Assays for the RNA chaperone activity of proteins

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Abstract
Proteins with RNA chaperone activity promote RNA folding by loosening the structure of misfolded RNAs or by preventing their formation. How these proteins achieve this activity is still unknown, the mechanism is not understood and it is unclear whether this activity is always based on the same mechanism or whether different RNA chaperones use different mechanisms. To address this question, we compare and discuss in this paper a set of assays that have been used to measure RNA chaperone activity. In some assays, this activity is related to the acceleration of monomolecular reactions such as group I intron cis-splicing or anti-termination of transcription. Hereby, it is proposed that the proteins release the RNAs from folding traps, which represent the kinetic barriers during the folding process and involve the loosening of structural elements. In most assays, however, bimolecular reactions are monitored, which include the simple acceleration of annealing of two complementary RNAs, the turnover stimulation of ribozyme cleavage and group I intron trans-splicing. The acceleration of these reactions most probably involves the unfolding of structures that interfere with annealing or folding and may in addition provoke annealing by crowding. Most assays are performed in vitro, where conditions might differ substantially from intracellular conditions, and two assays have been reported that detect RNA chaperone activity in vivo.

Introduction
RNA molecules are synthesized as single-stranded polyanionic chains and, to accomplish their diverse tasks, they must fold into defined three-dimensional structures. The folding pathways of RNA molecules are hierarchical, starting with the formation of short double-stranded helices through WC (Watson–Crick)-type base-pairs, which are part of the secondary structure. These short secondary-structure elements then organize themselves in space to form very diverse tertiary structures [1]. Apart from the canonical AU and GC pairs, RNA also supports GU pairs, so the number of possible pairing combinations is very high and the alternative conformations are often comparably stable. This structural versatility is a challenge for an RNA molecule, because it becomes more difficult to reach a single native folding state [2]. On the other hand, this structural promiscuity leads to an important attribute: the possibility for one RNA molecule to exist in different conformational states achieving different functions and rapid regulation through refolding.

Folding of large RNA molecules can be very slow, due to the formation of long-lived intermediates, which represent kinetic traps. Alternatively, folding can be inefficient due to the low stability of some structures and, very often, RNA populations are inhomogeneous, meaning that a variety of conformations coexist, making the characterization of RNA molecules rather difficult [3]. Proteins can assist in RNA folding by either binding and stabilizing specific structures or by RNA chaperone activity. The latter has been defined as an activity that accelerates folding through the resolution of misfolded structures or inhibition of their formation. Once the RNA is correctly folded, proteins with RNA chaperone activity are no longer required [4].

The RNA folding mechanism and the components that assist this process are less well understood than protein folding. It is unclear how often RNA misfolding occurs within cells and whether this represents a stress situation since, in contrast with denatured proteins, RNAs do not precipitate and might therefore not form destructive and invasive particles. Genetic evidence supports the idea that many RNAs require proteins for folding. For example, when translation of the pre-mRNA of the group I intron containing the td gene is inhibited by nonsense codons in the upstream exon, it cannot fold into a splicing-competent structure [5]. Folding of these pre-mRNAs can, however, be promoted by the overexpression of genes coding for proteins with RNA chaperone activity [6]. Thus this pre-mRNA is not capable of self-folding since its folding is strongly hampered by exon–intron interactions, which interfere with the formation of the active structure. In yeast, a mutation in a pre-tRNA renders the cell cold-sensitive and La protein-dependent [7]. La is a ubiquitous protein that binds to the 3′-end of RNA polymerase III transcripts and might exert RNA chaperone activity and display binding specificity to a class of RNAs and probably promote their assembly into RNP (ribonucleoprotein) particles. In an analogous manner, another yeast mutant that is impaired in the assembly of the U6 small nuclear RNP is La protein-dependent [8]. For a review on proteins with RNA chaperone activity and the strategies for RNA folding and assembly, see the recent reviews [9,10].

