Research Article

miRNA and tropism of human parvovirus B19

Olga Berillo [a], Vladimir Khailenko [a], Anatoly Ivashchenko [a], Lior Perlmuter-Shoshany [b], Alexander Bolshoy [b],*

[a] *Department of Biotechnology, Al-Farabi Kazakh National University, Al-Farabi prospect, 71, Almaty 050038, Kazakhstan*
[b] *Department of Evolutionary and Environmental Biology, University of Haifa, Mt. Carmel, Haifa 31905, Israel*

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ABSTRACT

Parvovirus B19 has an extreme tropism for human erythroid progenitors. Here we propose the hypothesis explaining the tropism of human parvovirus B19. Our speculations are based on experimental results related to the capsid proteins VP1 and VP2. These proteins were not detectable in nonpermissive cells in course of these experiments, although the corresponding mRNAs were synthesized. Our interpretation of these results is an inhibition of translation in nonpermissive cells by human miRNAs. We bring support to our hypothesis and propose detailed experimental procedure to test it.

1. Introduction

Parvovirus B19 (Anderson et al., 1983; Burton and Caul, 1988; Young and Brown, 2004) is pathogenic for humans and has a remarkable tropism for human erythroid progenitor cells (EPS). It was shown that aside from the cellular receptors required for virus entry into cells, successful accomplishment of the virus replication cycle is a key determinant of B19V tropism (Ozawa et al., 1988b; Liu et al., 1992; Wang et al., 1995). Nonstructural protein 1 (NS1) is produced in both permissive and nonpermissive cells but capsid protein synthesis appears to be restricted to permissive erythroid progenitors (Liu et al., 1992; Pallier et al., 1997). In nonpermissive cells, the capsid proteins (VP1 and VP2) are hardly detectable, although the corresponding mRNAs are synthesized (Pallier et al., 1997). Guan et al. (2009) hypothesized that translation of the capsid proteins either is inhibited in the cellular microenvironments of nonpermissive cell types (it may be also formulated as "absence of inhibition factors in permissive cells") or requires special cellular factors that are abundantly expressed in permissive cells.

A few explanations to the tropism of B19 appear in the literature: (i) an unusual promoter type of VP1 and VP2 (Wang et al., 1995), (ii) a sequence or a structure of the 3′ untranslated region of VP2 (Ozawa et al., 1988a; Pallier et al., 1997), (iii) an unusual RNA splicing pattern of viral mRNA (Brunstein et al., 2000), (iv) a block in full length transcript production in cells nonpermissive for B19 (Liu et al., 1992), and (v) a very recent explanation saying that a codon usage limitation is the reason to the tropism (Zhi et al., 2010). Zhi et al. (2010) reported cell type-specific expression of the B19V capsid genes and greatly increased B19V capsid protein production in nonpermissive cells by codon optimization. Moreover, they successfully generated B19 virus-like particles in nonpermissive cells by transient transfection of a plasmid carrying codon-optimized VP2 gene. Our hypothesis is related to the interpretation of the abovementioned results of Zhi et al. (2010) as inhibition of translation in nonpermissive cells by human miRNAs and absence of inhibition factors in permissive cells. However, neither the mechanism underlying the unique tropism of B19V genome replication in human EPCs, nor the cellular factors involved, have been identified (Chen et al., 2011). We propose our explanation to the tropism of B19 because, in our opinion, the above-mentioned theories are not sufficiently convincing.

We propose here a possible mechanism of the inhibition of translation of certain viral mRNAs in B19 nonpermissive cells that is related to the inhibition of translation initiation effected by miRNAs ((Mathonnet et al., 2007; Watanabe et al., 2007a) and references wherein). It is known that human miRNAs have a potential to regulate various human-infecting viruses via binding and down regulation of their target genes as a form of antiviral defense (Pedersen et al., 2007; Watanabe et al., 2007a; Mahajan et al., 2009); however, impact of human miRNAs on the regulation of translation of parvovirus B19 proteins is insufficiently

Table 1
64 isolates of the human parvovirus B19 (the synonym Erythrovirus B19) and their accession numbers.

