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The search for microRNA genes in the regions of two very late genes of *Bombyx mori* nuclear polyhedrosis virus

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***Aim**. B. mori nuclear polyhedrosis virus (NPV) codes two very late genes – polyhedrin (ph) and p10. Search for miRs genes in these regions is of interest because the polyhedra, formed at the very late stage of the virus development, include small RNA of 50–60 nt. The present work was aimed at search for potential precursors of miR transcribed from the late promoter element RTAAG and the TATA promoter elements located in the ph and p10 genes regions. **Methods**. The search was performed using the bioinformatic programs for miR prediction: MiPred, miRNA SVM, Micropoces- sor SVM, and RNAfold. **Results**. It has been predicted that the re- gion of ph gene encodes two predicted miRs (bmoNPV-miR-1ph, bmoNPV-miR-2ph) and one predicted potential (C) precursor bmoNPV- pre-miR-1Cph, which is not a Dicer substrate. The region containing p10 gene encodes one predicted miR – bmoNPV-miR- 3p10. **Conclusions**. A possibility of regulation of the genes orf 1629 and p74 expression by the predicted miRs, located in the same regions of a complementary chain, is assumed.*

Keywords: nuclear polyhedrosis virus, Bombyx mori, microRNA, bioinformatic method, prediction.

Introduction. MicroRNA (miRs) are among three most prevailing classes of small non-coding RNAs of 20–30 nucleotides (miRNAs, siRNAs, piRNAs), initiating RNA-interfering. miRs are bioregulators of gene expression in eukaryotic cells. The biogenesis, functioning, biochemical and bioinformatic approaches to miRs study, their participation in the regulation of various cell processes as well as their relation to some pathology have been previously described in [1]. siRNA, piRNA, and other small non-coding RNA are described in [2]. Besides eukaryotes, miRs are also revealed in viruses, in particular, in large DNA-containing ones [3]. Among RNA-containing viruses miRs were found in human immunodeficiency virus [4, 5]. However, little is

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known about miRs role in the virus–cell interrelations. A few experimental articles and reviews on this problem have been published [6–8].

Baculoviruses are attributed to the class of large DNA-containing viruses. Nuclear polyhedrosis viruses (NPV) are an independent serological group of baculoviruses, virions of which integrate into the inclusion bodies – polyhedra – at the very late stages of the virus development. Polyhedra-forming protein (polyhedrin) is the product of one of two very late genes. The second gene, *p10*, encodes protein p10. The expression of both genes is initiated by the late promoter elements – A/G/T/ TAAG [9].

Search for miR precursors (pre-miR) in RNAs, transcribed from two very late promoters of genome of *B. mori* NPV is of interest because the polyhedra of *B. mori* NPV, formed at the very late stage of the virus development, include not only virions, but also small RNA of 50–60 nucleotides [10]. This allowed us suggest their being pre-miR as these molecules are known to be of 50 - 100 nucleotides. Predicted pre-miR, included into polyhedra, is most likely to be processed from the very late transcripts and seized by polyhedrin in the process of polyhedra formation. Both mRNA of polyhedrin and p10 are attributed to these very late transcripts [9]. It is also possible that polyhedra may include either pre-miRs, processed from other late transcripts, or host pre-miR-let7. The rise of miR-let7 synthesis at the stage of larva transformation into pupa was observed by the authors of [11]. We used exactly this stage of the insect development to isolate polyhedra for their investigation (cocoons, containing dead larvae). Further biochemical investigation on RNA from polyhedra would help clearing out which small RNA is included into polyhedra.

The current work presents the results of bioinformatic approach to the search for pre-miRs and miRs not only in the transcripts, synthesized from the TAAG-promoter element for two very late proteins, but also in alternative transcripts (alts), synthesized from the predicted TATA promoter elements, located in the *ph* and *p10* genes regions of the *B. mori* NPV genome.

Materials and Methods. The nucleotide sequence of the genome of *B. mori* NPV was obtained from ICTVdB Management (2006) 00.006.0.01. Nucleopolyhedrovirus (ICTVdB – The Universal Virus Database, version 4. Columbia University, New York, USA (http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/00.006.0.01.htm).

