (Xue et al., 2016; Mathews et al., 2004).

It is currently known that highly ordered five-way junctions (5WJs), which consist of five duplexes joined at the binding point, (i) are structural elements of bacterial lysine-specific riboswitches that regulate lysine biosynthesis and transport (Serganov, Huang and Patel, 2008); (ii) participate in the assembly process of the small subunit of bacterial ribosomes, which starts from the interaction of 5WJ with the primary binding protein S4 (Chen et al., 2012); (iii) may be like as four-way junctions (4WJs) a component of hairpin ribozymes of plant viruses (Bajaj, Steger and Hammann, 2011).

4WJs are well-known as Holliday junctions. They are intermediate structures formed by DNA molecules during replication, repair of double-strand breaks, and mitosis. 4WJs contain four duplexes and one can have different configurations depending on the environmental conditions. These structures play an important role in the process of enzyme recognition and genome stabilization. 4WJs are functional elements of the internal ribosome entry site during translation and can be considered a target for anticancer therapy (Song et al., 2022; Melcher, Wilson, and Lilley, 2003). The involvement of these non-canonical structures in the processes of DNA replication and repair has facilitated the development of compounds that selectively bind to 4WJs with a therapeutic effect (McGorman et al., 2023). Currently, diagnostic platforms with electrochemical biosensors based on 5WJs and 4WJs have been developed to detect many dangerous pathogens, to analyze DNA and RNA molecules with the complete genome (Foguel et al., 2024; Lynch et al., 2019; Ojeda et al., 2024; Kashefi-Kheyrabadi et al., 2022).

The least complicated among known multi-helix junctions are three-way junctions (3WJs) representing labile flexible functional motifs. 3WJs consist of three duplexes connected at the binding point and

characterized by conformational mobility. They are typical for the secondary structure of RNA and DNA molecules. 3WJs play an important role in many biological processes, such as splicing, translation, recombination, and repair. 3WJs are associated with some human diseases (especially, degenerative disorders), which allows them to be considered as potential targets for drugs. They can be used as part of DNA molecules modified by certain compounds for inhibition and detection of some human pathogens, as well as to design nanoparticles to transport therapeutic agents too (Assenberg et al., 2002; Wu et al., 2004; Yamabe, Kaihatsu and Ebara, 2018; Shu et al., 2011).

3WJ is a dynamic structure of the RNA molecule of the procapsid shell of phi29 bacteriophage which is necessary for the self-assembly of this RNA and the spatial orientation of the helicase (Hill and Schroeder, 2017). 3WJs form the basis for the formation of more complicated 5WJs (Luo et al., 2010). They are typical structures of ribozymes, transport, ribosomal, and matrix RNA molecules of many microorganisms, especially human and plant viruses. 3WJs are known to be conserved motifs of the internal ribosome entry site (IRES) of hepatitis A and C viruses and play a role in the internal initiation of translation by a mechanism that is alternative to cap-dependent (Koirala et al., 2019; Ouellet et al., 2010). The non-canonical mechanism of translation is also facilitated by 3WJs localized in genomic RNA sequences of some plant viruses (Ojha et al., 2024).

Possibility of the 3WJ formation which may play a certain role in the cooperative binding of the Rev protein was shown by NMR relaxation dispersion techniques for the sequence of HIV-1 the Rev response element, which is considered as a target for antiviral therapy (Chu et al., 2019) (Fig. 1).