E U38546.1	E U38511.1	H/p – FoBe AY768535.1	E AF113323.1	H/p N8 AB030673.1	H/p – AN30 AB126264.1	H/p BN30.1 DQ408301.1	R0416 DQ234769.2
E U38518.1	E U38510.1	H/p – NAN AY504945.1	H/p – C39 from in vitro culture – FN598218.1	H/p – C39 from plasma FN598217.1	H/p Gh2768 NS1 – AY582124.2	H/p – BN32.2 DQ333427.1	H/p – KU1 FJ591158.1
E U38517.1	E U38509.1	H/p AB550331.1	H/p AN87 AB126271.1	H/p AN85 AB126270.1	H/p – AN23 AB126262.1	H/p BN33.2 DQ333428.1	Vn115 DQ357065.1
E U38516.1	E U38508.1	C39 DQ293995.2	H/p – Br543 AY647977.1	H/p – AN66 AB126269.1	H/p – P1 – FJ265736.1	H/p BN31.2 DQ333426.1	Vn147 DQ357064.1
E U38515.1	E U38507.1	H/p J35 – AY386330.1	H/p NC_000883.1	H/p – AN56 AB126268.1	H/p BN30.3 DQ408305.1	R0748 DQ234779.2	AnTo DQ225151.1
E U38514.1	E U38506.1	H/p Gh3051 AY582125.2	H/p – LaLi AY044266.2	H/p – AN41 AB126267.1	H/p BN60.3 DQ408304.1	R0693 DQ234778.2	OsFr DQ225150.1
E U38513.1	E U31358.1	H/p – AN28 AB126263.1	H/p – AB030694.1	H/p – AN40 AB126266.1	H/p BN59.3 DQ408303.1	D1599 DQ234775.2	SN807 DQ225149.1
E U38512.1	H/p – D91.1 AY083234.1	E strain HV AF162273.1	H/p – Mi AB030693.1	H/p – AN34 AB126265.1	H/p BN58.3 DQ408302.1	R0227 DQ234771.2	KyMa DQ225148.1

Abbreviations: E – Erythrovirus B19; H/p – Human parvovirus B19

known. miRNAs belong to a group of non-protein-coding small RNAs (Lee and Ambros, 2001; Ambros et al., 2003; Griffiths-Jones, 2004, 2006, 2010; Kozomara and Griffiths-Jones, 2011). They take part in important biological processes and negatively regulate gene expression at the posttranscriptional level (Bartel, 2004) through translational repression (Wightman et al., 1993; Mathonnet et al., 2007), direct mRNA cleavage (Hecht et al., 2005), or mRNA decay mediated by miRNAs deadenylation (Wu et al., 2006; Wu and Belasco, 2008). miRNAs are also involved in regulating stem-cell differentiation (Kanellopoulou et al., 2005), cell-lineage differentiation, and development (Naguibneva et al., 2006).

To check the hypothesis that inhibition of translation is caused by miRNAs we should demonstrate: (a) there are human miRNAs that bind to viral mRNA coding VP2 gene and silence it in nonpermissive cells; (b) these targets exist in all isolates of B19; (c) targets of these miRNAs are destroyed by codon optimization; and (d) the genes that these miRNAs have been originated from their introns are not expressed in permissive cells. By means of bioinformatics, we can check only points a, b, and c. We would check whether available data on human miRNAs and various isolates of B19 support the hypothesis.

2. Materials and methods

2.1. Data

Using NCBI resources 64 different coding sequences of B19 VP2 gene were found available at the time of writing this manuscript. Some isolates are named Erythrovirus B19, the others – Human parvovirus B19. The list of these sequences is in Table 1. All these coding sequences are equal in length and are very similar. The sequence (gi|291045153:3296–4960, B19 isolate F27) was used as a reference viral sequence and named further "normal". The natural sequences from different isolates were compared with a synthesized DNA sequence coding the same VP2 protein as the gi|291045153 sequence but with a different codon usage. This sequence that will be named further an "optimized" sequence was taken from (Zhi et al., 2010) and got its name because of the "optimized" codon usage.

Pre-miRNA sequences and miRNAs were received from the database miRBase ((Griffiths-Jones, 2006, 2010; Kozomara and Griffiths-Jones, 2011), http://mirbase.org).

