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Polypurine/Polypyrimidine Sequences with the Potential of Forming Triplexes in the Proviral DNA of Bovine Retroviruses

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Abstract—The perfect interstrand triplexes that could potentially arise in the proviral DNA of two widespread cattle retroviruses such as bovine leukemia virus (BLV) and bovine immunodeficiency virus (BIV) were determined. The fragments, which formed triplexes at acidic pH, were found in the genomes of both viruses; five fragments were found in BVL and 10 fragments in BIV. One of these fragments (it is localized in the BVL *gag* gene) might exist like a part of a cruciform structure. Existence of the triplexes was experimentally confirmed by visualization of supercoiled pGEMEX DNA with the use of atomic force microscopy; six fragments with mirror symmetry, which are necessary for formation of intramolecular triplexes, were found. Triplexes represent one of the elements of the signaling mechanisms of the genome function. Maps of triplex location in the cattle retroviral genome were built.

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INTRODUCTION

Genomic RNA and proviral DNA (integrated into the host cell genome of eukaryotes, prokaryotes, and viruses) are structurally flexible molecules. Depending on a nucleotide sequence (for example, in the presence of inverted repeats), double-stranded fragments (hairpins) could arise in single-stranded RNA. The formation of triplexes (which include a single-stranded fragment and a triple-stranded fragment) in double-stranded DNA under certain conditions (for example, in the presence of polypurine/polypyrimidine mirror repeats lengthwise along one DNA chain) is also possible [1].

Noncanonical structures formed in DNA and RNA are the "hot spots" of genomic instability in eukaryotes, prokaryotes, and viruses. Such capability is caused by the possibility of the formation of triplexes (or H-DNA), hairpins (in a single-stranded state) and cruciform structures (in a double-stranded state) in different genetic processes. Direct, inverted and mirror repeats are widely represented in the genome; they are involved in mutagenesis and in large genomic rearrangements. It was previously shown that inverted repeats block DNA replication in vitro [2–6] and also can serve as the stop sites in the elongation of the transcription by RNA polymerase [7].

Intramolecular inter-stranded triplexes are formed in homopurine (homopyrimidine) sequences with the formation of the triplets pur – pur: pyr (for example, AAT) or pyr – pur: pyr (for example, TAT and CGC^+) where pyr and pur are the pyrimidine and purine nucleotides, C^+ is a protonated cytidine phosphate. The sequence should contain a mirror repeat–it has to be the same in both the 3' ⟶ 5' and 5' ⟶ 3' directions lengthwise along one DNA chain. This is the difference between noncanonical and regular palindromes (inverted repeat), which forms a cruciform structure in the same sequence of a fragment in different DNA chains. Depending on the orientation of the third chain in relation to the central Watson–Crick chain, triplexes could be divided in two types, parallel and antiparallel. During the formation of parallel triplexes, the protonation of N3 cytosine for correct Hoogsteen pairing with N7 guanidine is needed. This is the reason why parallel triplexes are most stable at acidic pH. Unlike parallel triplexes, antiparallel triplexes do not need protonation and they are characterized by strong binding, which is not depended on pH.

In the parallel triplex, a triple helical pyrimidine motif binds in parallel orientation to a homopurine chain of double-stranded DNA by Hoogsteen hydrogenous pairing with the formation of triplexes similar to T–A:T or C^+–G:C, which contain the canonical pairs A:T and G:C, respectively. The binding of the third chain to a duplex is diminished at the physiological pH, because cytosine should be protonated to form a hydrogen bond. In the antiparallel triplex, a triple helical homopurine motif binds in antiparallel orientation to a homopurine chain of a DNA duplex by reverse Hoogsteen pairing with the formation of the A– A:T and G–G:C types of triplexes [8, 9].

