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Pathways of RNA folding and ribosome assembly from time-resolved footprinting

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Conspectus

Many non-protein coding RNAs fold into intricate three-dimensional shapes in order to act in protein synthesis, splicing, and many other facets of gene regulation and expression. Hydroxyl radical footprinting probes the solvent accessibility of the RNA backbone at each residue in as little as 10 ms, providing detailed views of RNA folding pathways in real time. Time-resolved footprinting of ribozymes showed, in conjunction with other methods such as solution scattering and single-molecule FRET, that stable domains of RNA tertiary structure fold in less than 1 s. However, the free energy landscapes for RNA folding are rugged, and individual molecules kinetically partition into folding pathways that lead through metastable intermediates, stalling the folding or assembly process.

Time-resolved footprinting was used to follow the formation of tertiary structure and protein interactions in the 16S rRNA during the assembly of 30S ribosomes. As previously observed in much simpler ribozymes, assembly occurs in stages, with individual molecules taking different routes to the final complex. Interactions occur concurrently in all domains of the 16S rRNA, and multi-stage protection of binding sites of individual proteins suggests initial encounter complexes between the rRNA and ribosomal proteins are remodeled during assembly.

Equilibrium footprinting experiments showed that one primary binding protein was sufficient to stabilize the tertiary structure of the entire 16S 5' domain. The rich detail available from the footprinting data showed that the secondary assembly protein S16 suppresses non-native structures in the 16S 5' domain. In doing so, S16 enables a conformational switch distant from its own binding site, that may play a role in establishing interactions with other domains of the 30S subunit. Together, the footprinting results show how protein-induced changes in RNA structure are communicated over long distances, ensuring cooperative assembly of even very large RNA-protein complexes such as the ribosome.

Non-coding RNAs play many roles in gene expression and genome maintenance. Not surprisingly, the folded structures of many non-protein coding transcripts are vitally important for their biological function, often more so than their primary sequence. Long non-coding RNAs fold into diverse and complex tertiary structures that allow them to act as enzymes or metabolic sensors, often in complex with one or more proteins.^{1,23–5} A confluence of biophysical and biochemical methods are now revealing the details of how

ribosomal subunits and other ribonucleoprotein complexes (RNP) assemble in real time from their molecular components.^{6,7}

In this review, I discuss how we have used hydroxyl radical footprinting and other experimental methods to study pathways of RNA folding and 30S ribosome assembly. The detailed information on RNA folding intermediates provided by footprinting experiments is providing evidence for induced-fit in RNA-protein recognition, and revealing how RNA-binding proteins can communicate RNA structural changes over long distances. In the future, a combination of in vivo footprinting, isotopic labeling, and genetic approaches should reveal how assembly is coordinated with RNA processing, intracellular trafficking, and quality control pathways.

Visualizing RNA folding pathways with hydroxyl radical footprinting

Over the past two decades, many laboratories have studied the folding pathways and dynamics of RNA, using a wide range of experimental methods including NMR, footprinting, fluorescence and absorption spectroscopy, single molecule techniques, small angle X-ray scattering (SAXS) and molecular simulations.^{8–10} Double helices are known to form rapidly, allowing stable elements of the RNA secondary structure to become established early during the folding process.¹¹ How these double helices pack together to form the final tertiary structure, however, is a more difficult problem, as the tertiary interactions are distributed through the RNA sequence, and intermediates can have diverse structures.

Hydroxyl radical is a particularly useful reagent for monitoring helix packing and protein binding in RNA, because cleavage of the RNA backbone in the presence of hydroxyl radical is proportional to the solvent accessibility of the C4' and C5' positions in the ribose (Figure 1).¹² Moreover, the strand scission chemistry is relatively insensitive to the base sequence, and can be determined for every residue. Consequently, protection from hydroxyl radical cleavage can be used to map the inside of a folded RNA with single nucleotide precision, and to quantify the extent of protein binding.^{13,14}

