FourU: a novel type of RNA thermometer in *Salmonella*

Torsten Waldminghaus,1 Nadja Heidrich,2 Sabine Brantl1 and Franz Narberhaus1*

1Lehrstuhl für Biologie der Mikroorganismen, Ruhr-Universität Bochum, 44780 Bochum, Germany.
2AG Bakteriengenetik, Friedrich-Schiller-Universität Jena, 07743 Jena, Germany.

Summary

The translation of many heat shock and virulence genes is controlled by RNA thermometers. Usually, they are located in the 5′-untranslated region (5′-UTR) and block the Shine-Dalgarno (SD) sequence by base pairing. Destabilization of the structure at elevated temperature permits ribosome binding and translation initiation. We have identified a new type of RNA thermometer in the 5′-UTR of the *Salmonella agsA* gene, which codes for a small heat shock protein. Transcription of the agsA gene is controlled by the alternative sigma factor σ32. Additional translational control depends on a stretch of four uridines that pair with the SD sequence. Mutations in this region affect translation in vivo. Structure probing experiments demonstrate a temperature-controlled opening of the SD region in vitro. Toeprinting (primer extension inhibition) shows that ribosome binding is dependent on high temperatures. Together with a postulated RNA thermometer upstream of the *Yersinia pestis* virulence gene *lcrF* (*virF*), the 5′-UTR of *Salmonella agsA* might be the founding member of a new class of RNA thermometers that we propose to name ‘fourU’ thermometers.

Introduction

The induction of heat shock proteins (Hsps) to protect the cell from heat-induced damage is a universal biological process (Gross, 1996; Yura et al., 2000). Like all other organisms, *Salmonella* species induce the expression of a large set of chaperones and proteases under heat stress conditions. Substantial cross-talk between the bacterial heat shock response and infection processes has been reported (Gophna and Ron, 2003; Goulhen et al., 2003). Several heat shock genes are consistently upregulated in pathogenic *E. coli* lineages (Takaya et al., 2002; 2003; Dowd and Ishizaki, 2006). Moreover, deletion of the ClpXP protease genes in an enterohaemorrhagic *Escherichia coli* strain impaired the secretion of virulence proteins (Tomoyasu et al., 2005). Virulence of *Salmonella enterica* serovar Typhimurium requires the DnaK/J chaperone system and the Lon protease (Takaya et al., 2002; 2003). Apart from the typical repertoire of molecular chaperones, *Salmonella* encodes a unique Hsp, named AgsA (aggregation-suppression protein), which is strongly induced at high temperatures (Tomoyasu et al., 2003). AgsA is a distantly related member of the small Hsp family, which comprises molecular chaperones that bind to unfolded proteins in order to maintain them in a folding-competent state (Narberhaus, 2002).

The majority of heat shock genes in *E. coli*, *Salmo-
ella* and related enterobacteria are under control of the alternative sigma factor σ32 (Yura et al., 2000). Immediately after a temperature upshift, the cellular concentration of the transcription factor increases rapidly and transiently by a complex regulatory circuit involving transcriptional, translational and post-translational control mechanisms. Only recently, the fundamental role of RNA in sensing of environmental parameters has been recognized (Winkler and Breaker, 2005; Narberhaus et al., 2006). Most RNA sensors are located in the 5′-untranslated region (5′-UTR) of mRNAs, fold into complex structures and control the expression of downstream genes by signal-induced conformational changes. While RNA thermometers sense temperature as a physical stimulus, riboswitches recognize chemical signals. Target molecules are bound with remarkable specificity and affinity. Gene expression is modulated either at the level of translation initiation, transcription termination or RNA processing (Mandal and Breaker, 2004; Nudler and Mironov, 2004).

RNA thermometers undergo temperature-induced conformational changes. All presently known RNA thermometers control translation initiation. In most cases, entry to the ribosome binding site is blocked by complementary base pairs at low temperatures. At increasing temperatures, melting of the structure permits ribosome access (Narberhaus et al., 2006). This simple regulatory principle can be realized by quite different RNA structures. Only a few distinct types of RNA thermometers have been discovered so far.

The first RNA thermometer described regulates development of phage λ by controlling expression of the cII

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Received 17 May, 2007. *For correspondence. E-mail: franz.narberhaus@rub.de; Tel. (+49) 234 322 3100; Fax (+49) 234 321 4620.

