Molecular basis for temperature sensing by an RNA thermometer

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Regulatory RNA elements, like riboswitches, respond to intracellular signals by three-dimensional (3D) conformational changes. RNA thermometers employ a similar strategy to sense temperature changes in the cell and regulate the translational machinery. We present here the first 3D NMR structure of the functional domain of a highly conserved bacterial RNA thermometer containing the ribosome binding site that remains occluded at normal temperatures (30°C). We identified a region adjacent to the Shine–Dalgarno sequence that has a network of weak hydrogen bonds within the RNA helix. With the onset of heat shock at 42°C, destabilisation of the RNA structure initiates at this region and favours the release of the ribosome binding site and of the start codon. Deletion of a highly conserved G residue leads to the formation of a stable regular RNA helix that loses thermosensing ability. Our results indicate that RNA thermometers are able to sense temperature changes without the aid of accessory factors.

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Introduction

Messenger RNAs apart from carrying their coding information for protein generation are also rapidly emerging as regulators of expression of the encoded message. With unique chemical and structural properties, sensory RNAs perform vital regulatory roles in gene expression by detecting changes in the cellular environment through interactions with small ligands (Mandal and Breaker, 2004; Winkler and Breaker, 2005), proteins (Brunel *et al*, 1995; Kaempfer, 2003;

Serganov *et al*, 2003) or small noncoding RNAs (Storz *et al*, 2005) or through physical parameters (Narberhaus, 2002). While the primary sequence carries the genetic code, the secondary and three-dimensional (3D) structures are utilised for diverse and complex regulatory mechanisms.

Temperature is one of the physical parameters under constant vigilance in living cells. A series of processes is temperature controlled in bacteria, among which are the expression of heat-shock, cold-shock and virulence genes (Narberhaus *et al*, 2006). RNA thermometers operate at the post-transcriptional level to sense selectively the temperature and transduce a signal to the translation machinery via a conformational change. They have usually a highly structured 5'-end that shields the ribosome binding site at physiological temperatures (Morita *et al*, 1999; Yamanaka *et al*, 1999; Nocker *et al*, 2001; Johansson *et al*, 2002). Changes in temperature are manifested by the liberation of the Shine–Dalgarno (SD) sequence, thereby facilitating ribosome binding and translation initiation.

We have closely examined temperature-induced conformational changes of a model thermometer, the ROSE (repressor of heat-shock gene expression) element present in the 5'-UTR of small heat-shock genes in many Gram-negative bacteria including rhizobia, *Escherichia coli* and *Salmonella* (Nocker *et al*, 2001; Chowdhury *et al*, 2003; Waldminghaus *et al*, 2005). Point mutations stabilising or destabilising the intramolecular interaction in the 5'-end of the UTR of the mRNA led to repression or de-repression, respectively. Interestingly, the SD sequence and the AUG start codon are embedded in the last stem-loop IV located at the 3'-end of the ROSE₁ sequence as seen from the predicted secondary structure (Figure 1A). G83, a highly conserved nucleotide, located in the opposite strand of the SD sequence is required for temperature sensing (Nocker *et al*, 2001; Chowdhury *et al*, 2003). Deletion of G83 represses translation at low and high temperatures. To obtain a detailed imprint of the temperature behaviour of the ribosome binding site, we determined here the 3D NMR structures of a portion of the stem-loop IV (wild-type (WT) MicroROSE) containing the SD region and of the mutant (Δ G83 MicroROSE) both at 30°C (Figure 1A). We also investigated the effect of temperature on the stability of the entire stem-loop IV (WT and Δ G83 MiniROSE, Figure 1A) by UV and NMR spectroscopy under different salt conditions. Our results together uncover the molecular basis of temperature sensing by an RNA thermometer.

Results

RNA constructs: MiniROSE and MicroROSE

Initial one-dimensional (1D) imino proton spectra showed that the lower stem regions of the WT and Δ G83 MiniROSE RNAs are identical and that the difference between the two molecules lies in the SD region. Therefore, the shorter MicroROSE version was chosen for further structural analysis. Both WT RNAs (MiniROSE and MicroROSE) were in

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Figure 1 The ROSE element. (A) The predicted secondary structure of the ROSE₁ element from *Bradyrhizobium japonicum* as determined by the mfold program (Jiang *et al*, 1997; Zuker, 2003). The Shine-Dalgarno sequence and the AUG start codon are underlined. The solid box indicates the MiniROSE and the dashed-lined boundary containing the SD sequence shows the MicroROSE. (B) Native PAGE (16%) analysis of the WT MicroROSE with natural CUUG tetraloop (lane 1) and with the UUCG tetraloop (lane 2). The hairpin and duplex conformations are indicated.

