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SEARCH FOR THE PUTATIVE RNA THERMOMETERS IN THE GENOME OF HEPATITIS E VIRUS

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Summary. Currently, some temperature-sensitive elements in bacteria are known. Structurally and functionally different RNA thermometers control a variety of cellular processes in bacteria including virulence. Up-to-date experimental confirmation of RNA thermometers functioning in viruses was obtained only for West Nile virus. But other, unknown yet, types of RNA thermometers may exist in nature. The goal of this study was the determination of conservative stem-loop structures in the swine, wild boars' hepatitis E virus (HEV) genome which may act as RNA thermometers. The search for putative RNA thermometers in the swine HEV which is a common pathogen in the pig population worldwide was executed. Bioinformatics analysis was used to predict the secondary structure of the linear RNA fragments and to determine the melting temperature of the potential hairpins in the HEV genome. 108 swine, wild boars genotype 3 and genotype 4 HEV isolates with complete genomes from the GenBank database were analyzed for the availability of stem-loop structures. Conservative hairpin with the putative thermoregulating function was found in genotype 3 HEV isolates from pig and wild boar for 64 HEV isolates from 108 analyzed ones. The stem of the hairpin with a length of 37 nt contains two AUG start codons of translation initiation and the melting temperature of the hairpin is equal to 38–42 °C for ionic strength of 0.165 M Na+. These hairpins contain a metastable element (one or two bulges) in the stem. Conservative secondary stem-loop structures with putative thermoregulating function for genomic RNA of 64 HEV isolates with complete genome were found by bioinformatics analysis. These hairpins contain a metastable element (one or two bulges) in the stem like an RNA thermometer of West Nile virus and satisfy the necessary and sufficient conditions of RNA thermometer formation. Determined stem-loop structures are proposed as putative thermoregulator elements because they are highly conservative uncanonical structures that are present in the genomes of 64 HEV isolates from 108 analyzed ones

Keywords: hairpin, stem-loop structure, pigs, wild boars

Introduction. Initiation of translation (protein synthesis) is one of the fundamental processes that is regulated through gene expression. Interaction between ribosome and mRNA sequence (Shine–Dalgarno sequence, SD sequence) is required for translation initiation in bacteria. As a rule, AUG start codon of translation initiation is located not further than 15 nucleotides from the SD sequence.

Pathogenic microorganisms often react to temperature increases by induction of expression of virulence genes. At low temperatures, the SD sequence is located within the stem-loop structure formed by the genomic RNA of a pathogen. An increase in temperature destabilizes the stem-loop structure so that the ribosome binding site (SD sequence) becomes available and the translation may be activated.

Currently, some temperature-sensitive elements in bacteria are known. Structurally and functionally different RNA thermometers control a variety of cellular processes in bacteria including virulence.

All the known RNA thermometers are structures with one or two extended stem-loop structure or several ones. Hairpins may be perfect or unperfect (i.e. hairpins may contain mismatched nucleotides in the stem of hairpin). Hairpin with mismatched nucleotides in the stem is shown on Fig. 1.

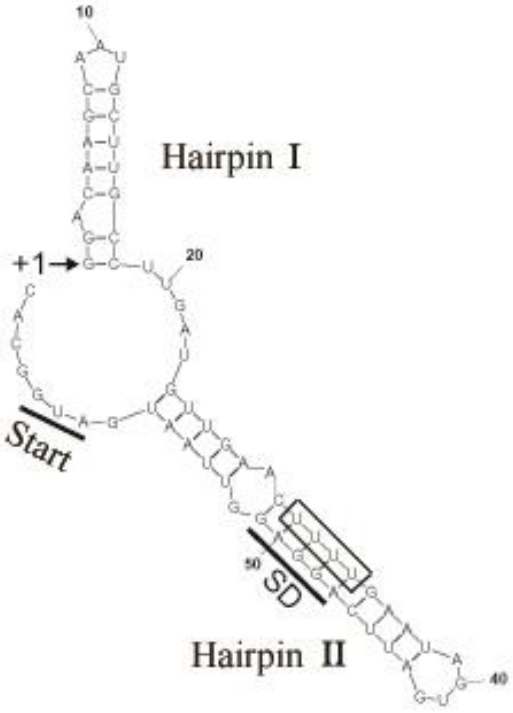

Figure 1. The secondary structure containing the predicted and confirmed 4U RNA thermometer in the *agsA* gene of *S. enterica* (Waldminghaus et al., 2007) at 20 °C. The Shine–Dalgarno sequence in the stem of the hairpin and AUG translation initiation site are highlighted.