Among existing programmes for microRNA prediction we selected the ones, the algorithm of which does not have the criterion of conservatism, since viral microRNAs are not conservative in contrast to microRNA of eukaryotes. The secondary structure of alts (hypothetical primary transcripts - h-pri-miR) was investigated using RNAfold programmes (http://rna.tdi.univie.ac.at/cgibin/RNAfold.cgi) [12]. The programme of predicting and processing pri-miR was used to search for alternative transcripts of sls (stem-loop structure) of 48–150 nt, which are Drosha and Dicer substrates (https://demol.interagon.com/miRNA/). Predicted pre-miR and mature miR were considered as substrates with the score, exceeding the intersection of curves of sensitivity (Se) and specificity (Sp) – >–0,55 [13]. The hairpins, processed by Drosha, but not processed by Dicer, were considered to be candidate (C).

The nucleotide sequences of hairpin structures, revealed in the alternative transcripts, were also studied using RNAfold programme. The processed hairpins were considered as sls with the value of free energy folding of –23.0 kcal/mol [12] or (in terms of kilojoules) –96.6 kilojoules/mol. Searching for miR in *B. mori* genome using RNAfold programme, Tong et al. [14] selected the value of free energy "exceeding 105 kilojoules/mol" as a "filter". The value, accepted by us, was 100 kilojoules/mol. The search for real and pseudo pre-miR was performed using miPred programme (http://www.bioinf.seu.edu.cn/ miRNA/index.html) [15].

The search for mature miR in the predicted pre-miR was performed using miRscan programme (http://genes.mit.edu/miRscan/) [16]. The nucleotide sequence of the investigated pre-miR was introduced in miRscan as the first and second sequences.

Results and Discussion. The late transcription in baculoviruses is initiated by TAAG-promoter element and terminated by polyT-sequence [9]. It is known that three polyhedrin transcripts of 1,16; 3,4, and 4,9 thousand b.p. [17] and two p10 transcripts of 0.75 and 2.5 thousand b.p. [18] are synthesized in *Autographa californica* NPV. There are no similar data regarding *B. mori* NPV. Since *B. mori* NPV is a genotypic variant of *A. californica* NPV, it is possible to assume the same

situation for the former. This was our basis for determining the boundaries of genome regions of *B. mori* NPV for the search of miRs. The regions, containing only two predicted polyhedrin transcripts (1.16 and 3.4 thousand b.p.) and both p10 transcripts, were selected for the investigation. This selection was conditioned by the fact that transcripts of 1.16 and 3.4 thousand b.p. cover gene *orf 1629*, and the transcript of 2.5 thousand b.p. – gene *p74*, located on the complementary chain. Polyhedrin transcript of 4.9 thousand b.p. was not investigated since it goes beyond the selected region. The location of the defined polyhedrin region in the genome of *B. mori* NPV is 128298–3404, and that of p10 region – 108411–110961. If A in AUG codon is taken for the reference point, these regions are –116–3404 (hereinafter ph) and –86–2565 (hereinafter p10), respectively. Transcripts of 1.16 and 3.4 thousand b.p. correspond to transcripts –51–1129ph and –51–3404ph; two p10 transcripts – to transcripts –71–630p10 and –71–2565p10.

As shown, the secondary structure of –51–1129ph transcript contains two stem-loop structures, one of which (slsph) is processed into the mature miR, and the other (sls2ph) does not pass the filters of the programmes, used. The secondary structure of the second transcript –51–3404ph contains 12 hairpins, sls1ph among them. Among the remaining stem-loop structures, three do not pass the programme filters and eight are processed only into pre-miRs. Since the figures of secondary structures are too lengthy, they are not presented in the current work, while sls characteristics will be considered further with regard to the discussion of alternative transcripts. –51–1129ph is likely to translate into polyhedrin [17].