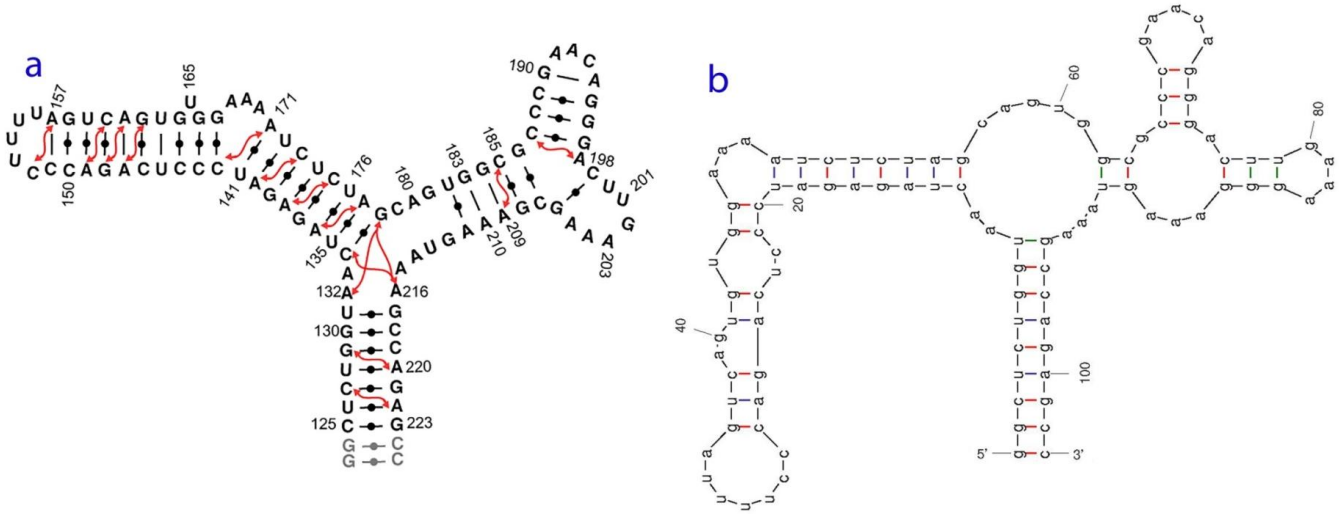


Figure 1. 3WJ structure with length of 103 nt hosting the binding site the primer from which HIV-1 reverse transcription is initiated (Chu et al., 2019) **(a)**. 3WJ structure with length of 103 nt in HIV-1 RNA, which was designed by Mfold software with the following parameters: forced formation of complementary pairs: F 12 56 8, F 20 45 2 F 65 75 3 (free energy ΔG is -28, 8 kcal/mol) **(b)**.

The conserved adenosine-rich three-way junction structure localized in the primer binding site of HIV-1 affects the efficiency of reverse transcription and the infectivity of the virus. It has been proven by several biophysical methods that the destruction of the specified 3WJ structure minimizes the formation of reverse transcription products and reduces the infectivity of HIV-1 (Song et al., 2021). There is currently no information on the possibility of 3WJ formation in the genome of animal viruses.

Spumaretroviruses, or foamy viruses, belong to the subfamily Spumaretrovirinae in the family Retroviridae. According to the updated and expanded in 2017 spumaretrovirus taxonomy and nomenclature, the existing genus *Spumavirus* was replaced by five genera titled *Bovispumavirus*, *Equispumavirus*, *Felispumavirus*, *Prosimiispumavirus*, and *Simiispumavirus*. The determined species bovine foamy virus, feline foamy virus, and equine foamy virus were included in the new genera *Bovispumavirus*, *Felispumavirus*, and *Equispumavirus*, respectively (Khan et al., 2018).

Objective. In the current study, putative 3WJ structures in the genomic RNA of bovine foamy virus are determined, since currently there is no appropriate information about 3WJ structures in the genome of this pathogen.

Materials and methods. Vfold2D software on the web server http://rna.physics.missouri.edu was applied to search for intramolecular 3WJs in the genomic RNA of BFV. The genomic RNA sequence of the BFV JX307861 isolates with the complete genome (which was isolated in Poland; the length is 12,010 nucleotides (nt)) from the GenBank database was cut into 114 fragments with a length of 145 nt, which overlap by 40 nt. Mfold (RNA Folding Form) software on the UNAFold web server (www.unafold.org) was used to confirm the secondary

structure and determine the thermodynamic parameters of 3WJs (Zuker, 2003). The multiple alignment of nucleotide sequences and the search for conserved motifs of 3WJ structures for 37 BFV isolates were carried out by MEGA software (version 6.06) (Tamura et al., 2013).

Nucleotide sequences of 37 BFV isolates with complete genomes were obtained by searching for taxonomic identifier (txid) 207343 in the GenBank database of the National Center for Biotechnology Information (USA).

Results. 3WJ motif is an alternative secondary structure of nucleic acid molecules consisting of three duplexes connected at the binding point (3WJ scheme is shown in the inset to Fig. 2). It is characterized by conformational mobility, affects the spatial orientation of the molecule and has a branched structure.