Based on bioinformatics analysis of 25 *Salmonella enterica* isolates with complete genomes, the algorithm and criteria for the search for new putative RNA thermometers in the genome of bacteria were developed by the author and coauthors in the paper (Limanskaya et al., 2013).

For *S. enterica* in addition to the known 4U RNA thermometer (Fig. 1) four stem-loop structures were proposed as putative thermoregulator elements (Limanskaya et al., 2013). These hairpins were located in 5'-UTR of virulence regulators *gltB* and *yaeQ*. Predicted elements (SeT1, SeT2, SeT3 (which can be in two different conformations) and SeT4) locate in 5'-UTR, contain SD sequence at a specific distance to translational start codon and their secondary structures are similar to U6 synthetic thermometer structure (Fig. 2).

Experimental checking of four putative thermoregulators was performed in *E. coli* which contained pBSUx plasmid with cloned 5'-UTR RNA thermometers. GFP was used as the reporter gene and the putative thermometer sequence was applied as 5'-UTR. The regulatory role of these 5'-UTR constructs at the translational level was proved by Nothern blot analysis (Fig. 3).

The analysis of RNA and protein accumulation under different temperatures was performed. All tested constructs have shown thermoregulatory function.

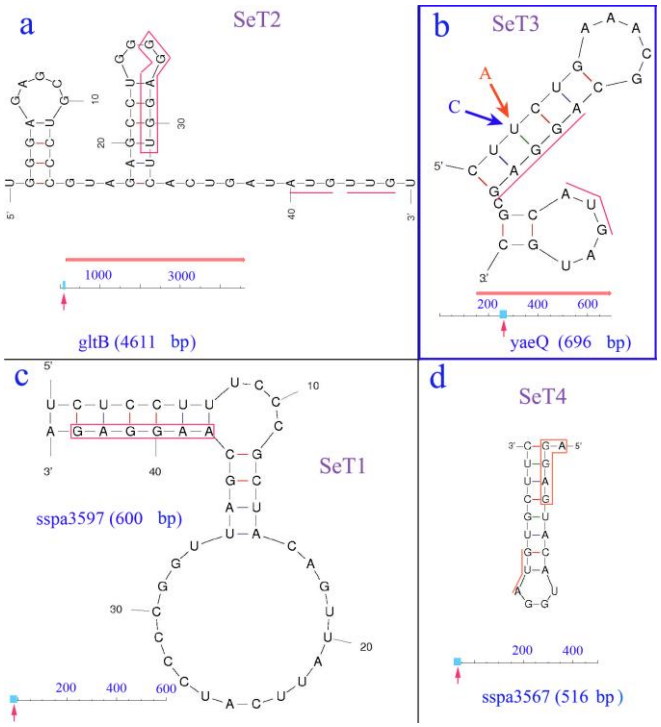

Figure 2. Putative thermoregulator elements in the genome of *S. enterica* are located in 5'-UTR of *gltB* (a — SeT2), *yaeQ* (b — SeT3), *sspa3597* (c — SeT1), *sspa3567* (d — SeT4) genes containing Shine–Dalgarno sequence (in stem-loop structure) and AUG start codon (Limanskaya et al., 2013).

They inhibit GFP expression at 22 °C but allow GFP expression at higher temperatures. All thermoregulators allow GFP expression at 37 °C. GFP expression is strongly induced at 42 °C (fever-related temperature) for all the *Salmonella* thermoregulators. SeT3a and SeT3b have shown clear induction of protein accumulation, but no a significant change in mRNA accumulation (Neupert et al., 2013). Up-to-date experimental confirmation of RNA thermometers functioning in viruses was obtained only for West Nile virus (WNV) (Meyer et al., 2020). But other, unknown yet, types of RNA thermometers may exist in nature.

The goal of this study was the determination of conservative stem-loop structures in the swine, wild boars' hepatitis E virus (HEV) genome which may act as RNA thermometers. The search for putative RNA thermometers in the swine HEV which is a common pathogen in the pig population worldwide was executed.

Materials and methods. The Mfold software package (www.unifold.org) (Zuker, 2003) and Blast (http://blast.ncbi.nlm.nih.gov) were used to predict the secondary structure of the linear RNA fragments and to determine the melting temperature (T_m) of the potential hairpins at the physiological ionic strength (I = 0.2 M Na^+, $[Mg]^{2+}$ = 0.0 mM or I = 0.15 M Na^+, $[Mg]^{2+}$ = 0.2 mM).