a hairpin to duplex equilibrium rendering NMR structure determination impractical (Figure 1B). The equilibrium of the corresponding Δ G83 RNAs was shifted towards a higher hairpin population compared to the respective WT RNAs. When the natural tetraloop CUUG was mutated into a more stable UUCG tetraloop, a homogeneous hairpin population was reached (Figure 1B). Hence, for structure determination of the WT MicroROSE and of the Δ G83 MicroROSE, we used the RNA constructs with a UUCG tetraloop. In addition, we added at the bottom of the stem G74, G75 and C102 to facilitate RNA transcription. None of these changes alter the SD sequence. When working with the WT MiniROSE RNAs, we used low concentrations to prevent duplex formation. For simplicity, we keep referring to the new engineered UUCG tetraloop RNAs as WT MicroROSE and Δ G83 MicroROSE.

NMR spectroscopy of the MiniROSE and MicroROSE RNAs

Imino assignment. For the MiniROSE and MicroROSE RNAs (WTs and Δ G83), the imino proton assignments were obtained with a two-dimensional (2D) ^1H - ^1H NOESY and a 2D ^1H - ^{15}N HSQC spectra in H_2O at 5°C or 2°C using unlabelled and isotopically labelled RNAs (Figure 2). As expected, an upfield shifted imino proton at 9.78 p.p.m. corresponding to

G90 was observed for both MicroROSE RNAs (Figure 2A and D). This indicated the formation of the UUCG tetraloop structure (Allain and Varani, 1995) and confirmed the presence of a monomeric stem-loop structure for both molecules. In Δ G83 MicroROSE, continuous sequential connectivity within the stem was observed from G90 in the tetraloop to G75 at the 5'-end in the 2D NOESY (Figure 2A). Formation of an U79-U97 and a G94-U82 base pair are indicated by the strong NOE correlations between the imino protons of the base pairs involved. U96 imino although broad is also observable in the 2D NOESY, indicating that U96-C80 is probably base-paired. The 2D ^1H - ^{15}N HSQC spectra (Figure 2B) confirm the assignments obtained with the 2D NOESY and the secondary structure proposed for Δ G83 MicroROSE, in which a continuous helix is formed from G74 to the UUCG tetraloop (Figure 2C).

In contrast to the Δ G83 MicroROSE RNA, sequential connectivity could only be observed from G75 to U79 imino proton and from G94 to the tetraloop G90 imino proton in the WT MicroROSE (Figure 2D). No imino protons for G83, U82, U81 and U96 were observable in the 2D NOESY or 2D ^1H - ^{15}N HSQC spectra (Figure 2D and E). Only weak NOEs appeared within the U79-U97 base pair (Figure 2D). The imino proton assignments were further confirmed using the 2D ^1H - ^{15}N HSQC spectra (Figure 2E) with labelled samples recorded at 2°C. These spectra indicate different base-pairing schemes in the central part of the stem as compared to the Δ G83 molecule and therefore a different secondary structure (Figure 2F).

Chemical shifts of imino ^1H and ^{15}N resonances observed for the larger RNA, namely the WT MiniROSE and the Δ G83 MiniROSE, were virtually identical to those in the equivalent MicroROSE molecules, except of course for the tetraloop (Figure 2G and I). This shows that the stem in the SD region has the same structure in the context of both the MicroROSE and MiniROSE RNAs. Furthermore, the imino proton chemical shifts for the bases in the bottom stem (G68 to A74 and U102 to G108) were identical in the WT and Δ G83 MiniROSE, indicating that the structural difference between the two RNAs lies essentially in the stem containing the SD sequence (Figure 2H and J).

Nonexchangeable proton assignment of the MicroROSE RNAs for structure determination. Assignments of the nonexchangeable protons and of their attached carbon resonances were obtained with homonuclear and heteronuclear NMR experiments using standard methodology (Varani *et al*, 1996). Sequential A-form RNA helical connectivity between anomeric ($\text{H}1'$) and aromatic protons could be established from G74 to C86 and G91 to C102 for both RNAs (see Supplementary Figures 1 and 2). Changes in chemical shifts were observed for G94 H8, G93 H8 and A95 H2 between the two RNAs, indicative of a different environment around these bases in the presence or absence of G83. In addition, G83 H8 in the WT MicroROSE has a strong NOE to its own $\text{H}1'$ indicating a *syn* conformation (see Supplementary Figure 2). Although several imino proton resonances are absent in the WT MicroROSE RNA, as discussed earlier, intrastrand base stacking is observed that confers an helical geometry to the RNA. The UUCG tetraloop in both molecules displays the characteristic spectral properties described earlier and could be assigned (Allain and Varani, 1995).