To follow RNA folding in real time, it is necessary to carry out the hydroxyl radical cleavage reaction in a short time, to obtain “snap shots” of the RNA tertiary interactions as they form. Mark Chance and Michael Brenowitz’s labs developed a synchrotron X-ray beamline for footprinting that in collaboration we used to follow the folding pathway of the Tetrahymena ribozyme.¹⁵ In “X-ray footprinting”, hydroxyl radical is generated by irradiating aqueous samples for 5–10 ms, which permits the dynamics of the structures to be examined.^{16,17} Brenowitz and co-workers subsequently developed a time-resolved footprinting protocol that uses the Fenton reaction (Fe(II)-EDTA) to produce hydroxyl radical, and a rapid quench instrument to mix reagents within a few milliseconds.¹⁸ Both methods produce comparable RNA cleavage patterns, although the X-ray beam can also be used to footprint RNA molecules in vivo.^{19,20}

Since footprinting methods probe the average conformation of the molecules in the solution at a given time, a clear footprint is only visible when >10% of molecules form exactly the same molecular contacts. Consequently, this method does not detect compact intermediates

that are dynamic in structure, or regions of the structure that are heterogeneously folded within the population. As discussed below, such compact intermediates can be detected by SAXS or by FRET, providing a more complete picture of the folding process.

Folding pathway of the Tetrahymena ribozyme

Hydroxyl radical footprinting of the Tetrahymena group I ribozyme after different folding times showed that tertiary interactions in its stable P4–P6 domain formed at a rate of 1 s^{-1} , while tertiary interactions around the periphery of the RNA structure formed at an intermediate rate, and interactions in the P3–P9 domain, which form the catalytic center of the ribozyme, appeared most slowly, at $\sim 1 \text{ min}^{-1}$ (Figure 2).¹⁵ These results agreed with the folding pathway revealed by equilibrium footprinting²¹ and antisense oligonucleotide probes,^{22,23} and showed that large domains of RNA tertiary structure can form in a short time.

As the domains of the Tetrahymena ribozyme become protected in stages, it is tempting to think of the folding process as a linear path towards the native state. However, other experiments showed that delayed protection of the P3–P9 domain is due to mispairing of the P3/P7 pseudoknot,²⁴ trapping the RNA in misfolded structures in which the P3–P9 domain does not pack correctly against its neighbors.²⁵ Folding times from misfolded structures are long because incorrect RNA structures must unfold before they can refold correctly.

Importantly, while 90% of ribozyme molecules become trapped in misfolded intermediates in vitro, a few molecules avoid this fate and fold correctly with a rate of $\sim 1 \text{ s}^{-1}$ at 37°C .^{25–27} Thus, the RNA population partitions among parallel folding pathways, some leading directly to the native structure while others detour through metastable intermediates.^{28,29} The footprinting results reflect the superposition of these folding pathways, or the probability that a particular region of RNA becomes ordered within a given time window (Figure 2). If the pathways are not too varied, the dominant folding intermediates can be inferred by deconvoluting the changes in backbone accessibility over time.³⁰

The “outside-in” folding pattern of the Tetrahymena ribozyme revealed by the time-resolved footprinting studies³¹ suggested that the late folding intermediates are compact, as later shown by SAXS experiments.³² The initial collapse transition to compact structures begins within 15 ms after Mg^{2+} is added to the RNA.³³ However, in the Tetrahymena ribozyme³⁴ and nearly all other ribozymes studied so far, the initial collapse transition, which is barely visible by footprinting, is followed by further conformational adjustments that allow the double helices to pack tightly and specifically with one another.¹⁰ This collapse transition is driven by electrostatic interactions with cations,¹⁰ and sequence-specific tertiary interactions that bring the helical domains together.^{34–37}

Free energy landscapes in RNA folding

In general, the overall folding time correlates directly with the fidelity of helix assembly and the heights of the energy barriers that separate the intermediates from the native state.^{28,29} For example, the Azoarcus group I ribozyme, which has the same core architecture as the