Therefore, according to our prediction the transcript –51–3404ph may be h-pri-miR. A similar situation is observed for two transcripts of –86–2565p10 region. Our data demonstrate that the secondary structure of a smaller transcript (0.75 thousand b.p.) contains the only sls1, processed into miR. Besides sls1, a larger transcript (2,5 thousand b.p.) contains six hairpins, which are processed in pre-miR, but do not pass the filters of other programmes. Similar to transcript –51–3404ph, transcript –71–2565p10 may act as h-pri-miR.

Logics of the approach to the search for miR in alternative transcripts are presented below. All the existing programmes of predicting candidate pre-miR are based on the search for some stem-loop structures, corresponding to specific requirements. In reality pre-miR hairpins are processed from the primary transcripts – pri-miR. The search for h-pri-miR among alts is complicated, because promoters, from which pri-miR transcription is initiated, are not determined exactly, though pri-miRs are known to be transcribed by RNA-polymerase II from TATA-promoters mainly [19]. The transcription of pri-miRs may take place from other sequences as well [20]. We decided to start the search for miRs from the prediction of h-pri-miRs among various alts. The boundaries of alternative transcripts were determined from predicted promoters TATA to polyT (at least four T) sequences.

The region –116–3404ph contains six predicted promoters and 33 polyT-terminating sequences, and the region –86– 2565p10 – nine promoters and 19 polyT-sequences. These data were the basis for our investigation of 148 alts-ph and 114 alts-p10. 148 alts-ph contain 19 unique sls-ph. Then unique alternative transcripts were selected according to the following principle: besides the required hairpin, alt, minimal in size, should contain a minimal number of other hairpins. Only 11 unique transcripts out of 148 alts-ph contained all 19 sls-ph, and 16 unique ones out of 114 alts-p10 contained 21 sls-p10. All the results of investigation of sls characteristics are presented in Tables 1 and 2.

As shown in Table 1, sls3-ph, sls6-ph, sls7-ph, sls10-ph, sls17-ph, sls19-ph are processed from unique alt-ph, while other sls-ph – from two or more alt-ph. Sls1-p10, sls5-p10, sls7-p10, sls9-p10, sls14-p10, sls16-p10, sls18-p10–sls21-p10 are also processed from unique alt-p10 (Table 2). All alts, containing the only processed sls, may be considered as candidates for h-pri-miRs.

The data of Table 1, column 5, demonstrate that 13 sls-ph (sls1–sls6, sls8–sls12, sls16, sls17) are Drosha substrates, while among 21 sls-p10 (Table 2, column 5) – 18 (sls1–sls4, sls6–sls13, sls15–sls17, sls19–sls21) are Drosha substrates. Among 32 selected sls, five sls-ph (sls1, sls3, sls11, sls12, sls16) and five sls-p10 (sls2, sls7, sls8, sls17, sls19) pass the “real” and “pseudo” filters (Tables 1 and 2, column 6). Among 10

Table 1.
The characteristics of stem-loop structures (sls) in alternative transcripts (alt), synthesized from region 128298-3404 of the B. mori *NPV genome encoding mRNA of polyhedrin*

Sls	Localization in alt	Start	Length, nucleotides	Drosha	Real	-E, kilojoules/mol.	Dicer
1	**–7–683**	**248**	**75**	**–0,421**	+	**114**	**–0,52**
2	–7–683	538	50	**–0,504**	–	64	–0,504
3	–51–1272	634	68	**0,024**	+	87	–0,661
4	744–970	775	67	**–0,219**	–	65	<–1,0
5	744–970	840	75	**–0,239**	–	73	<–1,0
6	–51–1003	941	60	**–0,33**	–	61	<–1,0
7	744–1331	1018	60	–0,88	–	36	<–1,0
8	744–2139	1046	56	**–0,204**	–	43	<–1,0
9	744–1672	1092	73	**–0,218**	–	70	–0,992
10	744–1407	1284	57	**–0,292**	–	58	<–1,0
11	**744–1672**	**1305**	**74**	**0,392**	+	**106**	**–0,425**
12	**744–2139**	**1540**	**69**	**–0,158**	+	**140**	<–1,0
13	744–2446	1607	65	нет	+	96	Нет
14	744–2446	1842	59	–0,729	+	80	<–1,0
15	744–2446	1905	65	–0,711	Pseudo	136	<–1,0
16	744–2446	2080	56	**–0,101**	**Pseudo**	71	–0,967
17	1851–2339	2169	50	**–0,22**	–	63	<–1,0
18	744–2446	2306	90	Нет	Pseudo	85	Нет
19	1851–3107	2724	56	Нет	–	73	Нет