Figure 2 Secondary structures and folding of MicroROSE RNAs. (A) Portion of a 2D NOESY, (B) portions of two ^1H - ^{15}N HSQC and (C) secondary structure of the ΔG83 MicroROSE RNA. (D–F) same as (A–C) but for the WT MicroROSE. (G, H) and (I, J) are the same as (B, C) and (E, F) but for ΔG83 MiniROSE RNA and the WT MiniROSE RNA, respectively. The 2D ^1H - ^1H NOESY spectra ($\tau_m = 250$ ms) and the ^1H - ^{15}N HSQC were recorded at 600 MHz in 90% $\text{H}_2\text{O}/10\%$ D_2O at 5 and 2°C for MiniROSE and MicroROSE RNAs, respectively. The assigned imino proton crosspeaks are labelled according to the respective base colours in the secondary structure. The UUCG tetraloop and the bases not present in the natural sequence of ROSE₁ are shown in khaki, the region affected by the deletion of G83 in ΔG83 MicroROSE in red and the bases that remain unaffected in blue. In the WT MicroROSE, the region affected by the presence of G83 is shown in green. For MiniROSE RNAs, additional regions showing bases involved in base pairing in the AUG start codon site are in pink, and natural CUUG tetraloop and remaining additional regions in black. The secondary structures in (C) and (F) represent the experimental observations of NOEs of imino protons at 2°C and H and J the same at 5°C . Accordingly, closed circles indicate Watson–Crick base pairing and the open circles represent noncanonical base pairs. The same colour code is maintained in the later figures.

Solution structure of the Δ G83 MicroROSE RNA

Using a total of 369 NOE distance and 108 torsion angle constraints, an ensemble of the 20 best conformers over 50 structures calculated were obtained for Δ G83 MicroROSE. The structure of the RNA is precise with a backbone r.m.s.d. of 1.01 ± 0.3 Å (Figure 3A and Table I). The Δ G83 MicroROSE RNA adopts a stable stem-loop structure with a well-defined stem stabilised by hydrogen bonding and base stacking interactions (Figure 3A and B). All the residues in the stem adopt a C3' *endo* sugar pucker conformation. The structure of the UUCG tetraloop is similar to that described earlier (Allain and Varani, 1995). In the central part of the stem, three noncanonical base pairs U79-U97, C80-U96 and U82-G94 stabilise the helix (Figure 3C).

Solution structure of WT MicroROSE RNA

To elucidate the molecular basis for thermosensing, the solution structure of the WT MicroROSE RNA was determined based on a total of 371 interproton NOE distance restraints and 134 torsion angle restraints (Table I). Based on the weak intensity of the H1'-H2' crosspeak in a 2D TOCSY spectrum, the sugar puckers of U82 and G83 were constrained to an open range between C2' *endo* and C3' *endo*. Hydrogen bonds were added for the noncanonical base pairs U79-U97, the imino protons being observed for these bases at 30°C (Figure 4G). G94 imino proton was observed in the 2D NOESY (H₂O) spectrum (Figure 2D and E), indicating that it is hydrogen-bonded. G83 is in a *syn*-conformation based on the strong NOE between its H1' and H8 resonances in the 2D NOESY (D₂O) (Supplementary Figure 2). No hydrogen bonds

Figure 3 Solution structures of the WT and Δ G83 MicroROSE element. (A) Ensemble of the 20 lowest energy conformers of the Δ G83 MicroROSE superimposed over all residues, sugar phosphate (in grey sticks), backbone (pink ribbon) and RNA bases (blue sticks). (B) Stereoview of the Δ G83 MicroROSE minimum energy structure shown as ribbon and stick model. The colour scheme of the bases is maintained as in Figure 2. (C) Close-up views of the Δ G83 MicroROSE structure showing the base pairing in the region affected by the deletion of G83. Hydrogen bonds are indicated by blue-dashed sticks. The hydrogen and the heavy atoms involved are displayed. (D) Ensemble of 20 lowest energy conformers of the WT MicroROSE superimposed over all residues. The overall r.m.s.d. is 0.84 ± 0.26 Å. (E) Stereoview of the lowest energy structure of WT MicroROSE shown as ribbon and stick model. Colour scheme of the bases are same as in Figure 2. (F) Close-up views of the MicroROSE structure showing the hydrogen bonding network in the region affected by G83. Hydrogen bonds observed from NMR experimental data are indicated by blue dashed sticks. Hydrogen bonds observed from the 3D structure are shown in broken magenta sticks. The hydrogen and the heavy atoms involved are displayed.