Tetrahymena ribozyme, largely avoids becoming trapped in non-native intermediates above 40 °C and folds in 5–30 ms.³⁸ The heterogeneity of the Azoarcus ribozyme collapse kinetics depends on the concentration of Mg^{2+} and interactions in the unfolded ensemble.³⁹

The stretched folding kinetics and heterogeneous folding intermediates in RNA can be described by free energy landscape models, in which the folding process is represented as a multi-dimensional search for low free energy structures.^{29,40,41} Non-native metastable structures in RNA are represented as local free energy minima in free energy landscapes that trap individual RNA molecules as they traverse the free energy surface. Individual molecules in a population can follow different routes across the folding landscape, and the kinetic partitioning of the population among different, parallel folding pathways has been visualized for several different RNAs using single molecule FRET and single molecule force denaturation.^{26,42,43} The same perspective is useful for understanding the assembly of RNPs, in which protein binding simply becomes an additional variable that alters the energy landscape of the system.⁷

Multistage assembly of 30S ribosome by time-resolved hydroxyl radical footprinting

As the rRNA structure defines the overall architecture of the ribosome, and its active sites are comprised almost entirely of RNA,⁴⁴ it seemed reasonable to expect that rRNA folding is a critical force in assembly of the ribosomal subunits. Few proteins contact each other directly in the mature bacteria 30S subunit.⁴⁵ Instead, the hierarchical addition of proteins to ribosomal subunits described by the Nomura assembly map⁴⁶ arises from structural changes in the rRNA due to protein binding.⁴⁷ Although each protein stabilizes the folded structure of its immediate binding site, it also stimulates structural changes in adjacent rRNA residues that help recruit other proteins to the complex.

The basis for cooperative assembly was illustrated by biophysical studies showing that protein S15 captures and stabilizes the junction between helices 20, 21 and 22 in the central domain, holding them in the correct orientation.^{48,49} S15 binding also stabilizes interactions with helix 23 that form the binding site of the S6–S18 dimer,⁵⁰ which in turn helps organize the binding site for S11. The thermodynamic cooperativity for binding of central domain proteins is associated with a reduction in the unfavorable entropy change associated with protein binding, consistent with increased pre-organization of the rRNA.⁵¹ As we shall see below, ribosomal proteins can smooth the folding and assembly landscape by preferentially stabilizing productive folding intermediates.⁵² Moreover, ribosomal proteins are positively charged and contain intrinsically disordered regions, features associated with RNA chaperones.^{53,54}

Although ribosomal proteins stabilize the folded structure of the rRNA, how do the RNA and protein interactions evolve over time as the RNP complexes come together? We used time-resolved hydroxyl radical footprinting to visualize the folding of the rRNA during 30S assembly in real time.⁵⁵ *E. coli* 16S rRNA was first refolded in 20 mM $MgCl_2$, then 30S subunit proteins were added using a rapid quench instrument. Complexes were exposed at various intervals to a synchrotron X-ray beam for 10 ms (Figure 3). This approach captured

“snapshots” of the emerging RNA tertiary interactions and RNA-protein interactions from 20 ms to 180 s of assembly.

As in the *Tetrahymena* ribozyme, some 16S residues became more than 65% protected within the first 50 ms of the assembly reaction, while other residues required several minutes to fold.⁵⁵ Not only were residues in different domains of the rRNA protected at different times, but individual residues were protected with more than one rate constant. For many residues, a burst of protection in the first 50 ms was followed by much slower saturation of the backbone interactions. These results suggested not only that assembly involves different intermediate complexes, but also that different rRNA molecules assemble along different paths, as inferred earlier from the binding kinetics of 30S proteins.⁵²

Residues in every domain of the 16S rRNA were protected rapidly, showing that the domains of the 16S rRNA assembly concurrently in vitro.⁵⁵ Some of the fast folding regions corresponded to helix junctions that are partly stable in the absence of proteins, while others mapped to protein binding sites, including primary assembly proteins S4, S8, S15, and S7. Regions of the 16S rRNA that were protected most slowly included irregularly folded regions of the rRNA, residues that form long-range interactions between structural domains, and residues that form the central pseudoknot and the mRNA decoding site in the mature 30S subunit. These last results agreed with reconstitution experiments showing that the pseudoknot forms in the late stages of 30S assembly.⁵⁶