2.2. Methods

The program miRNA-Finder (http://sites.google.com/site/malaheenee/software/mirna-finder) has been used for search

pre-miRNA inside human genes. At present there are many computational methods for (Lewis et al., 2003; Rehmsmeier et al., 2004; Kruger and Rehmsmeier, 2006; Watanabe et al., 2006, 2007a,b; Creighton et al., 2008; Kim et al., 2009; Maragkakis et al., 2009a,b, 2011). For calculation of energy hybridization to individual target predictions was used software RNAhybrid ((Rehmsmeier et al., 2004; Kruger and Rehmsmeier, 2006) http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/). The algorithmic core of program is one of variations of the classic RNA secondary structure prediction (Zuker, 1989a,b). RNAhybrid determines the most favorable hybridization site between two sequences miRNA–mRNA. This program allows choosing the characteristics of the hybridization sites search (Rehmsmeier et al., 2004). The length and position of seed can be defined (Kruger and Rehmsmeier, 2006). RNAhybrid uses thermodynamics-based algorithms as well as other techniques (DIANA-microT http://diana.cslab.ece.ntua.gr (Maragkakis et al., 2009b, 2011), PicTar http://pictar.mdc-berlin.de (Krek et al., 2005; Rajewsky et al., 2005; Rajewsky, 2006). Some of other programs use algorithms of seed complementarity (TargetScan (Lewis et al., 2003, 2005) http://targetscan.org), miRanda (John et al., 2004) http://www.microrna.org). Watson-Crick pairing of the mRNA target site with this seed region seems to be the most important factor for miRNA target prediction (Zhuo and Nikolaus, 2011). Apart from seed pairing, pairing with 3′ end of miRNAs also plays a role in target recognition (Bartel, 2009). Base pairing mRNA with 3′ region of the miRNA can also compensate for a mismatch in the seed region. It can supplement seed pairing and consequently improves specificity and affinity binding. However, these so called "3′-supplementary sites" are rare and only have weak effect (Bartel, 2009). Our script E-RNAhybrid (http://sites.google.com/site/malaheenee/software/) computes a score (ratio $\Delta G / \Delta G_m$), p-value. A ratio $\Delta G / \Delta G_m$ value (%), where ΔG_m equals binding energy for miRNA with perfectly complementary nucleotide sequence, is computed. Reliability degree (p-value) is estimated, that relies on ΔG and its standard deviation. Threshold significance is set to $p < 0.002$.

3. Results

64 isolate sequences of mRNA *VP2* gene of parvovirus B19 (Table 1) and the optimized variant of VP2 (Zhi et al., 2010) were aligned. The multiple alignments demonstrate that all isolates have identical length and that parsimonious explanation is that the only mutations are of "substitution" type and no indels happened (Fig. 1).