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protein (Altvia et al., 1989). At 37°C, the Shine-Dalgarno (SD) sequence and the AUG start codon are accessible in a single-stranded region flanked by two hairpins. In an alternative structure at 45°C, the AUG start codon and the SD sequence are hidden in a hairpin structure. This RNA thermometer is unique in switching on translation with decreasing temperature. Moreover, it is the only known RNA thermometer that does not seem to operate by gradual melting but switches between two mutually exclusive conformations.

The cellular level of the heat-shock sigma factor σ2 in *E. coli* is adjusted in part by the most complex RNA thermometer known to date. The RNA structure consists of two segments (region A and B) within the coding region of the *rpoH* mRNA (Morita et al., 1999a,b). While the SD sequence is accessible irrespective of the temperature, the AUG start codon and region A as part of the ribosome binding site are blocked at low temperatures by base-pairing with region B.

The expression of virulence genes can also be controlled by RNA thermometers (Johansson and Cossart, 2003). A temperature-labile stemloop structure blocking the SD sequence was predicted upstream of the *lcrF* gene in *Yersinia pestis* (Hoe and Goguen, 1993). Another RNA thermometer exists in the 5′-UTR of *prfA* in the food-borne pathogen *Listeria monocytogenes* (Johansson et al., 2002). The *prfA* gene is transcribed at 30°C. However, within an extended 120-nucleotide hairpin both the SD sequence and the AUG start codon are positioned in internal loop regions allowing only inefficient translation. Conformational changes in the RNA at body temperature (37°C) increase translation efficiency resulting in production of the virulence regulator.

By far the most common RNA thermometer is the ROSE (Repression Of heat Shock gene Expression) element, which was discovered in the nitrogen-fixing soybean symbiont *Bradyrhizobium japonicum* (Narberhaus et al., 1998). Meanwhile, more than 40 ROSE-like RNA thermometers have been predicted in diverse α- and γ-proteobacteria (Waldminghaus et al., 2005). They are located in the 5′-UTR of small heat-shock genes and between 70 and 120 nucleotides long. Their computer-predicted secondary structure is composed of three or four stemloops with the SD sequence being masked by imperfect base-pairing in the 3′-proximal hairpin (Nocker et al., 2001; Balsiger et al., 2004).

Here, we present an in-depth characterization of a new type of RNA thermometer that regulates translation of the *Salmonella enterica agsA* gene by four consecutive uracil residues that pair with the SD sequence. Our detailed *in vivo* and *in vitro* experiments demonstrate dynamic temperature-dependent conformational changes in the 5′-UTR of *agsA* that control ribosome access to the SD sequence.

### Results

**A new RNA thermometer candidate in Salmonella**

RNA thermometers are characterized by a short sequence with imperfect complementarity to the SD region. The systematic inspection of a database containing the sequences and predicted secondary structures of 5′-UTRs from annotated protein-coding genes of bacterial genome sequences (T. Waldminghaus, unpublished) revealed a potential RNA thermometer upstream of the *Salmonella agsA* gene. Unlike the widespread ROSE-like thermometers, the 5′-UTR of *agsA* consists of only two stemloop structures and the anti-SD sequence does not contain the typical U(U/C)GCU motif (Fig. 1). The SD sequence in the second hairpin (hairpin II) pairs with a stretch of four uracil residues. An internal loop (A29/G52), eight AU, four GU and only two GC out of 14 base pairs limit the free energy of this extended hairpin to −9.7 kcal mol⁻¹. The interesting novel features of this potential thermoregulator prompted us to investigate its structure and function in more detail.

**Fig. 1.** Secondary structure prediction of the 5′-UTR of the *Salmonella agsA* gene. The mfold program (version 3.2; Zuker, 2003) was used to predict the RNA structure. Nucleotides are numbered starting from the mapped transcription start site (+1). The Shine-Dalgarno sequence (SD) and start codon (START) are labelled. Hairpins I and II and the point mutations introduced in this study are indicated.

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Temperature-dependent transcriptional regulation of agsA

To assign the 5' end of the agsA transcript, primer extension experiments were performed. Total RNA was isolated from cells grown at 30°C and after heat shock at 45°C and reverse transcription was carried out. A strong signal was detected only in the heat-shocked samples (Fig. 2). The deduced -10 and -35 regions correspond well to the consensus sequence (CTTGAA-N_{13-18}-CNCCATAT) of σ^{32}-type promoters (Wade et al., 2006).