Table I Structural statistics

	Δ G83 MicroROSE	WT MicroROSE
<i>Number of experimental restraints</i>		
Distance restraints from NOEs:	369	371
Within the stem and loop		
Intranucleotide	166	155
Internucleotide	203	216
Number of NOEs per residue	20	20
Number of δ dihedral angles	28	29
Number of H-bond restraints (two per bond)	32	28
Total number of experimental restraints	429	428
<i>Structure calculation statistics</i>		
Number of refined structures	20	20
Number of distance violations > 0.2 Å	0	2
Maximum violation	0.2	0.27
Number of angle violation > 5°	1.65 ± 0.6	2.5 ± 0.6
Maximum violation (deg)	8.57	8.77
<i>Deviations from idealised covalent geometry</i>		
Bonds (Å)	4.7 ± 3.7 × 10 ⁻³	4.7 ± 1.6 × 10 ⁻³
Angles (deg)	2.14 ± 0.02	2.13 ± 0.02
<i>Pairwise r.m.s.d.'s for heavy atoms (Å)</i>		
Global	1.01 ± 0.3 Å	0.84 ± 0.26 Å
Lower stem (G74-A78, U98-C102)	0.47 ± 0.2 Å	0.19 ± 0.08 Å
Upper stem and loop (C84-G93)	0.25 ± 0.1 Å	0.36 ± 0.19 Å
Region of interest (U79-U82, G94-U97)	0.49 ± 0.2 Å	0.69 ± 0.34 Å
Region of interest (U79-G83, G94-U97)		

were used from bases C80 to G83 and G94 to U96 in the initial structure calculations. However, preliminary structure calculations were converging towards a noncanonical base pairing between G94 (*anti*) and G83 (*syn*), where G94 imino having a chemical shift at 10.4 p.p.m. would be hydrogen-bonded to G83 O6 and G94 amino to G83 N7 (Figure 3F). With such a base pairing, the imino proton of G83 is not hydrogen-bonded, which is in agreement with the spectra since it is not observed either in the 2D NOESY or the ¹H-¹⁵N HSQC spectrum. Such a G (*syn*)-G (*anti*) base involving the same two hydrogen bonds along with the G (*anti*) chemical shift between 10 and 11 p.p.m. pair has been reported previously (Burkard and Turner, 2000). Thus, in the final refinement, the two hydrogen bonds of the G94-G83 base pair were added as constraints. The final ensemble of the 20 lowest energy structures has a global r.m.s.d. for the heavy atoms of 0.84 ± 0.26 Å (Figure 3D and Table I). Beside the G94-G83 base pair, helical stacking is observed for the entire stem (Figure 3E). U79 and U97 form a base pair like in the Δ G83 RNA, and A95 forms a base pair with U82 (Figure 3F, instead of U81 as in the Δ G83 RNA), although U82 imino protons could not be observed experimentally (A95 was found base-paired with U82 in more than 90% of the 20 best calculated conformers, although no hydrogen-bond constraints were used in the calculation). Another interesting structural feature is a small internal loop involving C80, U81 and U96

(Figure 3F). C80 and U81 stack on each other and both interact with U96. Three hydrogen bonds stabilise this internal loop, C80 H41-U96 O4, U81 H3-U96 O2 and U81 O4-U96 H3 (the latter hydrogen bond is found in 50% of the conformers). This internal loop is stabilised by weak, and probably transient hydrogen bonds. This would explain the absence of experimentally observed iminos in the spectra in this region of the molecule. In conclusion, the WT MicroROSE has a stable helical structure at 30°C, therefore preventing access of the translation machinery to the SD region at this temperature. However, the presence of the G83-G94 pair in the WT RNA introduces an asymmetry (absent in the Δ G83 MicroROSE RNA) that decreases the strength of the hydrogen-bond network in the stem.