It was previously found that long homopurine tracts with the potential to form triplexes are arranged nonrandomly in the eukaryotic genome: it is typical of them to be located in the neighbourhood of gene promoters, "hot spots" of recombination [10]. There is experimental evidence of the involvement of intramolecular triplexes in the cellular processes like replication, recombination, and transcription [11–14]. In the study of different features of triplexes, synthetic nucleotides, or long polypurine tracts, which were extracted from microorganisms and cloned in plasmids, are commonly used as the model systems [15]. At the same time, the real arrangement of the genome fragments, which are potentially able to form triplexes, remains unclear for viruses and bacteria. In connection with this, I conducted a search for the structures, which could potentially form interstranded triplexes in the genome of two retroviruses, bovine leukemia virus (BLV) and bovine immunodeficiency virus (BIV). The retroviruses were found in various animal species in a wide taxonomic range.

All retroviruses possess a common feature; namely, the necessity to synthesize DNA copies on the genome RNA template by a reverse transcriptase enzyme [16]. Lentiviruses belong to a unique type of retroviruses with similar structural, biological, and pathological features. The immunodeficiency viruses of humans, cattle, and cats and also the visna-maedi virus of sheep, the arthritis-encephalitis virus of goats, the equine infectious anemia virus, and the Jembrana disease cattle virus are related to lentiviruses. Lentiviruses are nononcogenic viruses; they cause slow, chronic, and degenerative pathological changes in the infected host cell. Such changes are associated with the development of lesions of the immune system [17]. All lentiviruses infect monocytes and microphages. Unlike other retroviruses, lentiviruses can replicate in nondividing cells [18].

BIV, which is structurally similar to HIV, is the lentivirus that causes long-term infectious disease in cattle after a variable asymptomatic phase; this disease is similar to AIDS [19]. BLV, a different worldwide spread retrovirus, is associated with a lethal form of leukemia and persisting lymphocytosis [20].

In this study, the arrangement of potentially perfect triplexes in BIV and BLV proviral DNA was found with the use of computer analysis; the physical maps of the viruses with the localized triplexes were presented. Based on the analysis of the built maps of the location of the potential triplexes, it was shown that the arrangement of triplexes in the BIV and BLV genomes were quantitatively and qualitatively different. The possibility of *in vitro* triplex formation was confirmed by atomic force microscopy (AFM) visualization of the supercoiled pGEMEX DNA, which contains the homopurine/homopyrimidine sequences.

MATERIAL AND METHODS

Computer analysis. The sequences of the completely sequenced isolates of the cattle leukemia virus (GenBank No. AF 033818, length 8419 bp), the cattle immunodeficiency virus (No. M32690, length 8482 bp), and also of the pGEMEX plasmid (length 3993 bp, "Promega", USA) were used in the study. To find triplexes and to determine their parameters, I used the Site program of the GeneBee software package [21]. The search for the interstrand triplexes was conducted according to the formulas $R_n \cdot N_k \cdot R_n$ and $Y_n \cdot N_k \cdot R_n$, where R are the purines adenine (A) or guanine (G), and Y are the pyrimidines cytosine (C) and thymine (T). The n value varied from 4 to 10, and the k value varied from 4 to 7.

Atomic force microscopy. In the study, I used the atomic force microscope Nanoscope IY MultiMode System ("Veeco Instruments Inc.", USA) with E-scanner. The AFM images of DNA were captured by a tapping mode AFM in air in the regime "height", with the use of the OMCL-AC160TS cantilevers ("Olympus Optical Co.", Japan) in the resonant frequency of 340–360 kHz, and the spring constant was 42 h/m. The images were obtained in the 512 × 512 pixels format; they were flatten and analyzed with the use of the Nanoscope software (version 5.12r3) ("Veeco Instruments Inc.", USA). Specimen preparation was performed according to the technique presented earlier [22]. Briefly, I used the following procedure. To apply a solution of DNA onto amino mica, I used the TE buffer (10 mM HCl, 1 mM EDTA, pH 7.6). 10 μl of DNA solution in the TE buffer were dropped onto a band of mica (measuring 1 cm^2), exposed for 2 min, rinsed with ultra distilled RNase-free water, argon was blown-off, dried and, then, visualized. It should be noted that the freshly cleaved mica has a total insignificant negative surface charge. In the modification of the freshly cleaved mica by derivative aminosilane vapor, the properties of aminosilane groups that are localized near a surface of mica are changed under the influence of a mica surface. If in an aqueous solution the ionization constant (pK) for aminosilane 3-aminopropyltriethoxysilane (APTES) is ~10 [23], the APTES ionization constant on a surface of mica is decreased approximately by 3 units, and pK is ~7 [24, 25].