To confirm the presence of the putative σ^{32}-dependent promoter, translational fusions of bgaB to the agsA-promoter region were constructed (Fig. 3A). The bgaB gene codes for a heat-stable β-galactosidase from Bacillus stearothermophilus (Hirata et al., 1984). Promoter activity was measured in an E. coli ΔrpoH mutant deficient of the heat-shock sigma factor σ^{32} and in the isogenic wild-type (wt) MC4100. When cells were grown at 25°C, the β-galactosidase activity was low in both strains (Fig. 3B). After a temperature upshift to 40°C for 30 min, expression was induced sixfold in the wt strain but not in the ΔrpoH mutant. In the wt, 30-fold induction was observed 90 min after heat shock. Absent induction in the mutant strain demonstrates that the temperature-induced amount of agsA mRNA results from transcriptional control by the mapped σ^{32}-type promoter.

The 5'-UTR of agsA confers translational control in vivo

To separate the described transcriptional effects from translational events, translational agsA–bgaB fusions were placed downstream of the arabinose-inducible pBAD-promoter (Fig. 4A). Temperature-dependent translational control was monitored upon induction of the promoter with 0.01% (w/v) of L-arabinose.

Expression of the agsA–bgaB fusion in E. coli grown at 30°C showed a basal level of 29 MU that increased to 84 MU 30 min after a heat shock to 42°C (Fig. 4B). A similar fusion containing only hairpin II (mini-agsA) produced overall lower β-galactosidase activities. However, temperature-dependent induction was retained. As a control, we used a fusion to the 5'-UTR of the E. coli

Fig. 2. Mapping of the 5' end of the agsA mRNA. Primer extension experiments were carried out with total RNA from S. enterica cells grown at 30°C or from cells that were shifted from 30 to 45°C for 8 min. Primer STagsAPEv was used for reverse transcription. A corresponding sequencing reaction (ACGT) with plasmid pBO433 carrying the agsA region is shown. The 5' end of the mRNA (+1) is marked by an arrow and the deduced -10 and -35 regions of a σ^{32}-type promoters are underlined in the sequence to the right.

Fig. 3. Transcriptional regulation of agsA by σ^{32} (RpoH).
A. Schematic representation of the reporter gene fusion on plasmid pBO626.
B. Temperature-dependent expression of the agsA–bgaB fusion in E. coli grown at 25°C and heat-shocked to 40°C for 30, 60 and 90 min before β-galactosidase activities were measured. Results shown are the average of three independent measurements with the indicated standard deviations.
The gyrase gene (gyrA–bgaB), which is expected not to be thermally controlled. Starting from 4 MU at 30°C, β-galactosidase activity indeed increased only slightly (about 6 MU) with increasing temperature.

If the 5′-UTR of the agsA gene were a functional RNA thermometer, stabilizing and destabilizing point mutations should decrease and increase expression respectively. Point mutations in the full-length agsA–bgaB fusion affecting the stability of hairpin II are outlined in Fig. 1. The stabilizing mutations A29C, U32C and U33C reduced expression approximately 10-fold (from about 30 MU in the wt fusion to 3 MU) when cells were grown at 30°C (Fig. 4C). Moreover, induction at 42°C was abolished. In contrast, expression was elevated both at 30 and at 42°C in the presence of the destabilizing mutation CUU30-32AAA. Residual twofold induction at 42°C suggests that the bottom and top of hairpin II still have the potential to form base pairs, which melt at increasing temperatures.

The G21C exchange introduced a CAU triplet complementary to the AUG start codon, which might result in an extended hairpin II (see Fig. 1) and thus impair ribosome access. Indeed, β-galactosidase activity was reduced as compared with the wt fusion, in particular at 30°C (Fig. 4B) indicating that repressor was improved by this mutation. Efficient heat-induction (12-fold) was possible presumably because a mismatch (G58) immediately upstream of the AUG codon results in a loose temperature-labile structure. Taken together, these data demonstrate that the 5′-UTR of agsA from S. enterica mediates post-transcriptional control to the heat shock gene in a RNA thermometer-like fashion in vivo.