Direction of assembly during transcription

In nature, ribosomal subunits are assembled during transcription, and assembly is intimately coupled with processing and maturation of the pre-rRNA.⁵⁷ At 30 °C, the gradient of assembly flows in a 5' to 3' direction, presumably reflecting sequential assembly of the pre-16S rRNA during transcription.^{52,58,59} Our footprinting experiments suggested that the 5' to 3' direction of assembly correlates with the propensity of each 16S domain to misfold. While the 5' domain of the *E. coli* 16S rRNA can fold independently of proteins in a few seconds,⁶⁰ the central and 3' domains do not fold properly without their protein ligands. The folding time of the 3' domain became 100 times longer when the 16S rRNA was renatured at 30 °C instead of 42 °C, and was more sensitive to base modification,⁶¹ suggesting that assembly can proceed more rapidly if metastable conformations in the rRNA are overcome. The propensity of the 16S 3' domain to misfold may explain why many of the 3' domain proteins do not productively join the complex until after a heat activation step⁴⁶, and why several 30S assembly factors act on the 3' domain.

Induced fit in RNA-protein recognition

One of the most striking observations from time-resolved footprinting of 30S ribosome assembly was that the interfaces between the rRNA and individual ribosomal proteins form at different rates. For example, residues in helices 16 and 17 that contact the C-terminal domain of protein S4 were protected in 0.1 to 1 s, while residues in helices 16 and 18 that interact with the N-terminal region of S4 were protected more slowly (Figure 4).⁵⁵ This suggested that initial encounter complexes between the ribosomal proteins and the RNA contain only a subset of the native contacts and must undergo further structural changes. In

general, the slowest rates of protection from hydroxyl radical cleavage were most similar to protein binding rates measured by pulse chase mass spectrometry,⁵² suggesting that it is these slower steps that commit proteins to further steps of 30S assembly.

Interestingly, the N-terminal region of S4 is disordered in the free protein,⁶² raising the possibility that changes in protein structure occur after the initial binding event. Although RNA binding proteins may promote folding simply by selecting conformations already present in the ensemble of unliganded structures, non-specific protein binding may induce new conformations or increase the dynamics of the RNA, lowering the energy barriers to refolding.^{63–65} Most RNA binding proteins contain large numbers of basic amino acid residues, and electrostatic attraction to the RNA may drive the initial formation of non-specific and dynamic encounter complexes, as suggested for the RNA recognition motif (RRM) protein U1A.^{66,67}

Global stabilization of rRNA structure by ribosomal proteins

To understand in detail how proteins stabilize the tertiary structure of the rRNA, we footprinted the structure of the 16S 5' domain RNA in the presence of one or more proteins. The 16S 5' domain forms the body of the 30S subunit, and stably binds three primary assembly proteins (S4, S17 and S20), as well as one secondary assembly protein, S16.^{68–70} S4, S17 and S20 each organize a different helix junction in the 5' domain (Figure 5), while S16 binds at the interface between the “upper” and “lower” halves of the 5' domain. Protein S12 also forms part of 30S body, but binds 16S 5' domain fragments weakly.

The Mg^{2+} concentration required to refold RNA generally correlates with the stability of its tertiary structure. Therefore, by reconstituting 5' domain RNP complexes in a range of Mg^{2+} concentration, we could determine how much the proteins stabilize specific RNA tertiary contacts.⁷¹ Although the 16S 5' domain RNA can fold in the absence of proteins in high $MgCl_2$,⁶⁰ the ribosomal proteins stabilized the RNA structure as expected, allowing tertiary interactions in the 5' domain RNA to form in 5 mM $MgCl_2$.⁷¹