Instead of showing all 65 sequences of the performed multiple alignment only the "normal" sequence (gi|291045153:3296–4960)
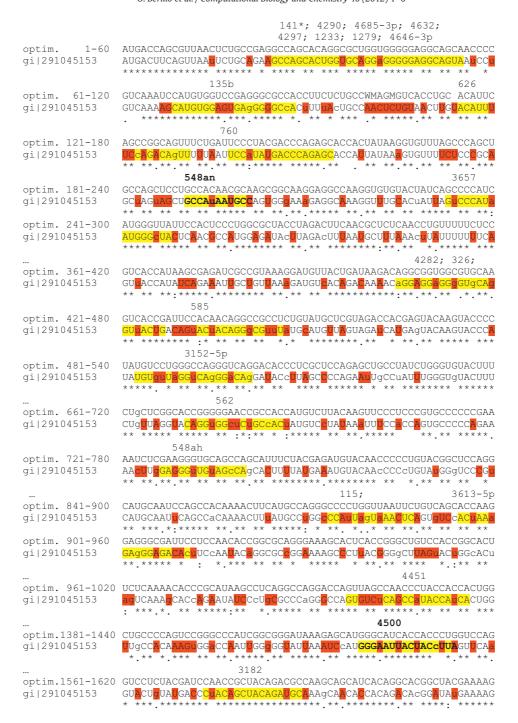
```
                          141*; 4290; 4685-3p; 4632;
                          4297; 1233; 1279; 4646-3p
optim.     1-60  AUGACCAGCGUUAACUCUGCCGAGGCCAGCACAGGCGCUGGUGGGGGAGGCAGCAACCCC
gi|291045153     AUGACUUCAGUUAAgUCUGCAGAAGCCAGCACUGGUGCAGGaGGGGGAGGCAGUAAuCCu
                 ************* ****** * *** ** *** ***** **** ***** ** ** ** *

                          135b                                      626
optim.   61-120  GUCAAAUCCAUGUGGUCCGAGGGCGCCACCUUCUCUGCCWMAGMGUCACCUGC ACAUUC
gi|291045153     GUCAAAAGCAUGUGGAGUGAgGGGGGCCAUUUnACUGCCAACUCUGUAACUUGUACAUUU
                 .      ************* .*** .***** *. ** * *** .* ***** ** ** ** **

                          760
optim. 121-180  AGCCGGCAGUUUCUGAUUCCCUACGACCCAGAGCACCACUAUAAGGGUGUUUAGCCCAGCU
gi|291045153     UCcAGACAgUUUUUAAUUCCaUAUGACCCAGAGCACCAUUAUAAaGUGUUUUCUCCCGCA
                 ** **.**. ** *** **.***** **** ** .  ** **.** ** ** ** **.***

                          548an                                     3657
optim. 181-240  GCCAGCUCCUGCCACAACGCAAGCGGCAAGGAGGCCAAGGUGUGUACUAUCAGCCCCAUC
gi|291045153     GCuAGuAGCUGGCCAuAAUGCCAGUGGgAAaGAGGCAAAGGUUUGCACuAUUAGuCCCAUa
                 ** ** **  **** ** ** ** * *.** **.*** **** *  ** ** * ** ** **.:

optim. 241-300  AUGGGUUAUUCCACUCCCUGGCGCUACCUAGACUUCAACGCUCUCAACCUGUUUUUUCUCC
gi|291045153     AUGGGCUACUCAACGCCAUGGAGAUACnUAGAcUUUAAUGCUUUAAAcgUAUUUUUUUCA
                 ***** ***** ** ** .******** ** .** ******:.**.** *.***** **

                          ...                                       4282; 326;
optim. 361-420  GUCACCAUAAGCGAGAUCGCCGUAAAGGAUGUUACUGAUAAGACAGGCGGUGGCGUGCAA
gi|291045153     GUuACCAUAUCAGAAAUUGCUGUGUUAAaGAUGUGCACAGACAAAACaGGAGGaGGGGUgCAg
                 ** ** **:*****.******* ** .** ** ** *********** .**.** * .**.

                          585
optim. 421-480  GUCACCGAUUCCACAACAGGCCGCCUCUGUAUGCUCGUAGACCACGAGUACAAGUACCCC
gi|291045153     GUuACUGACAGuACuACAGGgCGuuUaUGCAUGUUAGUAGAuCAUGAgUACAAGUACCCA
                 ** **.**.****.*  ** **  ** * .*  ** ** ***** **** ** *** ***

                          3152-5p
optim. 481-540  UAUGUCCUGGGCCAGGGUCAGGACACCCUCGCUCCAGAGCUGCCUAUCUGGGGUGUACUUU
gi|291045153     UAUGUguUaGGUCAgGGaCAgGAUACcUUAGCCCCAGAAuUgCCuAUUGGGGUgUACUUU
                 *****.. * ** **. ** ** ** ** **** * ** *** ******** ******

                          ...             562
optim. 661-720  CUgCUCGGCACCGGGGGAACCGCCACCAUGUCUUACAAGUUCCCUCCCGUGCCCCCCGAA
gi|291045153     CUgUUAGGUACAGGuGGcuCuGCCaCuAUGUCcUAUAAaAUUUCCaCCAGUGCCCCCAGAA
                 ** ***** ** ** ** .:*:.** **.*****.***** **.**.* **.** ** *****.

                          548ah
optim. 721-780  AAUCUCGAAGGGUGCAGCCAGCAUUUCUACGAGAUGUACAACCCCCUGUACGGCUCCAGG
gi|291045153     AACUUgGAGGGUUGUAGCCAGcGCACUUUUAUGAAAUGUACAAcCCCCUGUAgUGGgUCCCGu
                 ** **.**.** .** **.** **  ** **.******* ** ** * .** * ** ** *

                          ...                               115;          3613-5p
optim. 841-900  CAUGCAAUCCAGCCACAAAACUUCAUGCCAGGGGCCCCUGGUUAAUUCUGUCAGCACCAAG
gi|291045153     CAUGCAAUuCAGCCaCAAAACUUUuAUGCCuGGCCCAuUagUaAACUCAGUgUCcACuAAa
                 ** ****.*:.*** ** ***** **:** ** * .* .*.* ** **.** ** **.*:** *

optim. 901-960  GAGGGCGAUUCCUCCAACACCGGCGCAGGGAAAGCACUCACCGGGCUGUCCACCGGCACU
gi|291045153     GAgGGAGACACuUCcAAUACaGGCGCcGGAAAAAGCCCUuACGGGgCUUAGuAcUGGcACu
                 ** ***** * :.***.** .***** ** ***** **. ** * ** ** ** **

                          ...                                       4451
optim. 961-1020  UCUCAAAACACCCGCAUAAGCCUCAGGCCAGGACCAGUUAGCCAACCCUACCACCACUGG
gi|291045153     agUCAAAgCACCAGAAUAUCCCuGCGCCCaGGGGCCAGUGUCuCAGCCaUACCAuCACUGG
                 :  ***.*. ** *****:**.* * **** . ** .****** ** ** ** ** ***

                          ...                                       4500
optim.1381-1440  CUGCCCCAGUCCGGGCCCAUCGGCGGGAUAAAGAGCAUGGGCAUCACCACCCUGGUCCAG
gi|291045153     UUgCCACAAAGuGGaCCAAUUGGgGGUAUUAAAUCcAUGGGAAUUACUACcUUAGUUCAa
                 .*** .* *** ** ** ** *** ** ** ** **.*** **.**.** . *** ** **.

                          ...             3182
optim.1561-1620  GUCCUCUACGAUCCAACCGCUACAGACGCGCCAAGCAGCAUCACAGGCACGGCUACGAAAAG
gi|291045153     GUuACUgUAUGACCCuACAGCUACAGAUGCAAAgCAACACCACAGACAcGGAUAuGAAAAG
                 * **** *** ** ** ***** **** *** .. ** ** **** **.** *****:. *
```