To estimate the relative contribution of transcriptional and translational control at low, intermediate and high temperatures, expression of both fusions was measured after a shift from 30 to 30, 37 or 42°C. In case of the pBAD–agsA fusion, β-galactosidase activity increased with time at all three temperatures suggesting that the RNA thermometer permits some translation also at low temperature (Fig. 5A). However, expression strictly correlated with temperature and was highest at 42°C. The presence of the native s32 promoter prevented expression at 30°C completely and allowed only low expression at 37°C (Fig. 5B). Significant induction was observed at 42°C.

Structure probing experiments reveal temperature-mediated conformational changes

The secondary structure of the agsA thermometer was probed at different temperatures using RNases T1 (cuts 3′ of single-stranded guanines), T2 (cuts 3′ of unpaired nucleotides with a slight preference for adenines) and V1 (specific for double-stranded and stacked regions). Separation of the corresponding cleavage products on a denaturing 15% polyacrylamide gel gives an overview of the entire structure (Fig. 6A). For detailed analysis of hairpin II, samples were run on an 8% polyacrylamide gel (Fig. 6B).

The overall cleavage pattern at 30°C is in good agreement with the calculated secondary structure. Both terminal loops around positions 9 and 40 were cut by RNases T1 and T2 but not by RNase V1 (Fig. 6A, B and D). Complete protection of positions 2–8 and 11–18 against T1 and T2 cleavage and susceptibility of these regions to RNase V1 confirmed the stem region in hairpin I (Fig. 6A and D). This hairpin was not temperature-responsive as it
remained resistant against cleavage by RNases T1 and T2 at 45°C (Fig. 6A).

The experimentally defined structure of hairpin II is also consistent with the predicted structure (Fig. 6B and D). However, in contrast to hairpin I it appears to be in a dynamic, temperature-responsive conformation. The tetraloop (nucleotides 39–42) is readily accessible to RNase T1 and T2 at low and high temperature. The flanking SD and anti-SD regions are largely resistant to these enzymes at 30°C indicating a double-stranded RNA region that blocks ribosome binding. Residual cleavage by T1 and T2 suggests that a minor population is in an open conformation. The fraction of single-stranded molecules substantially increases at 45°C as illustrated by the accumulation of T1 and T2 derived products in the SD and anti-SD region (Fig. 6B and quantification of four independent experiments in Fig. 6C). Structure probing performed at 50°C showed further opening of stem II while stem I remained stable (data not shown). Our results suggest that hairpin II exists in equilibrium between open and closed structures, which is shifted towards the open conformation after exposure to high temperature.

Stabilizing mutations in hairpin II prevent release of the SD sequence

Point mutations potentially stabilizing hairpin II reduced expression of the reporter gene fusion (see Fig. 4). Structure probing of the U32C and A29C RNAs at 45°C indicates the presence of a thermostable structure in hairpin II (Fig. 7). Neither mutation affected the conformation of hairpin I. Consistent with the data presented in Fig. 6, the SD and anti-SD sequences in hairpin II of the wt RNA were accessible to T1 and S1 (cleaving single-stranded nucleotides) enzymes. In contrast, the U32C mutation resulted in a complete protection of G48, G49 and G50 in the SD region (Fig. 7). Moreover, in the A29C RNA all three guanines and G51, which is predicted to pair with C29, were protected against T1 attack. Impaired cleavage of nuclease S1 in the SD sequence supports the T1 results and demonstrates that in both stabilized RNAs the ribosome binding site is kept in a blocked conformation.

The similarity of the V1 cleavage pattern of variant U32C to the wt RNA shows that this mutation does not introduce major structural changes. In contrast, V1 cleavage of the A29C RNA is decreased in the SD segment but increased at nucleotides 26–29 suggesting pronounced changes in stem II geometry.

Binding of 30S ribosomes to the 5’-UTR of agsA depends on temperature

To examine the effects of the agsA-mRNA structure on formation of the translation initiation complex we performed toeprinting analysis (Hartz et al., 1988). After annealing of the primer, ribosomal subunits and initiator-tRNA were added to agsA mRNA (116 nt) and incubated at 30 and 45°C. As shown in Fig. 8, primer extension was not inhibited at 30°C. When samples were incubated at 45°C, a toeprint corresponding to position +17 relative to the translation start site was detected indicating formation of an mRNA-ribosome-tRNA\textsuperscript{Met} ternary complex. These results demonstrate temperature-controlled binding of the ribosome to the agsA translation initiation region in the absence of any other cellular factor.