N o t e. Both here and in Table 2 column 1 presents numbers of sls in the order of less proximity to the reference point. Columns 2 and 3 show localization of alt, containing the corresponding sls, with regard to the reference point and the position of the start nucleotide of sls, respectively. Columns 5 and 8 show the estimation of sls Drosha and Dicer substrates, respectively, in accordance to the programme of predicting and processing pri-miR [13]; "no" means absence of processing centres in the corresponding sls. In column 6 indications «+» – «real» hairpin, «–» – hairpin «cannot be real» is in accordance to miPred programme [15]. Column 7 shows the values of free energy of folding of sls in accordance to RNAfold programme [12]. The characteristics of sls, which passed the filters of corresponding programmes, are shown in bold.

selected real and pseudo hairpins, real sls1-ph, sls11-ph, sls12-ph and sls2-p10 pass the RNAfold filter (Tables 1 and 2, column 7). Among four selected "real" and "pseudo" hairpins, three (sls1-ph, sls11-ph, sls2-p10) are Dicer substrates. Therefore we accepted the hairpins, processed from them, as predicted pre-miR and indicated them as bmo-pre-miR-1ph, bmo-pre-miR-2ph and bmo-pre-miR-3p10, respectively, and we considered alts, containing them, as predicted h-pri-miRs (see their location in Tables 1 and 2). Sls12-ph does not pass Dicer filter. The authors of [13] consider hairpins that are not Dicer substrates to be candidate (C) miRs. Therefore, we indicated sls, passing all the filters, but for Dicer, as predicted candidate precursors of miRs – pre-miR-1Cph, and alts, containing them, as corresponding h-pri-miR (see Tables 1 and 2).

Fig. 1 presents secondary structures of three h-pri-miRs. All three h-pri-miRs contain two sls each.

Table 2.
The characteristics of stem-loop structures (sls) in alternative transcripts (alt), synthesized from region 108281-110842 of the B. mori NPV genome encoding mRNA of p10

Sls	Локализация в alt	Старт	Длина, нуклеотиды	Drosha	Real	–E, кДж/моль	Dicer
1	–86–144	–83	64	**–0,316**	–	40	<–1,0
2	**–30–301**	**66**	**65**	**0,637**	**+**	**112**	**0,009**
3	–30–301	214	54	**0,054**	–	53	<–1,0
4	–30–491	319	76	**–0,288**	–	52	<–1,0
5	323–1556	326	89	–0,616	–	65	<–1,0
6	–30–491	396	70	**0,326**	–	70	<–1,0
7	**387–611**	**423**	**95**	**–0,35**	**Pseudo**	77	<–1,0
8	323-1405	686	81	**–0,426**	+	88	<–1,0
9	550–980	742	57	**0,093**	–	51	–0,529
10	–30–980	808	48	**–0,521**	–	77	<–1,0
11	323–1405	817	62	**–0,142**	–	85	0,750
12	–30-980	926	51	**–0,477**	–	83	<–1,0
13	550–1307	960	47	**–0,178**	–	83	–0,943
14	–71–1112	989	65	Нет	Pseudo	91	Нет
15	550–1307	1084	50	**0,141**	–	60	–0,067
16	1203–1307	1203	105	**–0,008**	–	131	–0,902
17	323–1405	1222	60	**–0,008**	**Pseudo**	88	–0,902
18	1203–1556	1472	81	–0,793	Pseudo	102	<–1,0
19	1203–1870	1614	58	**–0,108**	+	84	0,618
20	411–2565	1952	52	**–0,024**	–	99	<–1,0
21	2233–2565	2364	83	**–0,188**	–	48	<–1,0

N o t e. See Table 1.