Fig. 1. Comparison of the reference VP2 sequence gi|291045153:3296–4960 of B19 parvovirus (isolate F27) and its optimized sequence, and consensus results of multiple alignment these sequences with other 63 VP2 mRNAs of B19 isolates. The sites of hybridization mRNA–miRNAs inside the reference sequence are yellow-highlighted. The differences between the optimized and reference sequences are red-highlighted. The nucleotides of 64 sequences, which are not conservative, are designated by lower case letter (there is no asterisk). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

and its optimized sequence are shown in Fig. 1. All nucleotide positions that are identical for all 64 viral isolates appear in capital letters and marked by asterisk below; while variable nucleotides are designated by lower case letter (there is no asterisk below as well). All positions different between the optimized and reference sequences are red-highlighted. Potential miRNA targets along the normal sequence are yellow-highlighted.

To find locations of miRNA sites along VP2 mRNA Database of 1424 pre-miRNA was studied using the program Finder 1.2. 686 sequences of pre-miRNA were found inside introns of human genes. The energies of hybridization miRNA–mRNAs of normal and optimized sequences were calculated using RNAhybrid program. Selection criteria for miRNA sites were chosen following two criteria: (a) to select only those hybridization sites of miRNAs that have no more than one internal loop, which size is less or equal to a predefined maximal size, and no more than one bulge loop, which size is less or equal to a predefined maximal size; (b) to select only five sites of each miRNAs with the lowest energy (highest negative energy). Maximal internal loop size is the maximally allowed number of unpaired nucleotides in either side of an internal loop,

Table 2
miRNA sites in 64 mRNA isolates of VP2 gene of parvovirus B19.