Surprisingly, S4 or S17 alone stabilized tertiary interactions throughout the 5' domain, allowing many of the expected RNA tertiary contacts to form in low Mg^{2+} .⁷² Indirect stabilization of an entire RNA tertiary domain has been observed for other non-coding RNAs, such as group I introns and RNase P, that form a strong network of interactions able to propagate the effects of protein binding to distant regions of the complex.^{73–75} In these examples, the RNA can usually form compact folding intermediates in the absence of protein. By contrast, RNAs with weaker tertiary interactions, such as signal recognition particle (SRP)^{76,77} or telomerase,⁷⁸ may recruit their protein ligands in a stricter hierarchy, because one protein is needed induce compact structures capable of being recognized by the other proteins.

A conformational switch in the 16S 5' domain

The detail in the footprinting data revealed how the ribosomal proteins change the structure of the rRNA, altering the free energy landscape for assembly. Although the primary assembly proteins S4, S17 and S20 each stabilized the 16S 5' domain globally, they selected

a different subset of RNA conformations that were revealed as different footprints in the intermediate Mg^{2+} concentrations.⁷² When all three proteins were added to the reaction, the RNA became more ordered, even in very low (0.01–0.1 mM) Mg^{2+} concentrations. However, in ~1 mM MgCl_2 , some residues were only partially protected, while residues in helix 18 and at the tip of helix 12, which both contact either side of helix 3 in the upper half of the 5' domain, were transiently exposed in moderate MgCl_2 concentrations.⁷¹ These results suggested first that assembly intermediates with different structures accumulate simultaneously, and second, that interactions surrounding helix 3 are remodeled during the assembly process.⁷¹

Nucleotides with similar folding behavior were clustered to yield a minimal equilibrium model for 5' domain assembly (Figure 5).⁷¹ When S4, S17 and S20 are present, at least two assembly intermediates have similar free energies: I_{nC} in which helix 3 interacts with helices 18 and 12 but the lower half of the 5' domain is in a non-native conformation, and I_N in which the lower half of the 5' domain is folded correctly but helix 3 is displaced from helices 18 and 12. The secondary assembly protein S16 depopulates I_{nC} , presumably by preferentially stabilizing the native configuration of the lower domain in I_N . As the Mg^{2+} concentration is raised further, the native RNP forms, containing the pseudoknot in helix 18, and normal tertiary interactions between helix 3 and helices 12 and 18. Thus, protein S16 smoothes the 5' domain assembly pathway, suppressing the I_{nC} complex and stabilizing the final, native RNP.

Remarkably, the S16 binding site straddles the interface between helices 15 and 17, ~30 Å away from helix 3. S16 binding may influence the conformational switch at helix 3 by correctly orienting helix 12 and helix 5, and by stabilizing a tertiary interaction with a C-loop motif in helix 15. The ability of S16 to influence the conformation of helices 3, 12 and 18 over this distance helps explain its important role in 30S assembly, and demonstrates how the architecture of the rRNA transmits ligand interactions over long distances, even in immature complexes.⁷⁹

Although our footprinting experiments were done on a fragment of the 16S rRNA containing only the 5' domain, helix 3 is likely to influence interdomain interactions late in 30S assembly. In the mature 30S subunit, helix 3 stacks co-axially with helix 1,⁴⁵ which in turn participates in the central pseudoknot that is critical for 30S function. The central pseudoknot and residues around the tip of helix 12 form the binding site for protein S12, and become protected from modification late in assembly, after the heat-dependent RI to RI* step of 30S reconstitution.⁵⁶ As helix 1 can exchange with an alternative helix in the pre-16S rRNA,⁸⁰ these folding steps may be linked to processing of the 5' end of the pre-16S rRNA.

Conclusions

Experimental methods and theoretical models for studying RNA folding pathways are being adapted to reveal how large cellular complexes are assembled from their molecular components. Given the many non-coding RNAs that function as cellular enzymes or that participate in regulatory pathways, methods for interrogating RNA conformation will remain in demand. Hydroxyl radical footprinting probes the solvent accessibility of individual

residues in RNA in a few milliseconds, allowing RNP assembly reactions to be followed in real time. The detailed information from footprinting studies complements results from fluorescence spectroscopy, microscopy, solution scattering and mass spectrometry.