RESULTS

It was found earlier that the possibility of *in vitro* formation of H-DNA in the homopurine/homopyrimidine fragments of a size less than 15 bp is problematic [26]. In the study [27], it was demonstrated that triplexes were formed for the $G_{16} \cdot C_{16}$ sequence; at the same time, they were not formed for the $G_{14} \cdot C_{14}$ sequence. Taking into account these data, for further analysis, I selected the BLV and BIV fragments that possessed a potential of triplex formation with parameters, such as, a repeat size of ≥11 nucleotides, stem length of ≥4 bp, and a loop size of up to 8 nucleotides.

Based on the determined potential triplexes in the genome of proviral DNA (Table 1), I built a diagram of their arrangement on the physical map of the BLV genome (Figure 1). The BVL proviral DNA approximately had a length of 8420 bp; this DNA was similar to the genome of other retroviruses with the typical location of the *gag*, *pol*, and *env* genes. The sequence and secondary structure of one of the found potential triplexes in the genome of BLV are presented in Figure 2. The proposed triplex model corresponds to a structure formed by perfect mirror repeats. At the same time, it was shown [28] that the presence of mirror symmetry was not necessary for the formation of the nucleotide triads in a triplex, and some nucleotides that did not coincide could be involved in interaction between the duplex and the third chain of a purine/pyrimidine sequence.

An analogical approach was used to obtain the diagrams of arrangement (Fig. 3) and the triplex parameters (Table 2) for the BIV. The BIV has the most complicated genome organization among lentiviruses (with the exception of the lentiviruses of primates). The length of the BIV proviral DNA is more than 8480 bp; the *vpw*, *vpy*, and *tmx* genes are unique bovine lentiviral genes.

The comparison of the diagrams of triplex arrangement in two BIV isolates (the BIV L04974 isolate was examined for the presence of triplexes lengthwise with the BIV M32690 isolate; the data are not shown) was carried out; it was shown that seven of nine potential triplexes (Table 2, positions 3–9) are the conservative structural motifs with identical sites of genome location of both BIV isolates. At the same time, the locations and sequences of two other triplexes were insignificantly different (both these triplexes are located in the *pol* gene, positions 1 and 2, see Table 2). The sequence and secondary structure of two triplexes found in the BIV are given in Figure 4. Joining the stem of one triplex with another neighbouring triplex may be flexible, depending on the length of the linker duplex between two triplexes. In the case of the double triplex found in the BIV genome, the length of the linker was 2 bp. Therefore, in this case, the formation only of the structure, a model of which is shown in Figure 4, a, is possible. At the same time, steric restrictions (Figure 4, b) make the formation of the U-like structure (a model of which was proposed in the study referred to here as 15) impossible.

Table 1. The triplexes that could be potentially formed in the proviral DNA of the cattle leukemia virus (AF033818 for the GenBank database)

No	Length		Sequence, genome location	Gene
	of a fragment	of a loop		
1	11	3	CTTTCTGTTTC 2373–283	*LTR*
2	18	6	TCTCCCTCGGCGCCCTCT 302–319	*LTR*
3	18	8	CCCCCTCCTATAACCCCC 452–469	*gag*
4	20	6	*CCCCCCCTTATGACCCCCCC* 740–459	*gag*
5	18	6	CCCGTACCCTCCTATGCCC 3621–3638	*pol*

Note: Here and in Table 2, the mirror-symmetrical repeats were underlined. The potential triplex highlighted by italics (a position 4) may be a part of a cruciform structure.