#	miRNA	Gene-host of miRNA	# of iso-lates	Positions in mRNA of isolates, nt.	Range of ΔG miRNA–mRNA, (kcal/mol)	Range scores (%)	Range of > p
1	miR-4500[2]	RP11	64	1417; 2097	$-25.6 \div -21.4$	$71.6 \div 85.6$	$0.0002 \div 0.0011$
2	miR-548an[1]	PLEKHJ1	64	190; 871; 680; 681	$-25.2 \div -22.5$	$71.7 \div 80.3$	$0.0006 \div 0.0016$
3	miR-3613-5p[2]	TRIM13	59	401; 1574; 894	$-23.4 \div -19.8$	$68.8 \div 81.3$	$0.0003 \div 0.0019$
4	miR-760[2]	BCAR3	55	136; 817; 979	$-36.2 \div -33.3$	$70.1 \div 76.2$	$0.0005 \div 0.0015$
5	miR-3182[1]	CDH13	53	1572; 427; 1107; 13	$-23.3 \div -22.2$	$71.4 \div 74.9$	$0.0006 \div 0.0012$
6	miR-4297[1]	EBF3	53	36; 717	$-26.4 \div -23.9$	$72.4 \div 80.0$	$0.0003 \div 0.0010$
7	miR-135b[2]	LEMD1	50	67; 748	$-28.1 \div -27.7$	$70.7 \div 71.7$	$0.0011 \div 0.0013$
8	miR-4632[2]	TNFRSF1B	50	31; 32; 712	$-43.6 \div -37.4$	$69.6 \div 81.2$	$0.0003 \div 0.0016$
9	miR-626[1]	SCP2	48	114; 795	-23.3	68.9	0.0018
10	miR-3115[1]	KDM1A	47	872; 873; 1553	$-24.4 \div -23.5$	$68.7 \div 71.3$	$0.0012 \div 0.0019$
11	miR-141*[2]	U47924	46	25; 760	$-29.7 \div -28.7$	$69.5 \div 71.9$	$0.0011 \div 0.0016$
12	miR-548ah[1]	DNM3	45	726; 1406	-22.6	71.1	0.0012
13	miR-562[1]	LOC100286979	43	670; 1350	$-27.9 \div -23.4$	$70.1 \div 83.5$	$0.0002 \div 0.0015$
14	miR-3657[1]	NAA25	41	232	-26.5	68.8	0.0019
15	miR-4451[1]	ARHGAP24	37	996	$-24.6 \div -24.5$	$69.0 \div 69.3$	$0.0017 \div 0.0018$
16	miR-4646-3p[1]	BAT5	35	32; 713	$-31.5 \div -31.4$	$69.6 \div 69.8$	$0.0015 \div 0.0016$
17	miR-1233[1]	GOLGA8B	34	35; 716	-33.1	69.2	0.0017
18	miR-4290[2]	RP11	34	33; 714	-30.7	75.4	0.0006
19	miR-4685-3p[2]	HPS1	33	27; 708	-37.6	74.0	0.0007
20	miR-585[1]	HDHD1	33	429; 1109	-26.4	69.3	0.0017

Abbreviations: superscript[1] (e.g., miR-1233[1]) designates a pre-miRNA that was found by program miRNA Finder 1.2; superscript[2] (e.g., miR-135b[2]) designates a pre-miRNA that was found in miRBase. Only miRNAs with significant hybridization sites appeared in more than 16 out of 64 isolates are presented.

and maximal bulge loop size is the maximally allowed number of unpaired nucleotides in bulge.

The value of maximal potential hybridization energy for each miRNA was calculated according to self-hybridization with its complementary sequence and it is dependent on GC-content. The miRNAs hybridization sites are characterized by scores in range of 68.5–85.0% of the maximum potential hybridization energy value. The sites of miRNA-mRNAs with high statistical significance $0.0002 < p < 0.002$ were selected. The 41 intron-originated miRNA were found using the program Finder 1.2, and 24 miRNA were obtained from the database miRBase.