Although sls2-ph is Drosha and Dicer substrate, it is not real, and does not pass the filter of folding free energy (Table 1). As for sls9-ph and sls3-p10, they are Drosha substrates, but they do not pass the filters of other programmes. Therefore, we indicated h-pri-miRs, presented in Fig. 1 as h-pri-miR-1ph, h-pri-miR-2ph and h-pri-miR-3p10. Fig. 2 presents the secondary structure of h-pri-miR-1Cph. Sls11 is processed to mature miR-2ph, and sls12 – to candidate pre-miR-1Cph. Fig. 3 demonstrates three sls, processed to mature miRs, and one sls, processed to candidate pre-miR-C.

Using the developed programme of predicting virus miRs (Vir-Mirdb), the authors of [21] revealed 11 pre-miR in the plus-strand of the genome of *B. mori* NPV, from which 22 miRs are cut out (one miR from each shoulder of pre-miR). It is hard to agree to these data as a mature miR is usually cut out from one 5'-shoulder. Besides, all miRs, predicted by the authors of [21], contain 26 nucleotides each, while miRscan has apparently the length of miRs, equal to 21 nucleotides, which is closer to the length of miRs *in vivo*. The same source of the genome nucleotide sequence was used by

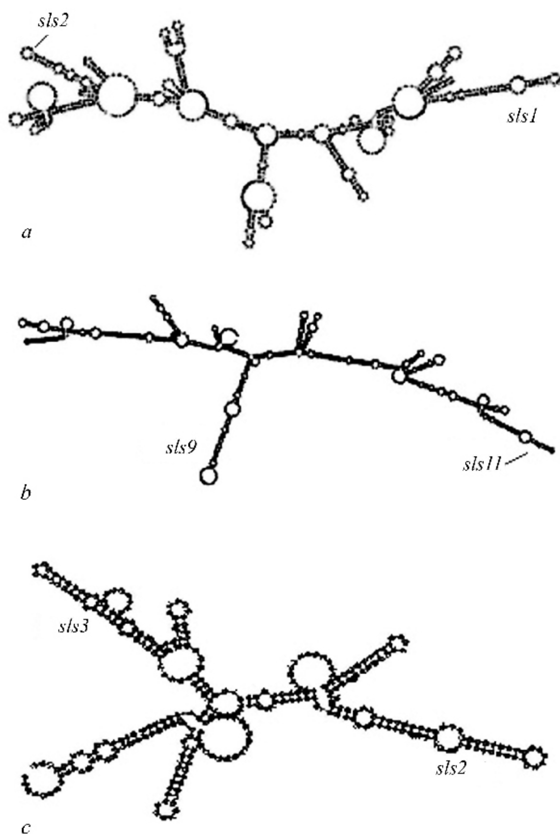

Fig.1 The secondary structures of minimal alternative transcripts, synthesized from putative TATA-promoter to polyT-sequence, accepted as hypothetical pri-miRs, containing a minimal number of stem-loop structures (*sls*): *a* - alt-ph - 7-763, accepted as h-pri-miR-1ph (hereinafter the reference point was AUG-codons of mRNA of polyhedrin or p10); *sls1* is processed to the mature miR; *b* - alt-ph 744-1672, accepted as h-pri-miR-2ph (*sls11* is processed to the mature miR); *c* - alt-p10 30-301, accepted as h-pri-miR-3p10 (only sls2 is processed to the mature miR)

these authors and us; nevertheless, they have not revealed pre-miR-1ph, pre-miR-2ph and pre-miR-3p10, predicted by us. However, they found a hairpin, which suits pre-miR-1Cph, predicted by us. Contrary to their data, this pre-miR-1Cph is not processed by Dicer as Table 1 (sls12) demonstrates.

It is noteworthy that while miR-1ph, miR-2ph and miR-3p10 are the only representatives among h-pri-miRs, predicted by us, they are also found in all investigated alts, containing the regions of their localization. It is possible that predicted mature miRs – miR-1ph, miR-2ph and miR-3p10 – will also be present, and therefore, processed in other unknown real alternative transcripts, synthesized in the cell (not only from promoters TATA and TAAG to polyT-sequence). miRs-ph, predicted by us, are completely complementary to mRNA *orf 1629*, and miR-3p10 – to mRNA *p74*. Therefore, if these miRs exist, they should function similar to si-RNA. In such case mRNA should split in the regions, complementary to the predicted miRs. Since miRs-1ph and miR-3p10 are complementary to 3'-UTR mRNA orf 1629 and p74, respectively, the participation of these miRs in the regulation of expression of genes *orf 1629* and *p74* t may be assumed.