The search of non-canonical 3WJ structures was performed for the individual sequence of the isolate JX307861, as well as for the consensus sequence of 37 BFV isolates. Further, a set of 37 BFV isolates was analyzed for the presence of the found 3WJ structures.

In the first stage, 17 putative 3WJ structures were found for the mRNA sequence of isolate JX307861 (Hechler et al., 2012) by Vfold2D software for 3WJ search. The sequence forming 3WJ in the RNA of phi29 bacteriophage in *Bacillus subtilis* was used as a control for a propriety of the 3WJ motifs search (Fig. 2a, Zhang et al., 2013). Small RNA molecule with a length of approximately 120 nt plays an essential role in phi29 DNA compaction *in vitro* (Guo, Erickson and Anderson, 1987). RNA is a component of the envelope of the viral precursor of DNA packaging machinery but is not a component of the natural virion (Guo, Erickson and Anderson, 1987). Small RNA molecules that act during genome packaging (pRNA) have been identified, in addition to phi29, in several podoviruses, including GA-1 (Bailey et al., 1990).

Three duplexes of this 3WJ motif the crystal structure of which was previously confirmed experimentally with a resolution of 0.3 nm (Zhang et al., 2013) contain 20 complementary nucleotide pairs (Fig. 2a). The free energy ΔG of this 3WJ structure is −20.9 kcal/mol, that we determined by Mfold software (but only with the forced formation of complementary pairs according to the following parameters F 1 78 1) (Fig. 2b).

At the second stage, such secondary structures were selected for further analysis among the identified 3WJ structures which corresponded to the parameters of the 3WJ structure, which was experimentally investigated (Zhang et al., 2013). For further analysis, 3WJ structures were selected, the free energy of which was less than −18 kcal/mol, and the total number of complementary nucleotide pairs was at least 20.

We found 6 putative 3WJ structures in the mRNA of the JX307861 BFV isolate which are stabilized by 20–26 complementary nt and localized in the 5'LTR, *gag* (2 structures), and *env* genes (2 structures), in *bel2* gene (Table 1), as well as three 4WJ structures, two 5WJ structures (results not shown).

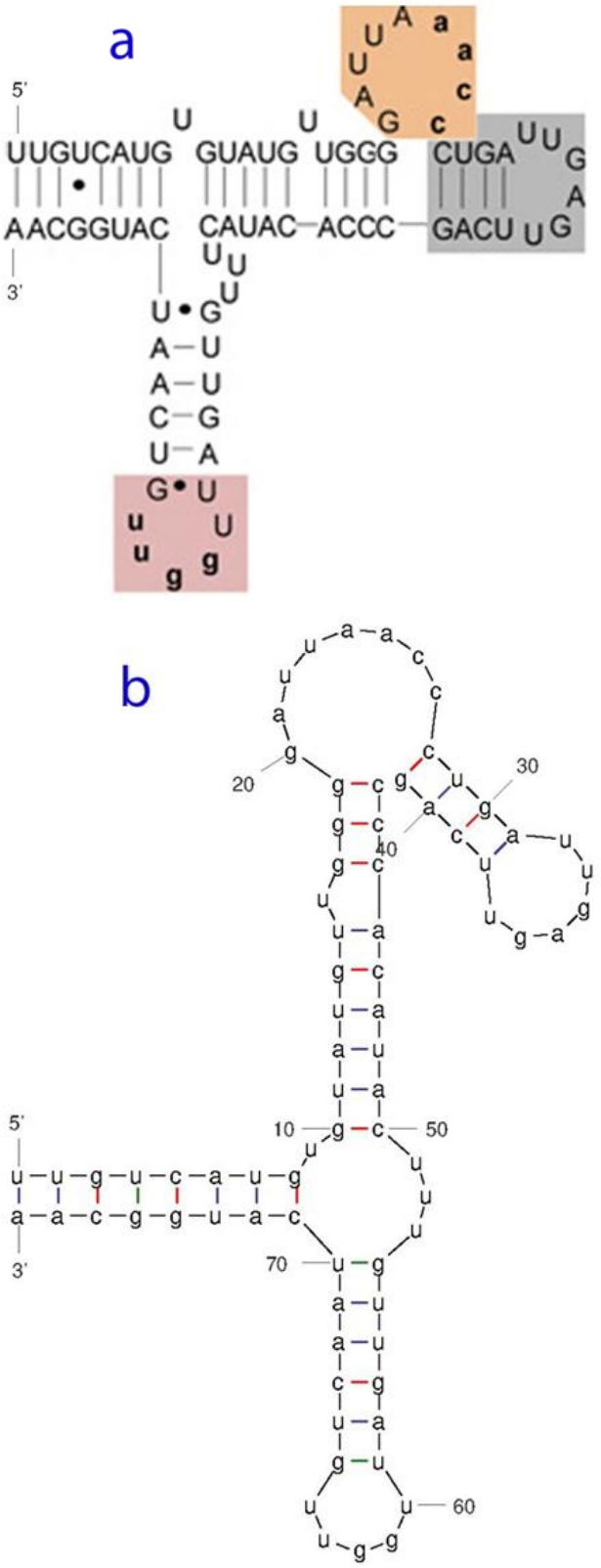
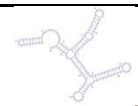
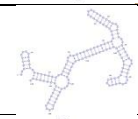
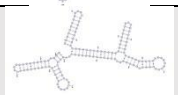
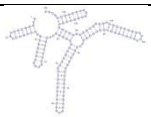
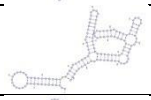
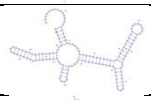
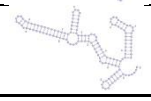


