Free-living microorganisms are subject to the fluctuations of environmental conditions, (temperature, pH, nutrient concentrations, etc.) which are continuously varying. In bacteria a complex network of protective mechanisms, which involves a range of cellular components from the membranes to proteins and DNA and RNA molecules, was developed as temperature-sensitive elements for effect prevention of temperature fluctuations.

Pathogenic microorganisms often react to a temperature of ~37°C by expression induction of the virulence genes. The regulation of expression of the environment-controlling genes may occur at the level of transcription by the interaction with the regulatory proteins. Several RNA-based posttranscriptional mechanisms were, however, recently discovered [1]. It was found out that some tRNA molecules, apart from providing the substrate for the ribosomes, contain certain control elements which modulate their expression depending on environmental conditions. Structural modifications in such sensor RNA depend on specific environmental variations.

Two fundamentally different classes exist, namely, cis-acting RNA elements, which contain the regulatory potential within mRNA sequences, and trans-acting ones, the small, non-encoding RNA molecules, acting by pairing of their nucleotides with the complementary mRNA sequences localized in other genomic loci [2]. Unlike the classical attenuators, which regulate the structure of the RNA leader sequence in accordance to the position of the translating ribosome, cis-acting RNAs change their conformation in response to the chemical or physical signals. The so-called riboswitches monitor the metabolic state of the cell by binding to metabolites with high specificity and affinity. They are localized in the 5'-UTR region (untranslated region) of the genes encoding for the biosynthesis, absorption, or degradation on minor metabolites and control the feedback for these metabolic pathways. Binding of a small molecule pushes the conformational switch, which changes the gene expression in one of three ways: (i) by untimely termination of transcription; (ii) by initiation of translation; or (iii) by mRNA processing. In the bound state, most riboswitches switch off expression. However, few riboswitches were found which switch on the expression of the genes.

Unlike highly specific riboswitches binding with metabolites, RNA thermometers, a closely related type of sensor mRNA, react to a general physical sig-
nal, i.e., to the intracellular temperature, which is an important parameter affecting, among others, expression of the genes encoding the cold and heat shock proteins and the virulence genes and is controlled by the duplexes (e.g., formed by the hairpin stem of the RNA thermometer). Nucleic acids are known to melt at increasing temperature. Thus, a temperature shift may modulate the conformation of the regulatory RNA molecules, i.e., transition of molecular fragments from a hairpin conformation to a single-stranded state.

A number of the presently known structurally and functionally different RNA thermometers control a variety of cellular processes. All the known molecular thermometers, both cis- and trans-acting, control translation by the isolation of the ribosome-binding site; most of them are localized in the 5'-UTR of heat shock or virulence genes. At low temperatures, the Shine–Dalgarno sequence (SD sequence; 5'-aaggag-3'; 5-rraggak-3' the prokaryotic consensus sequence; 5'-uygcu-3' the sequence for gram-negative bacteria) [2] is masked (located within the hairpin-loop structure). An increase in temperature destabilizes the hairpin-loop structure, so that the ribosome-binding site (SD sequence) becomes available and translation may be activated (AUG is the starting codon for initiation of translation).

The first RNA thermometer operating by the melting mechanism was found in the E. coli gene rpoH, which encodes the alternative sigma factor σ32, or RpoH [3]. The alternative RpoS sigma factor plays a central part in the regulation of the OspC and OspA external surface proteins associated with virulence in the Lyme disease caused by spirochete Borrelia burgdorferi. Temperature is one of the key environmental parameters controlled by RpoS, while DsrABb, a small noncoding RNA molecule, regulates the temperature-dependent increase in RpoS content. It was suggested [4] that DsrABb at 23°C forms a stable double-stranded structure, preventing pairing with the rpoS transcript. Increase in temperature results in melting of the RNA molecule and disruption of its secondary structure, which leads to the binding of the anti-SD region by the mRNA rpoS. This may stimulate translation by exclusion of the SD sequence and the site of translation initiation from the double-stranded structure in mRNA rpoS under virulence initiation conditions (37°C).