Discussion

Our knowledge on sensory RNAs, such as riboswitches and RNA thermometers, is just beginning to emerge. The estimation that as many as 2% of all genes in Bacillus subtilis might be controlled by riboswitches (Mandal et al., 2003) emphasizes that regulatory RNA elements play a
fundamental role in the genetic control of metabolic processes in prokaryotes. The recent identification of thiamine pyrophosphate-binding riboswitches in eukaryotes (Sudarsan et al., 2003; Thore et al., 2006) suggests that riboswitches derive from an ancient common ancestor of eukaryotes and prokaryotes (Vitreschak et al., 2004).

Given the importance of RNA-mediated genetic control, we set out to identify novel RNA thermometers that deviate from the few known examples. Unlike riboswitches, RNA thermometers are not characterized by a highly conserved ligand-binding domain (Narberhaus et al., 2006). All known RNA thermometers control

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**Fig. 6.** Temperature-dependent conformations of the RNA thermometer upstream of agsA. A and B. Enzymatic cleavage of 5′-end-labelled agsA RNA was carried out at 30°C or 45°C. The RNA fragments were separated on (A) 15% polyacrylamide or (B) 8% polyacrylamide gels. RNase T1 (0.1 and 0.01 U), RNase T2 (0.04 and 0.02 U) and RNase V1 (0.01 and 0.002 U) were used. Note that V1 creates 3′-hydroxyl ends so that the corresponding bands migrate somewhat slower than products derived from T1, T2 or alkaline hydrolysis. Lane C indicates the incubation control with water instead of RNase. Lane L: alkaline ladder. Selected nucleotides are marked with arrowheads. The SD and anti-SD sequences are labelled.

C. Quantification of temperature-dependent structural differences of the agsA thermometer. The results of four independent experiments with RNase T1 and T2 at 30 and 45°C were quantified and normalized to nucleotides 40 and 39 in loop II. Relative cleavage was calculated by division of the 45°C-values by the 30°C-values. Grey columns relate to nucleotides in the 5′-part of hairpin II, black columns to the 3′-part and white to the loop of hairpin II. Selected nucleotides are labelled.

D. Secondary structure model of agsA RNA and summary of the probing results at 30°C. Cleavage sites by RNases T1, T2 and V1 are shown by arrows as indicated. Dotted arrow lines represent moderate cleavage, full lines strong cleavage. Increased cleavage by T1 and T2 at high temperature occurred at the circled nucleotides (more than 1.8-fold induction from 30 to 45°C).

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**Fig. 7.** Comparative RNA structure analysis of the wt and stabilized 5′-UTRs of agsA. Enzymatic hydrolysis of 5′-end-labelled RNA of the wt and U32C and C29A variants was carried out at 45°C and fragments were separated on an 8% polyacrylamide gel. RNase T1 (0.01 U), nuclease S1 (1 U) and RNase V1 (0.02 U) were used. Note that V1 and S1 create 3′-hydroxyl ends so that the corresponding bands migrate somewhat slower than products derived from T1 or alkaline hydrolysis. Lane C: incubation control with water instead of RNase. Lane L: alkaline ladder. Selected nucleotide positions are marked with arrowheads. The Shine-Dalgarno and the anti-Shine-Dalgarno sequences are labelled.

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**Fig. 8.** Ribosome binding to the agsA 5′-UTR. Formation of a ternary complex on agsA mRNA at high temperature was shown by a primer extension inhibition assay (for details, see Experimental procedures, and Results). Addition of 30S ribosomal subunits and initiator tRNA at 30°C (Lanes 1–5) and 45°C (Lanes 6–7) is indicated above the gel. The corresponding DNA sequencing reactions (CTGA) were carried out with the same end-labelled oligonucleotide as in the toeprint experiments. The toeprint signal is located at position +17 relative to the A of the translation start codon.

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translation. In principal, masking of the ribosome binding site can be achieved by diverse RNA sequences and structures. de Smit and van Duin showed that mRNA hairpins incorporating the SD sequence affect translation efficiency only if their free energy is lower than -6 kcal mol\(^{-1}\) (de Smit and van Duin, 1990; 1994). Thermodynamic considerations argue against a complete unfolding of such a stable stemloop in the physiological temperature range. Known RNA thermometers are complex structures either consisting of one extended stemloop (Johansson et al., 2002) or of multiple hairpins (Waldminghaus et al., 2005). NMR studies on the functional hairpin of the RNA thermometer ROSE1 revealed a helical structure containing several non-canonical base pairs (Chowdhury et al., 2006). Irregular base stacking coupled with a network of weak hydrogen bonds facilitates liberation of the SD sequence in the physiological temperature range.