The thermometer

To assess the thermal response in molecular details, 1D proton spectra at different temperatures were recorded for the WT MiniROSE and Δ G83 MiniROSE mutant (Figure 4). The 1D spectrum of the WT MiniROSE RNA (Figure 4C) shows less imino peaks at 2°C than the Δ G83 MiniRNA where all imino proton peaks in the stem were observed even until 30°C (Figure 4A). The imino proton peaks corresponding to U81, U82, U79 and U98 are still present at 30°C for Δ G83 MiniROSE (Figure 4A) but not in the spectrum of its WT counterpart (Figure 4C). In the Δ G83 MiniROSE, most of the peaks remained from 37 to 42°C, with the broadening of only U81 and U70, indicating that there is no major conformational change of the mutated MiniROSE within this temperature range. For the WT MiniROSE, a severe broadening of G91, G93 and U85 was observed at 37°C and the peaks disappeared at 42°C. Interestingly, imino proton resonances from the bases in the start codon U104, G105 and those occluding it (G72, U73) were also lacking at 42°C (Figure 4C).

The temperature profiles of the MicroROSE RNAs show that the U79, U97 and G94 imino protons are still present at 50°C in the Δ G83 RNA (Figure 4E), whereas in the WT RNA, all the iminos of the internal loop (U79, U97 and G94) are absent at the same temperature (Figure 4G). This indicates that destabilisation of the WT MicroROSE upon temperature increase initiates at the region U79-G83 and G94-U97, and that this takes place at lower temperature than in the Δ G83 RNA.

UV melting curves performed under different salt conditions for WT and mutant MiniROSE (Figure 5A and B) and for WT and mutant MicroROSE (Figure 5C-F) further confirm a difference in stability between the WT and Δ G83 RNAs. The Δ G83 RNAs present, under all tested conditions, a 3 to 7°C higher melting temperature (T_m) than the WT counterparts. The RNAs were stabilised by 5 to 6°C (T_m) with increase in salt concentration (compare Figure 5E representing the NMR condition with Figure 5C) and by 7 to 10°C with increase in magnesium concentration (compare Figure 5A and B for the MiniROSE and Figure 5C and D for the MicroROSE RNAs). We therefore investigated by NMR if salt and magnesium had an effect on the structure and the temperature sensing of the MicroROSE RNAs (WT and Δ G83). We studied the RNA in 50 mM KCl and 3 mM MgCl₂, a concentration where the T_m is almost as high as in 100 mM KCl and 5 mM MgCl₂ (compare Figure 5F and D). The spectra of the MicroROSE RNAs in the presence and absence of magnesium showed the same peak pattern and only very little chemical shift differences were

Figure 4 Thermal response of MiniROSE and MicroROSE RNAs. (A, C, E, G) 1D ¹H spectra at increasing temperatures of the imino proton resonances of ΔG83 MiniROSE (1.2 mM concentration, 600 MHz NMR field), WT MiniROSE (0.7 mM concentration, 900 MHz NMR field), ΔG83 MicroROSE (1.2 mM concentration, 600 MHz NMR field) and WT MicroROSE (1.2 mM concentration, 600 MHz NMR field), respectively, at increasing temperatures. (B, D, F, H) Secondary structure of ΔG83 MiniROSE (at 37°C), WT MiniROSE (at 37°C), ΔG83 MicroROSE (at 42°C) and WT MicroROSE (at 42°C), respectively. The colour code is the same as in Figure 2.

observed (less than 0.1 p.p.m. for all pyrimidine aromatic resonances; Supplementary Figure 3), indicating that the structure of the stem loops is not affected by the presence of magnesium. Furthermore, the presence of magnesium did not prevent the local destabilisation of the WT MicroROSE RNA upon temperature increase (compare Figure 4 and Supplementary Figure 4), suggesting that thermosensing of the ROSE element is not affected by this cation. Thus, while we see an effect on the global melting (T_m) of the RNA in the presence of salts from the UV melting curves, NMR studies

show that the local thermal response remains unaffected under physiological conditions.

In conclusion, the central region of the WT MiniROSE and WT MicroROSE RNAs are more thermolabile at 37°C than the rest of the molecule from A78 to G83 and from G94 to U98 (Figure 4D and H). At 42°C, destabilisation of WT MiniROSE proceeds further into the SD region along the bases G91 to G93 and C84 to C86 together with the bases C71 to U73 and A103 to G105 in the translation start site region. The WT MiniROSE structure therefore undergoes a destabilisation of

Figure 5 UV melting curves of WT and ΔG83 ROSE RNAs. Absorbance (at 260 nm) versus temperature curves of both the WT and ΔG83 MiniROSE RNAs (**A**, **B**) and MicroROSE RNAs (**C**–**F**) in different salt concentration buffers at pH 7.0. The melting temperature T_m and the exact salt conditions are indicated in the legend.

the region embedding the ribosome binding and the translation initiation sites in the physiologically relevant temperature window. The structural basis of thermosensing lies in a network of weak hydrogen bonds in this internal loop created by the presence of the noncanonical G83-G94 base pair.