The ROSE (Repression Of heat-Shock gene Expression) element is probably the most widespread bacterial RNA thermometer. It suppresses expression of the heat shock genes. This element was found in many α and β-proteobacteria, including E. coli and Salmonella enterica [5]. The ROSE element (ranging from 60 up to 100 nucleotides) is usually localized in the 5'-UTR of the heat shock genes. It has a relatively complex secondary structure, including two to four hairpin-loop structures, one of which contains the SD sequence and sometimes also the AUG start codon. The 4U RNA thermometer, another common thermosensor, was originally found in the small agsA gene, the heat shock gene of S. enterica [6]. Its predicted structure contains two hairpins, with four uridine residues forming nucleotide pairs with the SD sequence (Fig. 1). Temperature-dependent melting of one of the hairpins was confirmed experimentally. Binding of the ribosome with the SD sequence was found to occur only at the heat shock temperatures.

The 4U RNA thermometer is often used for control of bacterial genes of heat shock and virulence, since it is able to bind with the 5'-agga-3' fragment of the SD sequence [6]. For example, the hypothesis of the RNA thermometer-mediated control was completely confirmed for the Yersenia sp. gene lcrF (virF), encoding the regulator of the virulence response [7]. Expression of the gene did not occur at 26°C, but it was induced at 37°C.

Analysis of the regulatory factors of the known RNA thermometers showed that their functioning requires several nucleotides to form complementary bonds with those of the SD sequence or of the flanking

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**Fig. 1.** The secondary structure containing the predicted and confirmed 4U RNA thermometer in the agsA gene of S. enterica [6] at 20°C. The Shine–Dalgarno sequence in the stem and the translation initiation site are highlighted.
The results of our computer analysis of the chromosomal DNA sequences of different S. enterica isolates (obtained from the databases) confirmed the absence of 4U RNA thermometer similar to those predicted in [6] (Fig. 1) for the agsA gene of S. enterica and experimentally confirmed in vivo and in vitro for three out of the 25 analyzed isolates (NC_011147, NC_011274, and NC_006511). Since pathogenic microorganisms need to survive and replicate under diverse conditions, including temperature fluctuations which may originate from the temperature differences between the environment and the host organisms, in pathogens the relevant mechanisms evolve, which were responsible for the regulation of gene expression and which have RNA thermometers among their elements. Since 4U RNA thermometers were not found in some Salmonella isolates, certain temperature-sensitive elements other than 4U RNA thermometers and probably other than the ROSE elements may exist in these strains.

In the present paper, the search for the new, previously unknown potential RNA thermometers was carried out in the sequences of the chromosomal DNA from the isolates of the gram-negative bacterium Salmonella enterica.

**MATERIALS AND METHODS**

**Search for hairpin-loop structures.** Complete sequences of the chromosomal DNA of 25 S. enterica isolates, which were available in the GenBank database at the time of investigation, were chosen for computer analysis.

The search for bacterial DNA fragments potentially able to form hairpin-loop structures was carried out using the formula:

5'-aaggag(n)katg-3',

where aaggag is the SD sequence, n is any of the possible nucleotides, k = 6–10, and atg is the initiation site (alternative initiation sites tgt and gtg were also studied).

For the search of the hypothetical 4U RNA thermometers in the sequences of the + and − strands of the S. enterica chromosomal DNA, the following formulas were also used:

5'-cat(n)ctctc(n)1aaa-3' and 5'-tttt(n)1aaggag(n)-atg-3'.

The sequences retrieved were analyzed in accordance with the above criteria.

The Mfold software package, version 3.2 [11] was used to predict the secondary structure of the linear DNA fragments and the respective RNA transcripts and to determine the melting temperature 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). Additionally, the RNA2 program from the GeneBee software package [12] was used to design the hairpin-loop structures.

**Atomic force microscopy (AFM).** The supercoiled structure of the pUC8 DNA molecule (with length of 2665 bp) in the air was visualized using a Nanoscope III atomic force microscope with a D scanner (Veeco Instruments Inc., United States). AFM images were recorded in the tapping mode in the air in the height regime using the standard unsharpened probes (NT-MDT, Russian Federation) at the resonance frequency of 300–360 kHz. Ami no mica was obtained by modification of freshly cleaved mica with amino groups in the vapors of distilled 3-aminopropyltriethoxysylane (Aldrich, United States). The procedure for modification was described previously [13].