Similar situation is possible for of *A. californica* NPV. As shown in [17], a transcript of 3.2 thousand b.p. is synthesized from the complementary chain in the region of *A. californica* NPV, containing the polyhedrin gene. It contains two open reading frames (*orf 1629* and *orf 603*) and covers the polyhedrin gene. The synthesis of transcript starts prior to mRNA of polyhedrin and it vanishes with the appearance of polyhedrin, though its fragments are still observed.

In 1990 the authors explained this phenomenon by three reasons: 1) destruction of promoter complexes from the 3'-end of polyhedrin gene by RNA-polymerase, transcribing polyhedrin; 2) formation of double-stranded RNA from polyhedrin mRNA; 3) negative regulation of promoter *orf 1629* by polyhedrin. At that time they could not assume the participation of microRNA in this process, as microRNAs were discovered only in the beginning of this century. We assume the participation of miR, encoded in polyhedrin gene, in the regulation of synthesis of the transcript, containing *orf 1629* and *orf 603*. In this case transcript of 3.2 thousand b.p. will be split. We plan further investigation on the detection of miRs in the region of the genome of *A. californica* NPV, containing polyhedrin genes *orf 1629* and *orf 603*.

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Поиск генов микроРНК в участках генома, содержащих два очень поздних гена вируса ядерного полиэдроза *Bombyx mori*

Резюме

Цель. Вирусы ядерного полиэдроза (ВЯП) B. mori кодируют два очень поздних гена – ph и p10. Интерес к поиску генов miR в этих участках генома обусловлен тем, что полиэдры, образующиеся на очень поздней стадии инфекции, включают в себя не только вирионы, но и малую РНК длиной 50–60 нуклеотидов. Цель настоящего сообщения состояла в поиске miRs в альтер-

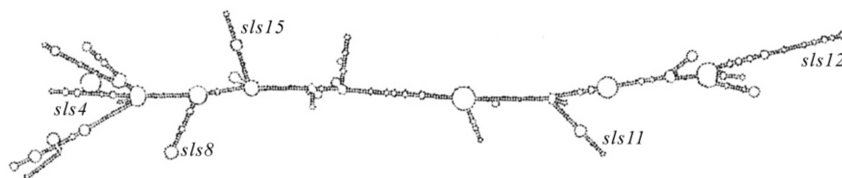

Fig.2 The secondary structure of alt-ph 744-2139, accepted as h-pri-miR-1Cph (only *sls12* is processed to pre-miR-1Cph)