The locations of the selected miRNA sites in VP2 mRNA of F27 (normal sequence) are shown in Fig. 1. Some miRNA have over-lapping sites: for example, the miRNA sites miR-141*; miR-4290; miR-4685-3p; miR-4632; miR-4297; miR-1233; miR-1279; miR-4646-3p are located at positions 25–56 of the normal sequence. We can observe 2–6 substitutions inside each miRNA hybridization site along optimized sequence (see Fig. 1). Codon optimization leads to appreciable increasing of hybridization energy in all 65 cases which means that binding between mRNA and miRNA becomes less stable. Thereby the miRNAs potentially can inhibit translation of the normal sequence, but not of the optimized. These miRNA sites in optimized sequence have either low statistical significance ($p > 0.002$) or these miRNAs sites have so low probability to serve as binding sites that they were not counted by the program RNAhybrid at all.

Than the energy values of these miRNA hybridization sites were calculated for the rest isolates and placed in Supplementary Table alongside with other data related to these miRNAs. The column 4 named "# of isolates" designates in how many isolates sites of this miRNA were found. A reduced version of Supplementary Table is shown in Table 2. miRNAs that appeared in less than 16 out of 64 isolates were excluded. From Table 2 we can learn that the sites of only 2 miRNAs appeared to be significant (miR-548an in all 64 isolates and miR-4500 in 63 isolates). These sites were predicted with high statistical significance ($0.0003 < p < 0.0008$ and $0.0002 < p < 0.0005$, correspondingly). Table 3 presents the miRNA hybridization sites of miR-4500 and miR-548an correspondingly for all 64 isolates. In concordance with the 'seed' theory, 6–8 nt. at the 5′ end of miRNA have to be complementary to 6–8 nt. at the

Table 3
Loci of miRNA hybridization sites along mRNA of VP2 gene of parvovirus B19 (isolate B19).

miRNA	ΔG energy, kcal/mol	Locus of hybridization miRNA-mRNA	p <	Score, %
hsa-miR-4500	-25.6	``` Target 5' U 3' GGGAAUUACUACCUUA UCUUUGAUGAUGGAGU miRNA 3' U 5' ```	0.0006	75.4
hsa-miR-548an	-23.3	``` Target 5' CU AGUG 3' AG GCCAUAAUGCC UU UGGUGUUACGG miRNA 3' UU AAAA 5' ```	0.0007	74.2

3′ end of mRNA. Hsa-miR-4500 has 16 complementary nucleotide pairs of 5′ end miRNA and high stable energy of hybridization.

In Zhi et al. (2010) the expression profiles of B19V genes, VP1, VP2, NS1, and genes coding 11-kDa protein, 7.5-kDa protein, and a putative protein X, were analyzed. VP1 and VP2 expression levels were high in CD36⁺ EPCs, but very low or undetectable in other cells. In contrast, NS1, 11-kDa, 7.5-kDa, and X proteins were produced at high levels in all cells tested (Zhi et al., 2010). In turn, we also decided to calculate the energies of hybridization of these 2 miRNAs with mRNAs of all viral genes: with VP1, VP2, NS1 and others. Only genes VP1 and VP2 have sites of the miRNAs hsa-miR-4500 and hsa-miR-548an with high statistical significance $p < 0.002$. miRNA sites of these two miRNAs are not significant in other gene mRNA sequences. Sites of hsa-miR-4500 and hsa-miR-548an are located at positions 1417 nt. (or 2097 nt.) and 190 nt. (or 680 nt., 871 nt.) for mRNA correspondingly (Table 3). Rest of parameters of sites is similar to those of the gene VP2.

The abovementioned calculations support our hypothesis which assumes that targets of those human microRNAs (miRNAs) that bind to viral mRNA coding VP2 gene exist in all isolates of B19 and they do not exist in optimized sequence because they are destroyed by codon optimization substitutions.

4. Discussion

Despite variability of 64 virus isolates, two miRNA sites are con-served in all 64 viral sequences. Thus, it is possible that these genetic

loci, being evolutionary conservative, are involved in the regulation of translation. Possible action of intronic miRNA on mRNA depends on expression of a source gene, in which intron pre-miRNA is situated, which, in turn, depends on a cell type and the stage of its differentiation.