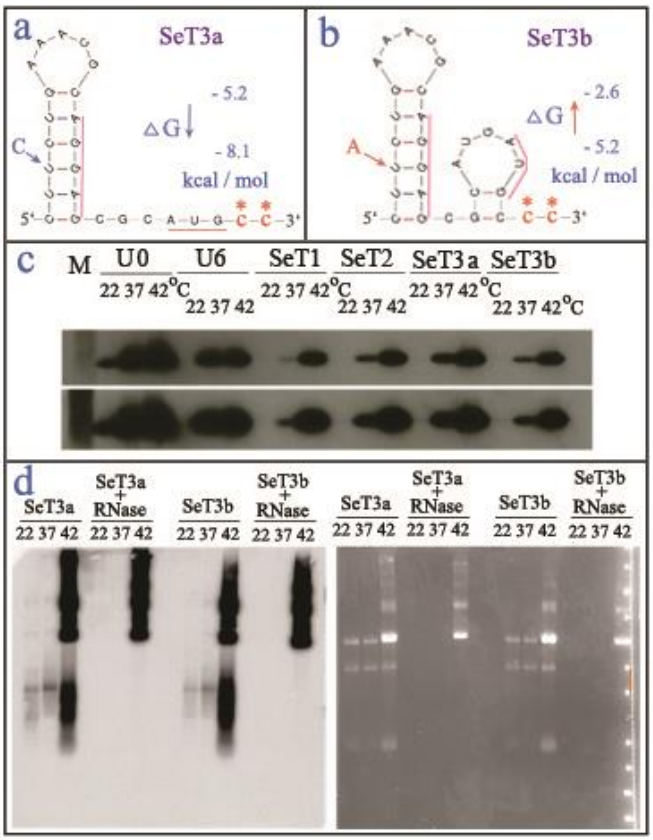

Figure 3. Putative thermoregulator elements from *S. enterica* (a, b) and results of their testing in *E. coli* by Western blot (c) and Nothern blot (d) at different temperatures (Neupert et al., 2013).

108 swine, wild boars genotype 3 and genotype 4 HEV isolates with complete genome from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank) were analyzed for the availability of stem-loop structures.

Previous computer and thermodynamic analyses of RNA thermometers in the *S. enterica* isolates have allowed us to determine the necessary criteria for a potential RNA thermometer. The search for HEV putative thermoregulators was performed for hairpins which are located in 5'-UTR (untranslated region), the melting temperature of the hairpin is within the 37–43 °C range (at the physiological ionic strength), AUG start codon is located in the stem of stem-loop structure.

Results and discussion. Conservative hairpins with putative thermoregulating function were found in genotype 3 HEV isolates from wild boar (Fig. 4a) and pig (Fig. 4b) for 64 HEV isolates from 108 analyzed ones. The stem of the hairpin with length of 37 nt contains two AUG starts codons of translation initiation and its melting temperature is 38–42 °C for ionic strength of 0.165 M Na+. These hairpins contain one (bulge; Fig. 4b) or two (Fig. 4a) metastable elements (buldges; Fig. 4b) in the stem.

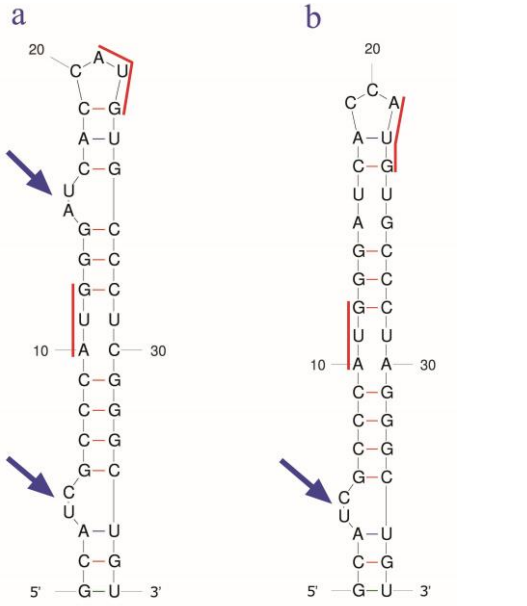

Figure 4. Putative RNA thermometer in the genome of hepatitis E virus with a length of 37 nt. Translation initiation sites AUG are highlighted. Sites AUG are located in the stem and loop of hairpin for genotype 3e HEV isolate from wild boar (a, accession number in the GenBank AB780450, position 5,135–5,171 nt) and genotype 3e HEV isolate from a pig (b, accession number in GenBank MH184579, position 5,156–5,192 nt). Arrows indicate bulges.

Conservative fragment of HEV genomic RNA with a length of 91 nt contains three AUG start codons of translation products (ORF3, ORF2S, ORF2C) of ORF3 and ORF2 viral genes (Fig. 5).