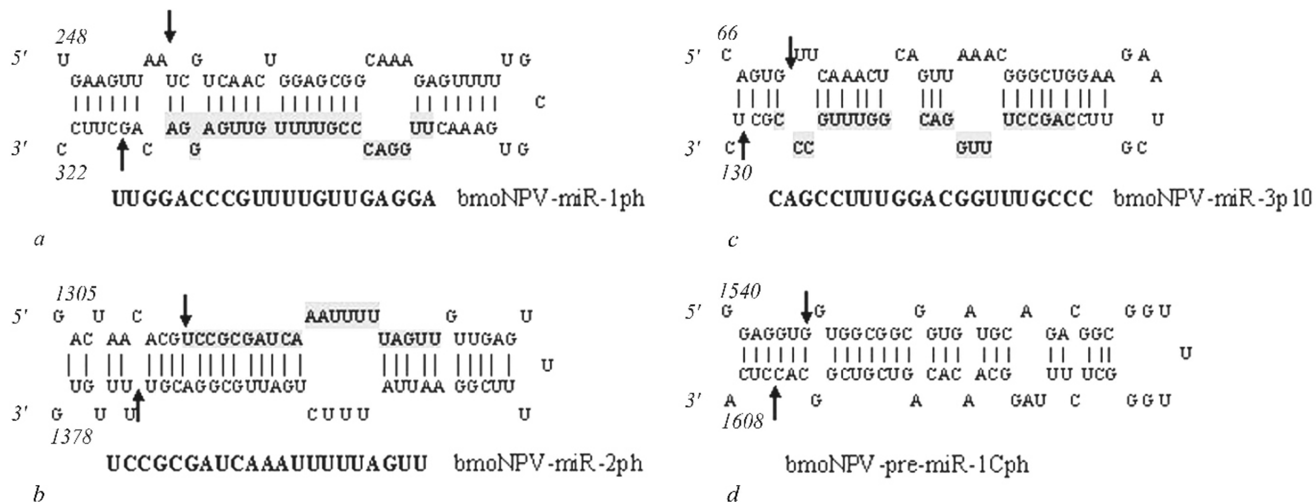

Fig.3 The secondary structures of sls1-ph (*a*), sls11-ph (*b*), sls2-p10 (*c*), sls12-ph (*d*) and predicted miRs. The centers of processing by Drosha to hairpins pre-miR-1ph, pre-miR-2ph, pre-miR-3p10, pre-miR-1Cph are indicated with arrows. The mature miRs, present in pre-miRs, are shaded.

нативных транскриптах, синтезируемых не только с TAAG-промоторного элемента, но и с TATA-промоторных элементов, расположенных в участках генома ВЯП B. mori, включающих гены ph и p10. ***Методы****. Поиск miRs осуществляли с помощью биоинформатических программ предсказания miR: MiPred, miRNA SVM, Microprocessor SVM и RNAfold.* ***Результаты****. Предсказано, что участок, содержащий ген ph, может кодировать две miRs (bmoNPV-miR-1ph, bmoNPV-miR-2ph) и один потенциальный (C) предшественник miR – bmoNPV-pre-miR-1Cph, не являющийся субстратом для фермента Dicer. Участок, содержащий ген p10, может кодировать одну предска- занную miR – bmoNPV-miR-3p10.* ***Выводы****. Обсуждается возможность регуляции экспрессии предсказанными miRs генов orf 1629 и p74, расположенных в тех же участках комплементарной цепи.*

Ключевые слова: вирус ядерного полиэдроза, Bombyx mori, микроРНК, биоинформатический подход, предсказание.

Т. В. Ширина, А. А. Висловух, М. Т. Бобровська, Е. А. Козлов

Пошук генів мікроРНК у ділянках геному, які містять два дуже пізніх гени вірусу ядерного поліедрозу *Bombyx mori*

Резюме

Мета*. Віруси ядерного поліедрозу (ВЯП) B. mori кодують два дуже пізніх гени – ph і p10. Інтерес до пошуку miR у цих ділянках геному обумовлений тим, що поліедри, які утворюються на дуже пізній стадії інфекції, містять у собі не тільки віріони, але й малу РНК довжиною 50–60 нуклеотидів. Мета даного повідомлення полягала в пошуку miRs в альтернативних транскриптах, синтезованих не лише з TAAG-промоторного елемента, а й з TATA-промоторних елементів, розташованих у ділянках геному ВЯП B. mori, що включають гени ph і p10.* ***Методи****. Пошук miRs здійснювали за допомогою біоінформатичних програм передбачення miR: MiPred, miRNA SVM, Micro- processor SVM і mFOLD.* ***Результати****. Передбачено, що ділянка, в якій локалізується ген ph, може кодувати дві miRs (bmoNPV-miR-1ph, bmoNPV-miR-2ph) та потенційний попередник miR – bmoNPV-pre-miR-1Cph, що не є субстратом для ферменту Dicer. Ділянка, у якій розміщений ген p10, може кодувати одну miR – bmoNPV-miR-3p10.* ***Висновки****. Обговорюється можливість регуляції експресії передбаченими miRs генів orf1629 і p74, розташованих на тих же ділянках комплементарного ланцюга.*

Ключові слова: вірус ядерного поліедрозу, Bombyx mori, мікроРНК, біоінформатичний підхід, передбачення.

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