**RESULTS AND DISCUSSION**

Presently, only several of the 40 ROSE thermometers predicted for proteobacteria have been confirmed experimentally. While the possible existence of 4U RNA thermometers was shown for the dnaJ gene of Brucella melitensis and for the region preceding the groES gene in Staphylococcus aureus, it is not clear whether they will act as RNA thermometers [6].

Analysis of the structural organization of the experimentally confirmed RNA thermometers made it possible to develop the algorithm for searching the relevant fragments in prokaryotic DNA sequences and therefore of the RNA transcripts. For the latter, one of the elements is the distance between the SD sequence within the hairpin-loop structure and the translation initiation site, which may be from 6 (for the ROSE elements) to 12 (for 4U thermometers) nucleotides. The following major criteria for potential RNA thermometers were used: (i) melting temperature of the hairpin is within the 37–43°C range (at the physiological ionic strength); (ii) the Shine–Dalgarno sequence is partially or completely located within the stem of the hairpin-loop structure; and (iii) the canonical codon AUG (ATG for DNA), initiating almost 90% of the prokaryotic coding sequences, as well as alternative codons GUG and UUG (GTC and TTG for DNA) are initiation sites [14].

The known RNA thermometers are the structures containing one lengthy hairpin or several hairpin-loop structures. Since the results of NMR investigations showed that the stem of the hairpin including the ROSE element contains several uncanonical nucleotide pairs [15], both the matched and mismatched hairpin-loop structures were analyzed during the search for the hypothetical RNA thermometers.
It was previously shown for palindrome-containing supercoiled DNAs that under physiological conditions hairpins could be formed as fragments of a cruciform structure with the stem of at least 7 bp and the loop not exceeding 4–5 nucleotides [16–18]. For example, the average stem length for the hairpins formed in 16S rRNA is 3–4 bp and can be as high as 10 bp [19], while the loop of 6–7 nucleotides is energetically preferable for the RNA hairpins [20]. Thermal denaturation revealed that the RNA hairpin-loop structures were more stable than these structures formed in DNA [21]. Based on the literature data obtained in both in vitro and in vivo experiments [22–25] and considering that SD-containing hairpin-loop structures efficiently affect translation only when their free energy is close to –6 kcal/mol [26], the hairpins with the loop usually not exceeding 8 nucleotides, the stem not exceeding 7 bp, and free energy (∆G) of about –6 kcal/mol were chosen for further analysis. Localization of potential hairpins relative to the hsp genes of heat shock protein was additionally considered.

Visualization of hairpin-loop structures in supercoiled DNA. Advantages of combining techniques of DNA immobilization onto amino mica and AFM capability made it possible to visualize cruciform structure of supercoiled pUC8 DNA formed by hairpins. On the AFM image of pUC8 DNA on amino mica (Fig. 2), the hairpins were visible as clearly delineated projections on the DNA strands, so that their length was measurable directly on the image. Analysis of our results showed that 11–12 bp were involved in hairpin formation, while thermodynamic analysis of inverted repeats confirmed that the hairpin was formed by 26 nucleotides and had the free energy ∆G = –17.8 kcal/mol.

RNA molecules visualized by AFM usually look like condensed structures in most studies. We have previously visualized RNA transcripts immobilized on mica, which formed rod-like condensed structures 122 ± 10 nm long with the 4.5–5 length-to-width ratio [27]. We think that visualization of stretched uncondensed RNA molecules containing hairpin-loop structures requires changing surface properties of the substrate (mica). The mentioned morphological features of the RNA molecules visualized by AFM may result from a significant effect of the surface properties of the mica on which the RNA transcripts were immobilized. The surface properties of the substrate, in turn, depend on the hydrophobicity and density of the cations localized on the mica surface. It should be noted that for visualization of RNA molecules, the same mica is used as for visualization of DNA molecules, i.e., the one with hydrophobicity and cation surface density preventing formation of condensed structures by linear and supercoiled double-stranded DNA molecules immobilized on the surface, but rather favors the uniform distribution of the DNA fragments. We have shown earlier that small variations in the hydrophobicity and cation density on the mica surface may result in significantly altered morphology of immobilized DNA molecules [28, 29].

Potential hairpin-loop structures in the S. enterica genome. Since the thermometers may be localized at any locus within the RNA molecule, the first stage of search for the hairpins (the major components of the new potential RNA thermometers different from the known 4U RNA thermometers) included analysis of the sequences of the chromosomal DNA of three S. enterica isolates not containing the 4U RNA thermometer.