Key words: FRET, group I intron, RNA annealing, RNA chaperone activity, RNA folding, trans-splicing.
Abbreviations used: CAT, chloramphenicol acetyltransferase; FRET, fluorescence resonance energy transfer; E3, relative FRET efficiency; IPRG, isopropyl β-D-thiogalactoside; RNP, ribonucleoprotein.
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It is still unclear whether, in analogy to protein-folding chaperones, proteins exist whose only function is to help RNAs fold correctly without recognizing or binding a specific RNA. A probable candidate is the bacterial Hfq protein, which binds to a very large set of RNAs, with strong or less strong affinities, and promotes annealing of RNAs [11]. The La protein might be an RNA chaperone for RNA polymerase III transcripts. However, most of the proteins that exert RNA chaperone activity have defined functions and are active in very specific contexts, e.g. (i) the HIV nucleocapsid-derived peptide NCp7 is involved in the dimerization and reverse transcription of the HIV genomic RNA and (ii) a relatively large number of ribosomal proteins are involved in the assembly and stability of the ribosome [12]. How they exert their individual RNA chaperone activities remains to be analysed and we will have to compare them carefully to understand the basic principles of the mode of action of these proteins.

The number of proteins for which RNA chaperone activity has been reported is growing steadily and there is no consensus as to what this activity is, how it is defined and how it should be measured and quantified. A diverse number of assays and systems have been described. In the present study, we want to discuss the different assays used to detect RNA chaperone activity, trying to focus on the differences between these assays regarding what type of reaction they might be revealing.

**In vitro assays for RNA chaperone activity**

**RNA annealing**

Assaying the effect of proteins on RNA hybridization reactions is a common method to test for RNA chaperone activity [13]. Single- and double-stranded fractions are usually analysed by gel electrophoresis, and radioactive labelling commonly provides flexibility to assay all kinds of RNA substrates *in vitro* (e.g. [14]). Figures 1(A)–1(C) show the annealing of fluorophore-labelled, 21 nt long RNAs lacking stable secondary structures in the presence or absence of *Escherichia coli* protein StpA, followed by separation on a native polyacrylamide gel. The introduction of the fluorescence labels also allows for a more sophisticated and accurate way to monitor nucleic acid hybridization (see [15] for the first DNA annealing assay). On duplex formation, the two fluorophores are close enough for FRET (fluorescence resonance energy transfer) to occur, with the increase in energy transfer being proportional to the fraction of double-stranded RNA (Figure 1A). This set-up enables measurements with a time resolution of milliseconds, which is vital when investigating the effect of RNA chaperones on an already fast reaction such as RNA annealing. At 37°C and a concentration of 10 nM each, the 21-mers anneal with a $k_{\text{obs}}$ of 0.0064 s$^{-1}$, which is accelerated 2.2-fold in the presence of 1 µM StpA in the sample (Figure 1C). In contrast, the ribosomal protein L9 of *E. coli* shows no RNA chaperone activity, as demonstrated previously in a trans-splicing assay [12]. Note that, in this set-up, the fluorescence was measured by two independent detectors and, hence, no absolute $E_{\text{FRET}}$ (relative FRET efficiency) values can be derived. Absolute fractions of annealed RNAs can be derived in a set-up using, for example, a monochromator switching between the donor and acceptor wavelengths or by comparing the result with values derived from gel-shift assays [16]. Choosing the substrate for RNA annealing assays is critical since secondary structures within one or both of the hybridizing strands introduce an unwinding step before annealing [17]. For molecular beacons, this is done deliberately to increase the signal-to-background ratio [18].

**Strand displacement**

RNA chaperone activity can be monitored more clearly in strand displacement reactions than in RNA annealing assays. Whereas RNA annealing also occurs without RNA chaperone activity, double-stranded RNA that is sufficiently stable at the assay temperature will usually not separate significantly *in vitro*. As shown in Figure 1(E), strand displacement with a double-stranded 21 nt RNA substrate depends on the activity of StpA. In contrast with RNA helicases, RNA chaperones also have not been shown to require 5’ or 3’ single-stranded overhangs for their RNA strand displacement activity. To decrease the reverse reaction of strand displacement, single-stranded sections can be introduced into the initial duplex. On strand displacement by a perfectly complementary competitor RNA, a thermodynamically more stable double-stranded molecule will be formed [19].