Triplex formation in the immobilization of DNA on the surface of amino mica from a solution at neutral pH was experimentally confirmed with the use of atomic force microscopy. I visualized several unusual single molecules of the supercoiled pGEMEX DNA; the fragments of these molecules formed a peculiar net (Fig. 5). Computer analysis showed that the pGEMEX DNA sequence contained six fragments with the potential of triplex formation. The stem length in three fragments was 5 bp, the stem length in two different fragments was 6 bp, and the stem length in one

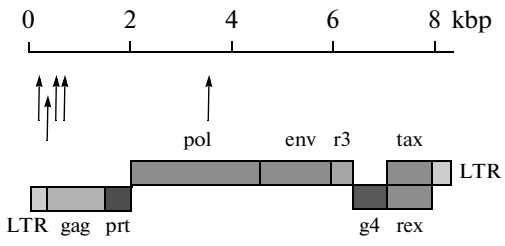

Fig. 1. A physical map of the genome of the cattle leukemia virus with indicated positions of the known genes. The positions of the determined mirror-symmetrical polypyrimidine sequences are shown by arrows.

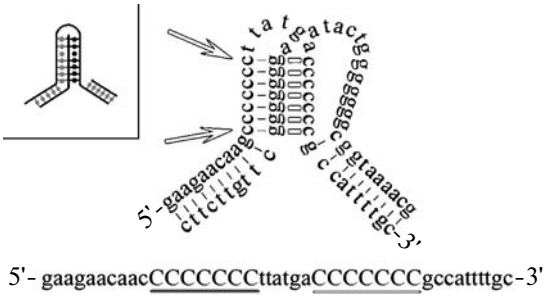

Fig. 2. The structure of the interstrand triplex (or H-DNA) for a fragment of proviral DNA of the cattle leukemia virus. The arrows indicate a triple helix. The highlighted fragments of a sequence "–" are the strands of the proviral DNA of the BLV indicate two symmetrical repeats, which are involved in triplex formation. The small rectangles show the interaction between the third strand of the triplex and a fragment of the double-stranded DNA caused by Hoogsteen pairing. A model of the triplex is given in the insert.

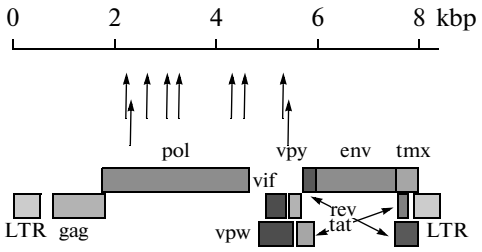

Fig. 3. A physical map of the genome of the bovine immunodeficiency virus with indicated positions of the known genes. The *rev* and *tat* genes consist of two encoding regions. The first encoding exon of the *rev* gene and the encoding region *tmx* have the same reading frame like the *env* gene. The encoding regions of the viral genome are shown by three horizontal bands that correspond to different reading frames. The arrows indicate positions of the determined mirror-symmetrical polypurine sequences.

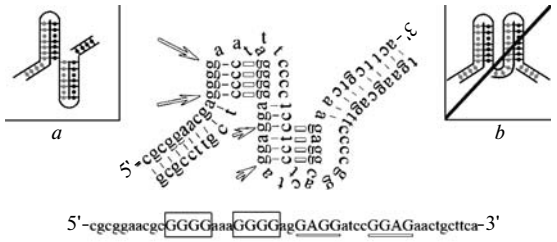

Fig. 4. The structure of two triplexes (or H-DNA) for a fragment of proviral DNA of the bovine immunodeficiency virus, which corresponds to the 5464–5488 position of the *vif* gene of the proviral DNA of the BIV. The arrows indicate a triple helix. In the rectangles and the highlighted by "+" mirror-symmetrical repeats are the strands of proviral DNA of the BIV, which form two triplexes. The small rectangles show the interaction between the third strand of the triplex and a fragment of double-stranded DNA caused by Hoogsteen pairing: insert *a* is the proposed double triplex model; insert *b* is a triplex model, which is impossible because of the steric restrictions that occur in the formation of the given secondary structure formed by two triplexes.

fragment was 7 bp. The loop size in all indicated fragments was 5 nucleotides.

Atomic force microscopy not only allows the visualization of molecules, but it also allows for the measurement of their geometric parameters. One such structural parameter (molecule height) was measured with the use of the construction of the pGEMEX DNA cross-section (Fig. 5, b). The height of a short interstrand triplex was determined from the cross-section profile and reached 0.75 nm.