Computer and thermodynamic analysis of the S. enterica isolate with complete genome (GenBank access no. FM200053) revealed four hairpin-loop structures (Figs. 3–6) satisfying the necessary criteria for a potential RNA thermometer, i.e., the presence of the following: (i) the Shine–Dalgarno sequence;
(ii) the translation initiation site located not further than 15 nucleotides; (iii) the relevant melting temperature of the hairpin (~40–42°C); and (iv) localization in the 5'-UTR region. The presence of the known 13 heat shock genes (with a 4U RNA thermometer associated with one of them) in the isolate FM200053 supports the suggestion that several types of RNA thermometers may exist in the *Salmonella* genome, rather than only one, which is presently known as a 4U RNA thermometer.

Importantly, the hairpin-loop structures (Figs. 3–6) not only satisfied the necessary and sufficient conditions of formation of RNA thermometers, but were also highly conservative uncanonical structures which were present in the genomes of all 25 analyzed *S. enterica* isolates.

For different RNA thermometers, melting is known to occur at different temperatures. For example, the ROSE thermometer is melted at 42°C, while the pfrA and lcrF thermometers, at 37°C [30]. The hairpin-loop structures with melting temperature within the 37–42°C range were therefore considered as potential RNA thermometers.

For many fragments of the 5'-UTR sequences, it is rather difficult to predict their possible functioning as RNA thermometers. To confirm the action of a hairpin-loop structure as a thermosensor, experimental confirmation is required, e.g., by testing the in vivo efficiency of expression of the reporter gene at different temperatures of by melting the hairpin-loop structure containing the SD sequence. The real melting temperature of specific RNA thermometers is determined, for example, by circular dichroism spectroscopy or UV spectroscopy. Since the melting temperature of nucleic acids depends on ionic strength, Mg$^{2+}$ concentration of 1–2 mM is accepted for determination of the thermodynamic parameters of the thermometers as the value corresponding to the physiological concentration inside bacterial cells.
The possible functioning of the found hairpin-loop structures as heat sensors in the *S. enterica* genome is confirmed by the fact that the hairpin structure completely analogous to the potential RNA thermometer no. 4 (Fig. 6a) was found to function as a heat sensor [9]. The authors of this work compared the efficiency of 12 synthetic RNA thermometers under physiological conditions and found that this thermometer (Fig. 6a) was one of the two which most efficiently controlled the temperature-dependent expression of the *lacZ* gene encoding *E. coli* β-galactosidase. The potential RNA thermometers found in the present...
work (Figs. 3–6) differ significantly in their secondary structure. While RNA thermometers (Figs. 3–5) are characterized by a small loop (4–5 nucleotides), the experimentally confirmed RNA thermometer no. 4 (Fig. 6a) has a relatively big loop (30 nucleotides); however, all of the hairpin structures found have a matched stem. Since it was found in [9] that significantly different hairpins were capable of the same efficient switching of gene expression, other hairpin-loop structures (Figs. 3–5) may probably also act as RNA thermometers.

Thus, based on the computer and thermodynamic analysis of 25 S. enterica isolates with complete genome, the algorithms and criteria for the search of potential RNA thermometers were determined. These results will make it possible to carry out a similar screening of RNA thermometers in the genomes of other socially significant pathogens. For S. enterica, apart from its known 4U RNA thermometer, the hairpin-loop structures determined may act as new RNA thermometers. They satisfy the necessary and sufficient conditions for formation of RNA thermometers and are highly conservative uncanonical structures.

**Fig. 5.** Potential RNA thermometer 3 localized in the region adjacent to the 5'-UTR region of the gene encoding a hypothetical protein. $T_m = 42.0^\circ$C at ionic strength $I = 0.2$ M Na$^+$; $\Delta G = -2.1$ kcal/mol (a). Localization of the hairpin structure relative to 5'-UTR of the gene encoding the hypothetical hydrolase similar to the haloacid dehalogenase in S. enterica (b).
which were found in the genomes of all analyzed 25 *S. enterica* isolates.

**ACKNOWLEDGMENTS**

The work was partially supported by the National Academy of Medical Sciences of Ukraine, grant AMN 95/2010.

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