Discussion

We present here the first high-resolution structure of the functional domain of an RNA thermometer. Moreover, we

identified the structural features responsible for thermosensing that explain the biological function of the RNA element. By using translational ROSE-*lacZ* fusions, a significant increase of β -galactosidase activity from 30 to 37°C has been reported previously (Nocker *et al*, 2001). *In vitro* melting studies showed conformational transitions of the ROSE element leading to its opening up with increasing temperatures (Chowdhury *et al*, 2003). This transition involves the SD sequence, which is occluded at 30°C but becomes accessible to antisense oligonucleotides at 42°C as was determined by RNaseH experiments with the full-length ROSE RNA. Our

Figure 6 Schematic representation of the temperature response of the RNA thermometer in translation. The Δ G83 MiniROSE is shown in (A) and WT MiniROSE in (B). [N] represents the nucleotides from the 5'-UTR after opening of the stem loop upon increased temperature. The red stars indicate binding to the 30S ribosomal subunit. The 30S ribosomal subunit is represented by an orange surface.

NMR studies support these previous observations and the 3D structure of the MicroROSE RNA shows precisely that the region adjacent to the SD sequence is the site where melting initiates at the onset of heat shock.

Another important observation of our NMR study is that the secondary structure of the temperature-responsive hairpin deviates considerably from the *in silico* prediction (Figure 1A). The universally conserved G83 which appeared bulged is actually engaged in base pairing with G94 in the opposite strand. G83 adopts a *syn* conformation, while G94 is *anti*. Moreover, the presence of a region containing a small internal loop (C80, U81, U96) with a transient hydrogen bonding network along with two noncanonical closing base pairs such as U79-U97 and G83-G94 renders the structure vulnerable to higher temperatures. Hydrogen bonds of the closing pair U79-U97 are affected at 37°C. Under heat shock at 42°C, the other closing pair G83-G94 along with A78-U98 is destabilised as well.

In the NMR studies with the MicroROSE RNA, we still observed the imino protons of G91, U85 and G93 at 42°C (Figure 4G) indicating only partial liberation of the SD

sequence at this temperature, whereas these imino proton resonances were already absent in the MiniROSE (Figure 4C). This discrepancy can be explained by a greater stabilisation of the stem imposed by the engineered UUCG tetraloop in MicroROSE, further strengthened by a favourable CG closing pair (Woese *et al*, 1990; Allain and Varani, 1995) in this context. Accordingly, introduction of the UUCG sequence into a ROSE₁-hspA-lacZ fusion prevented translation in the physiological temperature range between 30 and 37°C (data not shown). The CUUG tetraloop on the other hand is itself thermodynamically less stable and in addition has an unfavourable CG closing pair (Woese *et al*, 1990; Jucker and Pardi, 1995), which leads to further destabilisation of the WT like MiniROSE and explains the natural selection of the CUUG sequence in ROSE₁. In addition, hydrogen bonding in base pairs occluding the start codon also weakens at 42°C (Figure 4C), which should facilitate the liberation of the translation initiation site in the RNA thermometer. Thus, the fourth stem loop of the ROSE RNA is a sensitive thermometer in the physiologically relevant temperature range. Whether general RNA binding proteins or RNA helicases

contribute to efficient thermosensing *in vivo* is presently unknown.

It is noteworthy that there is one known example of a UACG tetraloop that belongs to the same UNCG tetraloop family as UUCG and occurs in the functional ROSE_{N1} element of *Rhizobium* sp. NGR234 (Nocker *et al*, 2001). Here the stabilising effect is probably compensated by replacing the closing base pair (C77-G99 in MiniROSE) by a weaker U77-A99 pair in ROSE_{N1}.

The 3D structure of the temperature-responsive region of the ROSE thermometer provides the platform to understand the functional significance of several conserved regions in this stem loop (Nocker *et al*, 2001). Apart from the SD sequence and start codon, the entire stretch of nucleotides from U81 to C86 complementary to the SD sequence is conserved in all ROSE-like elements present in α -proteobacteria, of which the rhizobial ones are a subclass (Waldminghaus *et al*, 2005). The consensus sequence **UYGCUY**, where Y stands for a pyrimidine deviates slightly from the γ -proteobacteria-like consensus **UYGCU**. Our NMR study suggests that the sequence conservation goes along with a structural conservation and is in accordance with our observations from mutational analyses. It provides a molecular insight as to why the Δ 83G RNA was not responsive to higher temperatures, why the A78U variant was derepressed and why the compensatory A78U/U98A mutant restored repression (Chowdhury *et al*, 2003). Although RNA can fold into independent 3D motifs (Brion and Westhof, 1997; Lukavsky *et al*, 2003) the role, if any, of the other stem loops of ROSE in opening of the SD region with increasing temperatures remains to be deciphered.