**Extended duplex formation coupled with ribozyme cleavage**

Dimerization of the HIV-1 genomic RNA is initiated by the interaction of two self-complementary stem loops called DIS (dimerization initiation site), followed by the conversion of this initial kissing loop into an extended duplex. This conversion requires the overcoming of an energy barrier created by the opening of the stems and cannot be detected at low temperatures unless the protein NCp7 derived from the nucleocapsid protein is present. An assay to monitor quantitatively this interconversion was designed in such a way that, when the extended duplex folds, a ribozyme is formed that cleaves one of the RNA strands (Figure 2A). The hammerhead ribozyme as well as a leadzyme were used, and both allowed us to monitor extended duplex formation [20]. This assay is clearly different from the RNA annealing assay, since no new annealing is required and the interconversion is a monomolecular reaction. The rate-limiting step in this assay is the opening of the stems, an activity that is probably analogous in the strand displacement assay. The irreversibility of the reaction is achieved by cleavage of one RNA strand. How NCp7 lowers the energy barrier is unclear, but in the presence of NCp7 at 37°C, the reaction is much faster than in its absence and at 55°C.
Figure 1 | RNA chaperone activity in RNA annealing and strand displacement assays

(A) The 21R+ oligoribonucleotide AUGUGAAACUUCUGACGU and the complementary 21R– oligoribonucleotide ACUGCUGAGAUAUUCACCACAU were obtained from VBC-Genomics (Vienna, Austria) with the 5’-ends labelled with Cy3 and Cy5 respectively [14]. On hybridization, FRET can occur as indicated by the shift in emission wavelength. (B) Oligoribonucleotides 21R+ and 21R– (100 nM each) were incubated at 37°C in annealing buffer [50 mM Tris, pH 7.5, 3 mM MgCl2 and 1 mM DTT (dithiothreitol)] in the absence or presence of 1 µM E. coli StpA. At the indicated time points, aliquots were removed and mixed on ice with stop buffer [final concentration of 0.7% SDS, 50 mM EDTA (pH 8.0) and 10% (v/v) glycerol]. Separation of 15 µl samples was on a 15% (w/v) native polyacrylamide gel at 4°C. Cy5 fluorescence was detected with a Molecular Dynamics Typhoon 8600. (C) Annealing of 10 nM 21R+ and 21R– at 37°C in annealing buffer in the absence or presence of 1 µM E. coli StpA and ribosomal protein L9 respectively. In a modified Applied Photophysics PiStar-180 spectrometer, Cy3 and Cy5 fluorescence was detected simultaneously with two photomultipliers. E\textsubscript{FRET} was calculated as the ratio of donor fluorescence to acceptor dye fluorescence and is given in arbitrary units. Owing to the usage of two independent detectors, in this particular set-up, no absolute E\textsubscript{FRET} values can be calculated. Therefore the amplitudes of the graphs are arbitrary and no absolute fractions of RNAs annealed can be derived. This does not affect the reaction rate; the RNAs annealed with and \( k_{\text{obs}} = 0.0064 \text{ s}^{-1} \) as computed from a single exponential fit, and the presence of 1 µM StpA accelerated this reaction 2.2-fold. (D) Strand displacement of a prehybridized 21-mer with a complementary competitor RNA results in the loss of FRET. (E) The band-shift assay of a strand displacement reaction with 100 nM double-labelled dsRNA was performed and analysed as stated in (B). Unlabelled 21R+ competitor RNA was present in 10-fold excess. (F) Stopped-flow analysis of the displacement reaction with 10 nM double-stranded RNA and 10-fold competitor RNA as described in (F). The \( k_{\text{obs}} \) as derived from a single exponential fit of 0.00014 \text{ s}^{-1} was increased by approx. 250-fold in the presence of 1 µM StpA.
Figure 2 | Ribozyme-coupled in vitro RNA chaperone assays