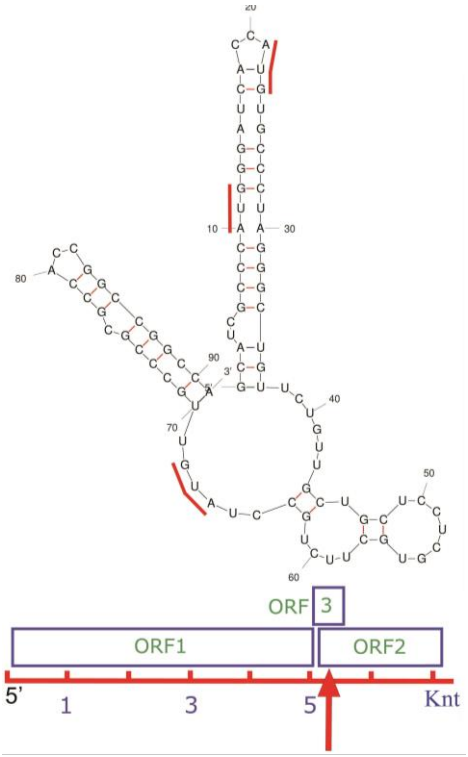

Figure 5. Secondary structure of 91 nt swine HEV fragment with two stem-loop structures including RNA thermometer. Location of putative RNA thermometer on swine HEV isolate F19 with complete genome with length of 7,239 nucleotides (nt; accession number in GenBank MN614429, position 5,147–5,237 nt) is shown by arrows. The viral genome contains three open reading frames (ORFs): ORF1 (position 6–5,132), ORF2 (position 5,167–5,497), ORF3 (position 5,129–5,497).

Determined HEV hairpin is similar to a viral RNA thermometer in WNV (Meyer et al., 2020). The calculated Tm of the hairpin (with a length of 59 nt and stem of 15 bp) of WNV RNA thermometer is equal to 54 °C for 0.2 M Na+ ionic strength. WNV RNA thermometer contains a metastable element, which consists of two conserved base pairs that are flanked by two symmetrical bulges. At the normal temperature of the human body, this RNA thermometer exists in stable conditions that lead to low kinetics of virus replication.

At the favored temperature, AUF1 p45 host protein interacts with the hairpin of WNV and destabilizes stem-loop structure with the following switching from the linear to the circular conformation of the viral RNA and fast pathogen replication. Both for human body and for pig, wild boar the temperature of 42 °C is fatal. We hope that HEV hairpin could interact in the swine body with host destabilizing protein in a similar manner. The authors of the paper (Meyer et al., 2020) explained that their hairpin works as an RNA thermometer that modulates flavivirus replication during host switching.

The Putative HEV RNA thermometer is located in the 5'end of ORF2 gene (ORF, open reading frame). The

ORF2 is located at the 3' end of the genome and encodes the major capsid protein that contains immunogenic epitopes, induces neutralizing antibodies, and is the target for vaccine development (Jameel et al., 1996; Xing et al., 2011). ORF2 contains an endoplasmic reticulum signal peptide (Graff et al., 2008). Three glycosylation regions have been identified in ORF2, but the biological relevance of these potential modifications is unclear (Pérez-Gracia et al., 2015).

The ORF2 capsid protein interacts with the 5' end of the viral RNA which probably plays a role in the viral RNA encapsidation (Surjit et al., 2004). Recent reports suggested that ORF2 protein is processed into at least two forms, including one or two forms of secreted glycoproteins that are not associated with infectious particles, and one unglycosylated form which is the structural component of infectious particles (Yin et al., 2018; Montpellier et al., 2018).

All ORFs are expressed during viral infection, as antibodies against these regions have been detected in naturally infected humans and experimentally infected monkeys (Khudyakov et al., 1994). Testing putative HEV RNA thermometer may be performed as in the paper (Meyer et al., 2020). Differential melting curves for HEV hairpins to determine melting temperature as of whole hairpin as of bulges at physiological ionic conditions may be performed for preliminary checking. To obtain more functional data, experiments with some mutants that stabilize or destabilize the thermometers may be performed.

Conclusions. Conservative secondary stem-loop structures with putative thermoregulating function for genomic RNA of 64 HEV isolates with complete genome were found by bioinformatics analysis. These hairpins contain a metastable element (one or two bulges) in the stem like an RNA thermometer of West Nile virus and they satisfy the necessary and sufficient conditions of RNA thermometer formation.

Importantly, the determined stem-loop structures (Figs 4–5) are highly conservative uncanonical structures that are present in the genomes of 64 HEV isolates from 108 analyzed ones. They may control translation initiation in the eukaryotic host like a similar RNA thermometer of WNV controls virus replication in the host.

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