Finally, based on the structure–function correlation obtained from our NMR analysis along with fully consistent previous *in vivo* and *in vitro* studies, we illustrate the principle of temperature sensing in Figure 6. The SD sequence and start codon of the mRNA remain occluded by base pairing with a complementary sequence in its 5'-UTR, thereby repressing translation at growth temperature of 30°C. The first step in temperature sensing initiates destabilisation of the region adjacent to the SD sequence at 37°C, making it partially accessible to the 30S ribosome subunit (Figure 6B) leading to binding and low levels of translation as demonstrated by significant reporter gene activity *in vivo* (Nocker *et al*, 2001; Chowdhury *et al*, 2003). A further increase in temperature strengthens the mRNA-30S interaction by full liberation of the SD and AUG sequence providing better access of the fMET-tRNA to the A-site of the 30S particle (Figure 6B). For the Δ G83 mRNA, stronger intramolecular protection of these two sites impairs translation (Figure 6A). It has been observed that both the start codon and the SD sequence are important for translation initiation to meet the conformational demands required by the fMET tRNA/mRNA/A site interaction on the 30S subunit (Yoshizawa *et al*, 1999). Also, the SD sequence remains bound to the 16S RNA anti-SD site until the addition of the first few amino acids (Zavialov *et al*, 2005). Translation elongation frees the SD and AUG sites where new 30S subunits can bind leading to polysome formation and finally producing large levels of the encoded protein. Thus, bacterial RNA thermometers detect temperature changes by a simple and efficient mechanism that appears sufficient to control the gene expression of several heat-shock genes.

Materials and methods

RNA sample preparation

Unlabelled, ¹⁵N-¹³C AU-only and ¹⁵N-¹³C GC-only labelled RNA were prepared by *in vitro* transcription of synthetic DNA templates using T7 RNA polymerase (Milligan *et al*, 1987). The RNA was purified either on denaturing polyacrylamide gel according to the protocol of Price *et al* (1998) or by HPLC under denaturing conditions at 85°C (6 M urea buffered at pH 7.4 by Tris-HCl solution at 12.5 mM final concentration). In this last case, the oligonucleotides were first separated on an anionic exchange column (DNAPac PA-100 from DIONEX, Switzerland) using sodium perchlorate (0.5 M) as exchange salt and purified on a Sep-Pak C18 cartridges (supplied by Waters, Switzerland) in the presence of 0.1 M triethylamine/acetic acid at equimolar concentration (pH 6.5 adjusted by adding NaOH solution) in order to get rid of urea. After washing of the cartridge with water and RNA elution with acetonitrile/water (80/20 in volume), the eluted oligonucleotide was lyophilised. The RNA was suspended in 1 ml of water and treated by a slightly excess of 1 M aqueous sodium bicarbonate solution with respect to the RNA phosphate equivalence. Four successive chloroform extractions of this treated solution were performed to remove triethylamine traces (10 ml of chloroform each time) and then the final solution was lyophilised. Finally, the RNA dissolved in 5 ml of water was annealed at pH 6.0 (with NaOH) by heating to 95°C and snap cooling in liquid nitrogen to ensure the formation of a homogeneous population of monomeric stem loops. RNA was lyophilised and dissolved in 100% D₂O or 10% D₂O/90% H₂O in a final concentration range of 0.7–2 mM for NMR spectroscopy.

UV melting studies

Absorbance versus temperature was recorded for both WT and Δ G83 MiniROSE and MicroROSE RNAs at 260 nm on Varian-Cary UV-visible spectrophotometer equipped with a Peltier temperature control device. Standard 1 cm path length quartz cuvettes were used for the measurements. RNA melting curves were recorded at a heating rate of 0.5°C/min. The desalted RNA stock solutions at 1 mM concentration were adjusted at pH 7 by adding KOH 1 M and then diluted (2000 dilution factor) in different buffers: 30 mM NaCl (close to salt concentration of NMR samples), 100 mM KCl, 100 mM KCl plus 5 mM MgCl₂ (simulating physiological conditions) and 50 mM KCl plus 3 mM MgCl₂. The diluted samples were heated at 100°C for 10 min followed by immediate immersion in liquid nitrogen for 3 min. Then, before UV measurement, the samples were degassed by sonication for 15 min. Fitted RNA melting curves calculated from experimental data and melting temperatures were derived from five parameters sigmoidal equation using Sigmaplot software. Each UV measurement was repeated at least twice.