(A) Principle of the ribozyme-coupled dimerization assay. Heterodimers are formed by a kissing interaction between the loop bases of two RNA stem-loops, each containing one part of a ribozyme (white and grey boxes). Only structural rearrangements into a double-stranded extended dimer leads to the formation of a functional ribozyme and cleavage of one RNA strand. Addition of the NCP7 peptide, which has strong RNA chaperone activity, accelerates this transition. (B) Cis-splicing of a group I intron pre-RNA occurs when the intron is correctly folded into an active structure. The pre-RNA is transcribed in vitro and purified. Folding is achieved by denaturing the RNA for 1 min at 95°C and incubating at the desired temperature. The percentage of correctly folded molecules is monitored through splicing, which can be initiated by the addition of the guanosine cofactor. The addition of a protein with RNA chaperone activity leads to an increase in the amount of RNA molecules that can react with a high reaction rate. (C) The hammerhead ribozyme assay: the presence of an RNA chaperone leads to increased reaction rates of the ribozyme. By monitoring the changes in reaction rates, it is possible to distinguish between the different activities of the protein, namely strand annealing and strand displacement. (D) Trans-splicing assay: two separately transcribed parts of a group I intron are hybridized. Splicing is initiated by the addition of exogenous guanosine. In the first trans-esterification reaction, the 5′-exon is cleaved and the exogenous guanosine is ligated to the 5′-intron. In a second trans-esterification reaction, the 3′-exon-intron junction is cleaved and both exons are ligated. The presence of RNA chaperones increases trans-splicing at lower temperatures.
Group I intron splicing in cis
Splicing of a group I intron is dependent on the folding of the RNA strand into a defined three-dimensional conformation. In vivo proteins usually assist and promote folding of the RNA, leading to a splicing reaction that is fast and efficient. In contrast, in vitro splicing is mostly slow and inefficient due to the misfolding of the RNA molecule, which occurs in the absence of assisting protein factors. The cis-splicing assay takes advantage of this fact. In vitro-transcribed and radioactively labelled RNA of the thymidylate synthase gene of the bacteriophage T4 is used to monitor splicing, which is initiated by the addition of GTP as the splicing cofactor (Figure 2B). This enables monitoring of the folding and splicing events separately. In the absence of proteins, most of the RNA molecules are in a slow reacting conformation. The addition of potential RNA chaperones to the folding reaction significantly decreases the proportion of slow reacting molecules and the amount of fast reacting RNA molecules increases [21,22]. This assay is most likely monitoring the unfolding of misfolded RNAs, and since it is a monomolecular reaction, it is independent of the RNA concentration.

Acceleration of the hammerhead ribozyme activity
Another model system that allows the examination of the RNA chaperone activity of a protein is the acceleration of the cleavage reaction of the hammerhead ribozyme (Figure 2C). In this system, the annealing of the substrate RNA, the unwinding of RNA molecules and the release of the cleavage product are rate-limiting steps. The presence of an RNA chaperone can help the ribozyme to overcome these barriers and thus lead to an enhancement of the reaction rate. Since the individual rates and equilibrium constants for the reaction of the hammerhead ribozyme are well known, this system allows the interpretation of the effect of the respective proteins, such as strand annealing or strand-exchange activities. The hammerhead system has been used to show the RNA chaperone activity of a wide variety of proteins such as the NC protein of HIV-1 [21,23,24].

Group I intron splicing in trans
Similar to the cis-splicing assay, in vitro splicing of the thymidylate synthase group I intron is tested in the trans-splicing assay. The splicing construct is split into two halves: construct H1 consists of the full-length exon 1 (549 nt) and 131 nt of the 5′-part of the intron, so that H1 ends with sequences in L6 (loop 6 of the P4–P6 domain) of the intron; construct H2 consists of 147 nt of the remaining intron and a short exon 2 (23 nt) [25]. For measurement of the RNA chaperone activity of a protein, both constructs are transcribed separately in the presence of [35S]α-CTP so that the transcripts become internally labelled (Figure 2D). Then, the two RNAs are combined, denatured and annealed. In vitro splicing is initiated by the addition of [32P]GTP. At 37 °C, trans-splicing is significantly decreased and only at high temperatures (55 °C) can efficient trans-splicing occur, suggesting that misfolding prevents trans-splicing at lower temperatures. The addition of a protein with RNA chaperone activity may rescue the folding into a splicing-competent group I intron and, as a result, an increase in splicing is detected. This assay was used to demonstrate the RNA chaperone activity of several ribosomal proteins [12,21].

In vivo assays for RNA chaperone activity
In vivo folding trap
The thymidylate synthase group I intron of the E. coli bacteriophage T4 self-splices in vitro and even more efficiently in vivo. The intron is embedded in a protein-coding gene; consequently, transcription, splicing and translation are coupled. Surprisingly, the uncoupling of translation results in a significant decrease in in vivo splicing. This splicing deficiency was interpreted as a result of the formation of a folding trap due to interactions between exon and intron sequences that interfere with intron folding [5]. Folding of these trapped pre-RNAs can be rescued by overexpressing genes coding for proteins with RNA chaperone activity (Figure 3A) [6].