Table 2. The triplexes that could be potentially formed in proviral DNA of the cattle immunodeficiency virus (M32690 for the Gen Bank database)

No	Length		Sequence, genome location	Gene
	of a fragment	of a loop		
1	13	5	GAAATTGTGAAAG 2169–2181	*pol*
2	13	3	AAGGGAACGGGAA 2259–2271	*pol*
3	12	4	AAGGTCCAGGAA 2713–2724	*pol*
4	13	5	AGGAGTAAAAGGA 3096–3108	*pol*
5	14	4	AGGGAAGAAAGGGA 3363–3376	*pol*
6	14	4	GGGGGAATAGGGGG 4489–4502	*pol*
7	15	3	GAAAGGAATGGAAAG 4616–4630	*pol*
8	11	3	GGGGAAAGGGG 5464–5474	*vif*
9	12	4	GAGGATCCGGAG 5477–5488	*vif*

DISCUSSION

The BLV and BIV cycle of replication is similar to the cycle of replication of other retroviruses. After the efflux of RNA molecules to the cytoplasm of the infected cell, the viral reverse transcriptase, which is encoded by the *pol* gene, transcribes RNA in a double-stranded DNA molecule (it is also known as the proviral DNA). Next, the proviral DNA is integrated into the host cell genome by the viral integrase. The proviral DNA may remain in the latent state or in the presence of certain signals it may serve as a DNA template for synthesis of new viral RNA molecules.

Retroviruses use diversity of cis-regulatory signals and auxiliary regulatory proteins for the modulation of different aspects of their replication and infectious effect. The retroviral genes' expression depends on numerous factors, including the interaction between transcription factors of the host cell with cis-regulatory elements located in long terminal repeats (LTR) and the conformation of genome regulatory fragments.

In the study referred to as 28, it was shown that an inverted repeat cloned in the plasmid (the stem length was 7 bp) was capable of forming a cruciform structure with a neighbouring fragment; however, at acidic pH the cruciform structure was destroyed and the indicated fragment formed a triplex. Only one fragment among all analyzed potential triplexes in the BLV and BIV proviral DNA (Table 1, position 4) may be involved in the formation of a cruciform structure (the results of analysis of the BLV and BIV genome RNA on the presence of hairpins, which were capable of forming cruciform structures in proviral DNA, were not shown). The data concerning the possibility of a trigger switch of a BLV proviral DNA fragment from the canonical double helix conformation to the triplex

conformation or the cruciform structure conformation indicate the specific role of the indicated fragment in a genome structure of BLV. In addition, the absence of perfect potential triplexes in the *env* gene of both retroviruses becomes noticeable. On the one hand, this confirms that the *env* gene is the most variable gene among the known BLV and BIV genes [29, 30] and, on the other hand, it gives additional information for the designing of primers in creating molecular-genetic test-systems for the detection and typing of cattle retroviruses.

It is well-known that parallel triplexes are more stable under physiological conditions compared to antiparallel [31]. The formation of parallel triplexes (in contrast to antiparallel triple helixes [9]) depends on the pH: homopurine/homopyrimidine sequences in DNA duplex form triple helixes at acidic pH (approximately pH 4) [32]. Under these conditions, the transition into H-DNA was observed in relaxed, nonsupercoiled DNA [32]. For DNA molecules, the indicated conditions can be modeled during their immobilization on the mica surface, which is used as a substrate in the studies by atomic force microscopy.

At the same time, the surface features of the mica effect on both the pK of APTES and the pK of nucleotides during DNA immobilization on the mica surface. This means that some nucleotides under conditions of DNA immobilization on modified amino mica can be protonated. One of the reasons for the formation of a visualized structure (Fig. 5) could be the intramolecular interstrand triplex formation. A triple DNA helix is formed for the fragments of purine/pyrimidine DNA sequences at pH ~4 in an aqueous solution; that is, under the condition of a decrease in the pH level by 3 units compared to a neutral pH value, the structural transition of B-DNA into H-DNA occurs [33]. The values of the ionization constants of the molecules immobilized on the mica shift by 3 units to an acidic pH.