Here, we describe a comprehensive structure-function analysis of an unusually short RNA thermometer. It consists of only 57 nucleotides, is folded into two hairpins and controls expression of the small heat shock gene agsA in *Salmonella enterica* serovar Typhimurium. The sequence of the 5′-UTR bears no similarity to the widespread ROSE element that controls the expression of many small heat shock genes in α- and γ-proteobacteria (Waldminghaus et al., 2005). Despite its short sequence and its simple structural design, the *Salmonella* agsA thermometer is fully functional as demonstrated by a complementary set of *in vivo* and *in vitro* experiments. Owing to its simple architecture it might serve as an excellent model for future studies, for example aiming at the structure determination of a full-length RNA thermometer.

*In vivo* evidence for a functional RNA thermometer was provided by a reporter gene assay, in which the isolated 5′-UTR of agsA permitted temperature regulation independent of its natural promoter. Like other RNA thermometers (Johansson et al., 2002), the agsA leader region alone induced gene expression roughly threefold. Hence, it is unlikely that the thermometer alone accounts for the massive induction of AgsA protein after temperature upshift and in response to protein aggregation (Tomoyasu et al., 2003). Apparently, rapid and efficient induction under stress conditions is achieved by the combination of two separate modules, a σ\(^{32}\) promoter and an RNA thermometer. Dual control by a σ\(^{32}\) promoter in concert with a ROSE-like RNA thermometer was also shown for the ibpA genes of *E. coli* and *Salmonella* (Waldminghaus et al., 2005). The equivalent set-up of the agsA promoter suggests that many other σ\(^{32}\)-controlled genes might possess a hitherto undiscovered RNA thermometer as additional layer of control. It is a matter of speculation which regulatory mechanism was invented first in evolution. Given the relatively simple mechanistic principle of RNA thermometers one might argue that they evolved earlier than the more complex and multifactorial transcriptional control by the alternative sigma factor σ\(^{32}\).

Complex control circuits involving several layers of control are common in bacterial stress responses. They allow the integration of multiple signals as was shown for the general stress response in *E. coli* (Hengge-Aronis, 2002). Cross-regulation of the heat shock response by σ\(^{32}\) and the repressor protein HrcA occurs in many proteobacteria (Permina and Gelfand, 2003). Using various control elements in a modular composition is predicted to increase the robustness of stress responses (El-Samad et al., 2005). Implementing a heat sensing device downstream of a σ\(^{32}\) promoter provides at least one additional advantage. Transcriptional control by σ\(^{32}\) primarily responds to the accumulation of unfolded proteins in the cell whereas RNA thermometers are not expected to react to any other signal than temperature. By its modular architecture, the regulatory region of *agsA* has obtained the capacity to integrate at least two separate signals, misfolded proteins and temperature.

Are additional factors involved in translational control by the agsA thermometer? The function of *trans*-acting small regulatory RNAs often requires the Hfq protein (Muffler et al., 1996; Schumacher et al., 2002; Zhang et al., 2002). Hfq is a RNA chaperone that assists in RNA–RNA interactions (Valentin-Hansen et al., 2004). It is required for virulence of *Salmonella* (Sittka et al., 2007). In *in vitro* experiments with the ROSE element suggested that RNA thermometers act without the aid of Hfq or any other accessory factor (Chowdhury et al., 2003; 2006). According to our data on the agsA thermometer, it seems unlikely that additional cellular factors are required for temperature sensing. First, there was a clear correlation between the computer-calculated free energy and the respective *in vivo* expression of mutated *agsA*-bgaB fusions showing that the intrinsic stability of hairpin II determines the temperature response. Secondly, structure probing experiments revealed that hairpin II of the synthetic 5′-UTR responds to temperature changes in the absence of any additional factors. Hairpin I was not absolutely required for regulation. However, it might play a structural role during cotranscriptional folding of the RNA thermometer *in vivo*. Finally, toeprinting analyses demonstrated that access of isolated 30S ribosome subunits to naked RNA is temperature-controlled.