To test RNA chaperone activity in vivo, we introduced a stop codon in the upstream exon of the td gene. This uncouples splicing from translation and results in decreased splicing. The td mutant is expressed from a high-copy plasmid with IPTG (isopropyl β-D-thiogalactoside)-inducible promoter in the E. coli strain C600 (thy−). A second IPTG-inducible plasmid carries the putative RNA chaperone. E. coli cells are induced with IPTG and grown to exponential phase, after which the cells are harvested, cracked and total RNA is extracted. RNA chaperoning activity, which rescues the misfolding that occurs in the stop codon mutant, is tested indirectly by measuring the increase in splicing efficiency. The limitation of this assay occurs when overexpression of the RNA chaperone becomes toxic for E. coli; however, a wide collection of proteins ranging from bacterial proteins (Hfq, StpA and S12), viral proteins (NCp7) and human proteins (La and Ro60) were evaluated in this assay.

Transcription anti-termination in vivo
The temperature-shift of an exponentially growing E. coli culture from 37 to 15 °C results in the temporary stopping of cell growth, during which expression of cold-shock genes is induced. In E. coli, nine proteins of the Csp family have been described. RNA chaperone activity was demonstrated for CspA, CspC and CspE [26,27]. In vivo, the RNA chaperone activity of the cold-shock proteins was tested using the transcription anti-termination assay [28]: the CAT (chloramphenicol acetyltransferase) gene is preceded by a ρ-independent trpL terminator. Transcription of the terminator results in the formation of a stem–loop structure of the transcribed RNA, which is followed by a poly-U stretch. Formation of this structure within the transcript causes
**Figure 3 | In vivo RNA chaperone assays**

(A) Folding trap assay: In the absence of translation in the context of a stop codon mutant, 3'-terminal intron sequences preferentially pair with exon 1 sequences, resulting in a splicing-deficient fold (misfolded ribozyme). Overexpression of RNA chaperones in the living cell can partly alleviate this splicing deficiency by 'melting' the misfolded structure. Correct folding can occur (ribozyme) and the properly folded ribozyme can undergo cleavage. (B) Transcription anti-termination assay: The formation of a terminator stem-loop structure in the transcribed RNA prevents the RNA polymerase from continuing transcription. The downstream CAT gene cannot be expressed and the cells are chloramphenicol-sensitive (CmS). Overexpression of RNA chaperones 'melts' the terminator stem; transcription can continue, and the cells become chloramphenicol-resistant (CmR).

**Conclusions**

Several proteins have been reported to accelerate RNA folding reactions based on a variety of assays. This acceleration of folding is also known as RNA chaperone activity. Since the assays monitor quite diverse folding events, it is not clear whether the types of reaction and mechanism observed are different. The *in vivo* assays, as well as the group I intron *cis*-splicing assay, clearly monitor monomolecular reactions, where it is believed that the rate-limiting step during folding is the resolution of aberrant conformations, which interfere with correct folding. The activity of the RNA chaperone in these assays is most probably unfolding of the RNA. It has indeed been shown that the presence of the *E. coli* protein StpA results in increased accessibility of bases to dimethyl sulphate, a small chemical that methylates accessible positions in adenines and cytosines [29]. We interpreted this increase in accessibility as the result of a looser conformation of the intron RNA. In strand displacement and in ribozyme-coupled extended duplex formation, the activity that is detected is also clearly an acceleration of the unfolding of RNA helices. When these assays are quantified to compare the RNA chaperone activities of proteins, it has to be...
ensured that helices with identical stabilities are used. In the
different assays, the structural stability of the RNAs differs
considerably, making it difficult to compare the activities of
different proteins. A detailed comparison of proteins with
RNA chaperone activity remains to be done systematically
and the different activities need to be related to different
protein motifs. It is however already clear that the term ‘RNA
chaperone activity’ will require a more detailed classification.

Research in our laboratory is supported by the Austrian Research
Fund, FWF grants P16026, F1703 and 272.

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Received 21 January 2005