Figure 2. The secondary structure (3WJ structure) which was found in the RNA of the packaging motor of phi29 bacteriophage connecting with DNA packaging (Zhang et al., 2013) **(a)**, and that was designed by the Mfold (RNA Folding Form) software **(b)**. 3WJ is characterized by 20 complementary nt (7, 4, 9 complementary nucleotide pairs in the direction 5'→3' for three duplexes, respectively).

However, only two 3WJ structures (in *gag* (position 1,388–1,460) and *env* (position 8,112–8,174) genes) the above mentioned above six ones designed by Vfold2D software coincide with the 3WJ structures determined by Mfold software (without forced formation of complementary pairs). 5 of 6 identified 3WJ structures are not conserved for the set of 37 BFV isolates. Only highly conserved 3WJ motif with a 100% level identity (Fig. 3) is localized between the 5'-LTR (1–1,312) and the 5'-end of the *gag* gene (1,420–3,054) and partially covers the 5'-end of the *gag* gene (positions 1,388–1,460 for JX307861 BFV isolate). In addition, a longer fragment with a length of 103 nt (1,382–1,484 for JX307861 BFV isolate) also has a 100% identity for 37 BFV isolates.

Table 1 — Parameters of 3WJ structures in the genome of JX307861 bovine foamy virus isolate with a number of complementary nucleotide pairs for three duplexes forming 3WJs that is more or equal to 20 (the conserved 3WJ structure for all 37 BFV isolates is highlighted)

Number	Structure	Genome position	Free energy ΔG, kcal/mol	Gene/ element	Number of complementary pairs
1		72–135	−18.2	5'LTR	6-7-4
2		136–217	−21.5	5'LTR	8-8-6
3		1,388–1,460	−19.8	between 5'LTR and *gag*	9-5-6
4		2,138–2,224	−25.2	*gag*	4-12-10
5		8,017–8,119	−14.9	*env*	8-8-5
6		8,112–8,174	−19.8	*env*	9-5-6
7		10,644–10,717	−28.2	*bel2*	5-9-10

We did not use 3WJs in HIV-1 RNA (Fig. 1a, Chu et al., 2019) as a control for determining putative 3WJs because this structure is energetically relatively unfavorable and it is formed only under conditions of forced formation of complementary pairs (Fig. 1b). The free energy of this 3WJs is equal −28.8 kcal/mol, while free energy of a more favorable secondary structure that does not form 3WJs for this HIV-1 RNA fragment is equal −37.8 kcal/mol.

There are sequences of the *gag* gene with a length of 1,620–1,665 nt with complete cds (protein coding sequence with a start codon atg and a stop codon taa are present) for 48 BFV isolates in GenBank database in addition to 37 BFV isolates with the complete genome, which are 40 nt overlap with the nucleotide sequence of the definite 3WJ conserved structure. Proviral DNA of 43 BFV isolates was extracted after a passage series of Madin Darby Bovine Kidney (MDBK) cells infected with the Riems BFV strain (Bao et al., 2020).