Consistent with studies on smaller ribozymes, time resolved footprinting of the 16S rRNA showed that 30S subunit assembly is multiphase, suggesting that individual complexes take different routes to the final structure. Each domain of the 16S rRNA assembles concurrently, with many stable RNA and RNA-protein interactions forming within the first 50–100 ms. Individual proteins protect their binding sites with more than one rate constant, suggesting that induced fit of RNA-protein contacts contributes to the hierarchy of 30S assembly. Finally, the connectivity in the rRNA structure allows bound proteins to influence the structure of the rRNA over long distances, driving specific conformational changes that help create the mRNA decoding site of the 30S subunit. In the future, in vivo footprinting methods promise further insight into how the folding and assembly of RNA-protein complexes coordinate with each phase of RNP biogenesis.

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Figure 1. Hydroxyl radical footprinting of RNA and protein interactions

Ribose sugars buried by RNA tertiary interactions or protein contacts are protected from attack by hydroxyl radical, producing a gap or “footprint” in the distribution of cleaved products.¹² Radicals are produced by the Fenton reaction or X-ray photolysis of water; strand scission products are analyzed and quantified by primer extension with reverse transcriptase. Base oxidation products are not readily detected by this method.

Figure 2. Folding pathway of the Tetrahymena ribozyme

Time-resolved footprinting^{30,31} showed that tertiary interactions in the P4–P6 domain (green) form in ~1 s, more rapidly than contacts in peripheral helices (pink and grey) and in the P3–P9 domain (blue), due to mispairing of the P3 helix (gold).²⁴ The ensemble of unfolded structures (U) partitions among parallel pathways, with 5–10% of molecules folding directly to the native state (N).²⁵

Figure 3. X-ray footprinting of 30S ribosome assembly

The evolution of RNA and RNA-protein interactions was probed from 20 ms to 120 s, using a 10 ms X-ray pulse.⁵⁵ Native *E. coli* 16S rRNA was pre-folded in reconstitution buffer before addition of native *E. coli* 30S proteins (TP30). Quantitation of primer extension products revealed multi-stage folding of individual residues throughout the 16S rRNA.

Figure 4. Induced fit in rRNA-protein interactions

Different residues in a single 30S protein binding site are protected with different rate constants, when probed by X-ray footprinting. (a) Residues contacted by protein S4 in mature 30S subunits colored according to the rate of protection: red, 20 s^{-1} ; orange, $2\text{--}20\text{ s}^{-1}$; green, $0.2\text{--}2\text{ s}^{-1}$; blue, $0.01\text{--}0.2\text{ s}^{-1}$. (b) Residues contacted by protein S16, colored as in (a). Reproduced from Ref. ⁵⁵ with permission.

Figure 5. S16-dependent conformational switch

(a) Proteins that bind the 16S 5' domain (body) stabilize RNA tertiary interactions, lowering the Mg^{2+} concentration required for protection from hydroxyl radical cleavage.^{71,72} Fitted curves for protection of residues in helix 15 (nt 378; dashed lines) and helix 18 (nt 501–502; solid lines) in the presence of S4, S17 and S20, without S16 (pink) or with S16 (blue). (b) Exposure of helix 18 can be explained by movement of helix 3 (purple) during assembly. Ribbon (pdb 2avy)⁸¹ shows 16S nt 24–562 with proteins as pastel surfaces; S4 (pink), S17 (green), S20 (yellow), S16 (blue). (c) Minimal model for 5' domain assembly, involving intermediate RNPs with native (I_N) or non-native (I_{nC}) configuration in the lower half of the domain near helices 6, 6a, 10, 15. Helix 3 is displaced in I_N , then moves back into position in the native complex (N). S16 smooths the path of assembly by favoring I_N over I_{nC} , and by stabilizing N. Reproduced from Ref. 71 with permission.