Another fascinating aspect of the *agsA* thermometer is its simple structural design. The SD sequence in hairpin II is blocked by a consecutive stretch of four uridine residues. A similar structure composed of four U residues that pair with SD sequence (AGGA) has been predicted but never proven experimentally upstream of the lcrF gene in *Y. pestis* (Hoe and Goguen, 1993) (Fig. 9). To distinguish this new class of thermosensors from the widespread ROSE-type thermometers, we suggest the
Experimental procedures

Strains and growth conditions

*Escherichia coli* cells were grown at 30 or 37°C in Luria–Bertani (LB) medium supplemented with ampicillin (Ap, 200 µg ml⁻¹) or kanamycin (Km, 50 µg ml⁻¹) if appropriate. Strain KY1612 (ΔrpoH) was grown at 25°C in LB. For induction of the pBAD promoter in strains carrying translational *bgaB* fusions, 0.01% (w/v) L-arabinose was added. *Salmonella enterica* serovar Typhimurium M556 was grown in LB medium at 37°C.

Plasmid construction

Recombinant DNA work was performed according to standard protocols (Sambrook *et al*., 1989). Polymerase chain reaction (PCR)-generated fragments were inserted into pUC18 or pK18 digested with Smal to generate plasmids pBO433 and 471 (Table 1). Site-directed mutagenesis to generate pBO653 to 657 was performed according to the instruction manual of the QuikChange mutagenesis kit (Stratagene, La Jolla, USA). Plasmid pBO471 served as template for PCR with mutagenic primers (Table 1). The inserts containing the mutated *agsA* fragment were isolated upon NheI/EcoRI digestion and cloned into the corresponding site in pGF-bgaB. To construct pBO627 primers miniAgsAfw and miniAgsArv were annealed and cloned into the NheI/EcoRI site of pBAD-*bgaB*. The *agsA* promoter fusion (pBO626) was constructed by inserting a PCR generated fragment cut with EcoRI and BamHI into the corresponding site in pGF-*bgaB* (Stoss *et al*., 1997). The correct nucleotide sequence was confirmed by automated sequencing.

RNA isolation and primer extension

*Salmonella* cells were grown at 30°C to exponential growth phase before 10 ml where transferred to prewarmed flasks at 45°C for 5 min. Isolation of total RNA using hot phenol was performed as described previously (Waldminghaus *et al*., 2005).

Primer extension was carried out as described previously (Babst *et al*., 1996). Primers STagsAPErv and STagsAPErv2 were used to map the transcription start site of *agsA* (Table 1).

β-Galactosidase assays

*Escherichia coli* cells carrying *bgaB* fusions were grown in 25 ml cultures at 25 or 30°C to exponential growth phase before samples of 10 ml where transferred to prewarmed flasks at 40 or 42°C. After the indicated time periods, β-galactosidase activity was measured according to standard protocols (Miller, 1972) except that enzyme activity was measured at 55°C.

In vitro transcription and structure probing experiments

RNAs were synthesized in *vitro* by runoff transcription with T7 RNA polymerase from PCR-generated DNA templates and 5’ end labelled as described (Brantl and Wagner, 1994). Partial digestions of 5’ end-labelled RNAs with ribonucleases T1, T2, V1 and nuclease S1 were carried out as follows. RNA corresponding to 30 000 cpm was mixed with 1 µl of 5× TMN buffer (100 mM tris acetate, pH 7.5; 10 mM MgCl₂; 500 mM NaCl) and 0.4 µg tRNA, and distilled water was added to a volume of 4 µl. Samples were pre-incubated for 5 min at the indicated temperature before 1 µl of different concentrations of nucleases were added. After 5 min of cleavage, 5 µl formamide loading dye were added, samples were denatured for 5 min at 95°C and aliquots were separated on a denaturing 8 or 15% polyacrylamide gel. Alkaline ladders were generated as described (Brantl and Wagner, 1994). The Aida Image Analyzer v. 4.03 software was used for densitometric quantification. Band intensity for T1 and T2 cleavage were measured at 55°C.

FourU: a novel RNA thermometer

The *mfold* program (version 3.2; Zuker, 2003) was used to predict the RNA structures of sequences upstream of the AUG start codon of the indicated heat shock or virulence genes. The typical ‘fourU’ sequence in the anti-SD region is marked.

**Fig. 9.** Secondary structure models of confirmed and predicted fourU thermometers. The *mfold* program (version 3.2; Zuker, 2003) was used to predict the RNA structures of sequences upstream of the AUG start codon of the indicated heat shock or virulence genes. The typical ‘fourU’ sequence in the anti-SD region is marked.