Infection by B19V is restricted exclusively to EPCs (Takahashi et al., 1990; Young and Brown, 2004); thus, expression of genes that may be related to inhibition of translation should be checked in EPCs and in nonpermissive cells. For such gene expression comparative study one may use so-called semi-permissive cells (Shimomura et al., 1993; Wong and Brown, 2006): human bone marrow cells (Ozawa et al., 1987), megakaryoblastoid cells (Leruez et al., 1994; Morita et al., 2001), and erythroid leukemia cells (the KU812Ep6 cell line (Miyagawa et al., 1999)). Similar microarray studies were performed in the past: Zhan et al. (2007) analyzed expression profiles of 295 miRNAs using microarray before and after their erythroid differentiation induction. They used 295 miRNAs in their experimental study and more than 100 miRNAs of them were found to be expressed in erythroid cells.

We have studied the database of 686 intronic miRNAs in silico and have determined 2 miRNAs, which probably are the best candidates to participate in translation inhibition in nonpermissive cells. Hsa-miR-4500 is encoded in RP11 gene on chromosome-13. Hsa-miR-548an is encoded in Cxorf57 gene on chromosome-X. (By the way, protein functions of these genes are unclear at present.) The question is: in which conditions these genes are expressed? Thereby possibility of translation regulation will be identified. If the genes encoded miRNAs hsa-miR-4500 and/or hsa-miR-548an are expressed in nonpermissive cells and are not expressed in permissive cells, then one or both of these miRNAs can negatively regulate on posttranscriptional level depending on a cell type.

Alternatively, a simpler experiment could be performed: synthesis of VP2 containing synonymous substitutions, which knockout hybridization sites, and check whether VP2 protein will appear in enlarged amounts in nonpermissive cells. Zhi et al. (2010) synthesized new sequences coding the original viral proteins but containing multiple synonymous substitutions. They constructed eight plasmids using codon-optimized VP2 (optVP2) and wild-type VP2 (VP2). Briefly, VP2 or optVP2 genes with or without the 3′ untranslated region of the B19V VP2 gene were inserted into a pcDNA(p6) vector. Likewise, VP2 or optVP2 with or without the 3′ UTR was inserted into pcDNA3.1 in which expression of the B19V capsid gene was controlled by the CMV promoter. Using these plasmids, expression levels of VP2 were evaluated in different cells by immunoblotting with anti-VP2 antibody. To check whether certain fragments of VP2 are responsible for inhibition of translation specific mutations would be planned. These mutations are of synonymous substitutions type and are neutral from the "protein point of view" but destroy a hybridization site.

4.1. Proposal to experiment 1

A new VP2 sequence with the site of hsa-miR-4500 (located at position 1417) UGGGAAUUACUACCUUAGUU that has been changed to UGGGCAUCACCACCCUGGUC would be synthesized and used instead of optVP2 in experiments like in (Zhi et al., 2010).

4.2. Proposal to experiment 2

A new VP2 sequence with the site of hsa-miR-548an (located at position 190) GC**U**AG**UAG**CUGCCA**U**AA**U**GC**C**AGUGG that has been changed to GCCAGCUCCUGCCACAACGC would be synthesized and used instead of optVP2 in experiments like in (Zhi et al., 2010). If it will not happen, then, may be, other miRNAs are involved in

translational regulation of VP1–VP2 gene expression and new high-throughput techniques or microarray analysis should be applied.

4.3. Hypoxia

What is the key event of the B19V life cycle: capsid assembly, replication of the B19V RF DNA or generation of the ssDNA B19V genome? In the recent study, Chen et al. (2011) expressed their belief that replication of the B19V RF DNA is the key event elevated in B19V-infected EPCs under hypoxia. The results of Pillet et al. (2004) clearly demonstrate that B19 erythrovirus protein expression and replication in EPS are upregulated by reduced oxygen supply. Hypoxia leads to higher proportion of viral proteins, and this could be due to a higher level of expression in each cell. May be, expression of Cxorf57 or RP11 gene is hypoxia related?

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.compbiolchem.2012.06.002.

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