NMR spectroscopy

NMR data were acquired at 275, 278 or 303 K on Bruker DRX-600 and Avance 900 spectrometers. 1D temperature profiles were obtained at the different temperatures mentioned in the text. Data were processed with XWINNMR (Bruker) and analysed with Sparky (<http://www.cgl.ucsf.edu/home/sparky/>). ¹H, ¹³C and ¹⁵N assignments were made using a combination of homonuclear and ¹³C and/or ¹⁵N heteronuclear experiments. Exchangeable proton resonances were assigned from 2D NOESY and ¹H-¹⁵N HSQC spectra of ¹³C-¹⁵N AU-only and GC-only labelled samples of the WT MicroROSE, Δ G83 MicroROSE, WT MiniROSE and Δ G83 MiniROSE. For the WT MicroROSE and of the Δ G83 MicroROSE, most of the sequential resonance assignments were achieved using 2D and 3D ¹³C-edited NOESYs taking advantage of the helical structure of the RNAs (Varani *et al*, 1996). Sugar spin systems were completed using 2D TOCSY, ¹H-¹³C HSQC and 3D HCCH TOCSY. Assignment ambiguities were further resolved with 2D F1-edited, F2-edited NOESY and 2D F1-filtered, F2-edited NOESY (Peterson *et al*, 2004). NOE restraints of nonexchangeable protons were obtained from 2D NOESY experiments with mixing time of 100, 250 and 350 ms and characterised based on their intensities as either very strong (1.8–2.5 Å), strong (1.8–3.0 Å), medium (1.8–3.7 Å), weak (1.8–4.5 Å) and very weak (1.8–6.0 Å). Upper limit restraints of 5 Å were used for aromatic–aromatic interresidues NOEs. G83 H1'-H8 distance was constrained to 3.0 Å typical of a *syn* conformation based on the strong NOE between these protons. Assignments were made for all

NOE crosspeaks to the imino protons observed at 2 or 5°C, except for U81, U82, G83 and U96 of the WT MicroROSE and of the WT MiniROSE that could not be observed in 2D NOESY in H₂O probably owing to exchange with the solvent. Hydrogen bond restraints were not introduced for these protons. For both RNAs, the hydrogen bonds relative to the U79–U97 base pair were established according to the peak intensity of the sequential H5 correlations with the two preceding H2' and H3' sugar protons observed in the 3D (¹H–¹³C) NOESY-HSQC. The intensity of U97 H5 NOE crosspeaks were similar to those observed in the A–U Watson–Crick base pair, whereas U79 H5 NOE crosspeaks, which has its O₂ hydrogen-bonded to the U97 imino, had much weaker intensity because of the base shifting. Dihedral angle constraints conferring to C3' *endo* (50° ≤ δ ≤ 110°) or C2' *endo* (130° ≤ δ ≤ 190°) were determined from the H1'–H2' crosspeak intensities in the 2D TOCSY spectra.

Structure calculation

The final set of constraints consisting of NOE distances, hydrogen bonds and dihedral angle restraints was used to calculate a total of 250 structures for both the WT MicroROSE and the ΔG83 MicroROSE in DYANA (Güntert *et al*, 1997) starting from random structures. Loose RNA A-form torsion angle restraints were used in the canonical part of the stem to impose better convergence of the final ensembles; no such restraints were used in the region of the stem without Watson–Crick base pairing. The 50 structures with the lowest target function were then used as starting structures for further refinement using AMBER 7.0 software (<http://www.nersc.gov/nusers/resources/software/apps/chemistry/amber/>) with the generalised-Born solvation model (Cornell *et al*, 1995; Bashford and Case, 2000) having (Cornell *et al*, 1995) force field, in implicit water with a refinement protocol as described in Stefl *et al* (2001) and

Padrta *et al* (2002). Chirality constraints were introduced in the protocol to avoid any atom flipping during the heating step. The relative weightings and the values of the force constants of the various energy terms were set as in previously determined RNA structures (Maris *et al*, 2005). Based on minimum energy criteria, the 20 best structures were selected out of 50 for final analysis and viewed in MOLMOL (Koradi *et al*, 1996). The hydrogen bonds not observed experimentally were deduced from the final ensemble of the structures based on distance between the donor (proton) and acceptor.

Atomic coordinates and NMR restraints of the WT MicroROSE and ΔG83 MicroROSE RNAs have been deposited to the Protein Data Bank under the accession codes 2GIO and 2GIP, respectively.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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