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**Table 1.**

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**Fig. 9. Secondary structure models of confirmed and predicted fourU thermometers.**
Table 1. Strains, plasmids and oligonucleotides used in this study.

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<td>F- araD139 Δ(argF-lac)U169 rpsL150 relA1 b5B5.01 fraA25 deoC1 ptsF25 e14-</td>
<td>Peters <em>et al.</em>, 2003</td>
</tr>
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<td>Escherichia coli KY1612</td>
<td>MC4100 ΔpoH30 Δzhf 50::Tn10 Δimm21pF13</td>
<td>Zhou <em>et al.</em>, 1988</td>
</tr>
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<td>Salmonella enterica servovar Typhimurium M556</td>
<td>sseD::aphT</td>
<td>Hapfelmeier <em>et al.</em>, 2005</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pK18</td>
<td>Cloning vector, Km’</td>
<td>Pridmore, 1987</td>
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<tr>
<td>pBAD-bgaB</td>
<td>Translational bgaB fusion vector with pBAD-promoter and araC, Ap’</td>
<td>This study</td>
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<td>pBO433</td>
<td>S. enterica agsA upstream region in pk18</td>
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<tr>
<td>pBO471</td>
<td>S. enterica agsA 5’-UTR in puc18</td>
<td>This study</td>
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<tr>
<td>pBO472</td>
<td>S. enterica agsA-bgaB fusion in pBAD-bgaB with full 5’-UTR</td>
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<tr>
<td>pBO626</td>
<td>S. enterica agsA-promoter fusion to bgaB</td>
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<tr>
<td>pBO627</td>
<td>S. enterica agsA-bgaB fusion in pBAD-bgaB with 36 bp upstream of AUG start</td>
<td>This study</td>
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<tr>
<td>pBO653</td>
<td>S. enterica agsA-T32C-bgaB fusion in pBAD-bgaB</td>
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<td>pBO654</td>
<td>S. enterica agsA-A29C-bgaB fusion in pBAD-bgaB</td>
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<td>pBO655</td>
<td>S. enterica agsA-CTT30-32AAA-bgaB fusion in pBAD-bgaB</td>
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<td>pBO656</td>
<td>S. enterica agsA-T33C-bgaB fusion in pBAD-bgaB</td>
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<td>pBO657</td>
<td>S. enterica agsA-G21C-bgaB fusion in pBAD-bgaB</td>
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<td>pBO496</td>
<td>E. coli gyrA-bgaB fusion in pBAD-bgaB</td>
<td>Gaubig, unpublished</td>
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<td>STagsAPEfw</td>
<td>GAAAAATTGTAAGATGAGGAGCA (PE of S. enterica agsA)</td>
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<td>STagsAPEev</td>
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<td>STagsAPEev2</td>
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<tr>
<td>agsAT33Cfw</td>
<td>CTTCCGTAATCATTACATTCAATTTCCTGCAAATCAGAAG (construction of pBO655)</td>
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</table>

* Introduced restriction sites are underlined. PE, primer extension.
region. Mean values of four independent experiments were calculated and the ratio of band intensities at 45°C versus 30°C was plotted for each cleaved base position.

Toeprinting analysis

Toeprinting experiments were carried out using 30S ribosomal subunits, target mRNA and tRNA\textsuperscript{Met} basically according to Hartz et al. (1988). The 5′-[\textsuperscript{32}P]-labelled agsA-specific oligonucleotide STagsAPEv complementary to nucleotides +58 to +36 of the agsA mRNA was used as a primer for cDNA synthesis. An aliquot of 0.04 pmol of agsA mRNA annealed to the oligonucleotide was incubated at 30°C and 45°C for 10, 20 and 30 min without or with 0.4 pmol of 30S subunits and the oligonucleotide was incubated at 30°C and 45°C for 10, 20 and 30 min without or with 0.4 pmol of 30S subunits and 8 pmol of uncharged tRNA\textsuperscript{Met} (Sigma-Aldrich, St. Louis, Missouri) before addition of 1 µl of M-MuLV-RT (80 units; Fermentas, Burlington, Canada). cDNA synthesis was performed at 37°C. Reactions were stopped after 10 min by adding formamide loading dye.

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References


Narberhaus, F. (2002) α-crystallin-type heat shock proteins: socializing minichaperones in the context of a multi-

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