It should be mentioned, that the diameter of a helix of double-stranded DNA is approximately 2 nm for the B-form according to the data of X-ray structural analysis [34]. At the same time, under the effect of the surface features of amino mica, the height of a DNA molecule immobilized on amino mica is decreased and it reaches 0.4–0.5 nm. The difference in the height values of B-DNA and triplex indicates that the structures, which are visualized with the use of AFM, are intramolecular triplexes. The effective formation of such structures arises due to protonation of nucleotides on the amino mica surface with a high density of a positive charge, which is necessary for triplex formation. A 1.5-fold increase of the triplex height compared to the B-DNA was obtained by me; these data coincided with the results of study referred to as 15 and aimed at the determination of structural parameters of triplexes during immobilization onto amino mica.

A similar net of plasmid DNA molecules, immobilized on freshly cleaved mica, was visualized [35] only

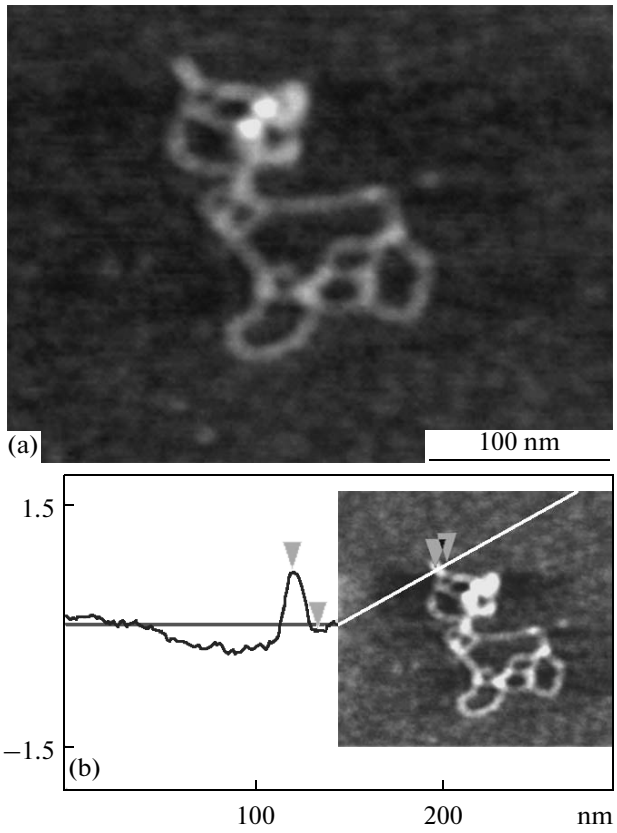

Fig. 5. The image of a single supercoiled pGEMEX DNA molecule obtained with the use of an atomic force microscope (a) (segments of a molecule form an original net; the image size is 345 nm × 345 nm), and the cross-section profile of the pGEMEX DNA fragment that formed a triplex (b) (the cross-section was made lengthwise along a line, perpendicular to the surface of the plan; the determined value of the triplex height, indicated by the two triangles, is 0.73 nm)

for a certain value of Mg^{2+} ion concentrations. Thus, the obtained results confirm that, firstly, triplex formation is possible in the presence of mirror purine/pyrimidine triplexes in the DNA sequence; secondly, supercoiled DNA form triple helixes only in a narrow interval of both the surface charge density of the mica and the DNA concentration.

Ascertainment of the arrangement of potential triplexes in the animal retroviral genome is one of the stages on the path to understanding the main components of the signal mechanism of the functioning of the virion genome. Intramolecular triplexes in the genome of cattle retroviruses may indicate the polymerase stop sites during replication and transcription.

Moreover, the determined triplexes may find use in gene therapy of the cattle retroviruses, namely, for modulation of the biological functions associated with DNA. The knowledge of specific targets in the DNA with a potential of triplex formation could be applied to create the molecules that are complementary to triplexes; this makes possible the further manipulation of

genes (i.e., a selective inhibition of genes). Such an approach is based on inhibition of the transcription of the appropriate genes, caused by complex formation with triplex motifs in the target DNA. Further basic research at the level of single molecules and cells will answer numerous questions, such as, the role of the indicated interstrand triplexes and how they function and interact with other key molecules.

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