To determine the passage influence of BFV-infected cells on the conformation of the found 3WJ structure, we analyzed the localization of point mutations for the above-mentioned set of 43 *gag* gene fragments. 100% identity was determined for 17 fragments from 48 ones that we examined, including 43 fragments from the study (Bao et al., 2020) of *gag* gene with a length of 40 nt, which are a part of the found 3WJ structure with a length of 73 nt. For 31 fragments of the *gag* gene, we identified point mutations after passages. For 27 fragments of the *gag* gene, single nucleotide polymorphisms (SNPs) were found, which are localized in the hairpin loop (position 45 on the sequence of the 3WJ structure, Fig. 3). 3 *gag* gene fragments have SNPs in the base of the hairpin stem (positions 58 (for two fragments) and 62 on the sequence of the 3WJ structure, Fig. 3). One isolate (MF105962) is characterized by the presence of SNP in the base of the hairpin stem (position 62 on the sequence 3WJ structure, Fig. 3) and SNP in the loop (position 45 on the sequence 3WJ structure, Fig. 3). SNP in the stem of the hairpin (replacing AT pair with AC pair) results in a small decrease in the melting point of the hairpin and, therefore, of the whole 3WJ, but does not significantly affect the conformation of this alternative structure.

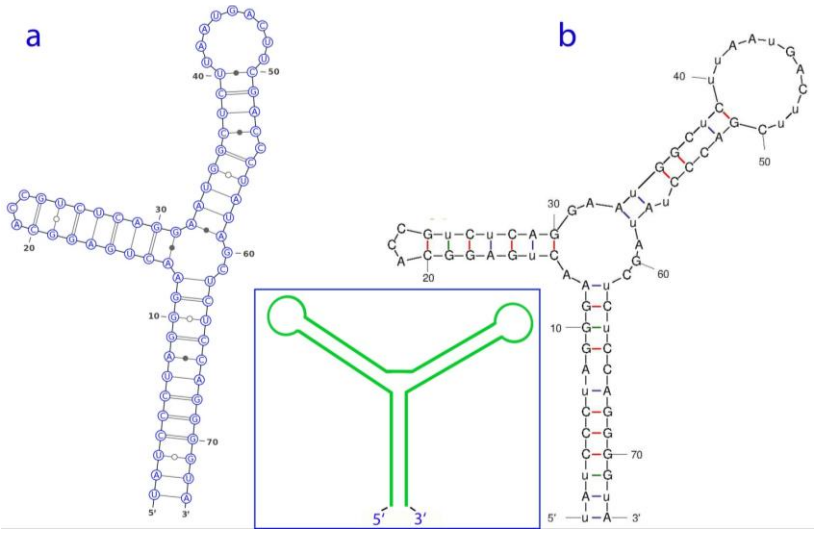


Figure 3. Conserved for 37 BFV isolates 3WJ structure which was found at the 5'-end of BFV *gag* gene (position 1,388–1,460 for isolate JX307861) and was determined by Vfold2D **(a)** and Mfold **(b)** software. 3WJ is characterized by 20 complementary nucleotide pairs, which are shown by segments with a dot (9, 6, 5 complementary nucleotide pairs in the 5'→3' direction for the three duplexes, respectively). The inset shows the 3WJ structure, which consists of three duplexes formed by two hairpins and a stem.

Conclusions. As shown here, in the mRNA of bovine foamy virus, a highly conserved 3WJ structure with a length of 73 nt was found, characterized by 100% identity for 37 BFV isolates with a complete genome. The mentioned intramolecular secondary structure which is localized between the 5'-LTR and 5'-end of the *gag* gene and partially covers the 5'-end of the *gag* gene is formed by three duplexes and stabilized by 20 complementary nucleotide pairs with free energy of −19.8 kcal/mol. The determined conserved RNA structural motif is energetically preferable to other putative 3WJ structures for this BFV sequence because it was calculated without forcing the formation of complementary pairs in the three duplexes forming this secondary structure. The data presented here support the conclusion that BFV is a unique and unconventional virus and its *gag* gene displays a significant difference in BFV molecular structure compared to the other retroviruses (Lindemann et al., 2021).

Our analysis of single-nucleotide polymorphisms in the paper (Bao et al., 2020), which arose after culture passages of BFV-infected cells has shown that found 3WJ structure is retained, indicating its importance for BFV functioning.

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