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Perspective

Cell

The Ribosome Emerges from a Black Box

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Although the basic facts about the ribosome were already known 40 years ago, elucidating its atomic structure and molecular mechanisms required sheer persistence and the innovative use of new technology and methods. These advances have transformed our understanding of translation in the cell.

Two years after *Cell* was born, I left theoretical physics to become a graduate student in biology at UCSD, and two years after that, I joined Peter Moore's laboratory at Yale to begin working on the ribosome. So by a strange coincidence, my life as a ribosome scientist has largely overlapped with the life of *Cell*. It is interesting to look back at what was known about the ribosome at *Cell's* inception, and the remarkable progress that has been made since.

That progress can be visually encapsulated in Figure 1, which shows the structure of the ribosome 40 years ago (Lake, 1976) compared to a recent atomic structure of a translating ribosome bound to the translocon (Voorhees et al., 2014). The latter was obtained by single-particle electron cryomicroscopy (cryoEM), a technique that not only did not exist in 1974, but which only recently has been capable of producing high-resolution structures of asymmetric objects like the ribosome.

The state of the ribosome in 1974 was captured in a comprehensive book, entitled simply "Ribosomes," with contributions by many leading scientists of the day (Nomura et al., 1974). It marked the end of what is commonly referred to as the "golden age" of molecular biology, during which the salient facts about the ribosome had already been established. Ribosomes from all species consist of consist of two subunits (30S and 50S for bacteria and 40S and 60S for eukaryotes) and in most species are two-thirds of RNA by mass. They contain over 50 proteins, and three large ribosomal RNA (rRNA) molecules. The small subunit binds mRNA and the large subunit carries out peptidyl transfer. The tRNAs bound in the P and A sites hold the nascent peptide chain and the new amino acid to be added, respectively. Protein factors act at each stage of the process, and many of these are GTPases. These basic facts have been textbook material ever since.

Given the complexity of the ribosome, to go beyond that basic level of understanding seemed a daunting task, so only a few aficionados persisted in working on it. During the subsequent decades, three qualitative advances have changed our understanding of the ribosome. The first was the emergence of the idea that the ribosome is primarily an RNA-based machine. The second was the atomic structure of the ribosome, which not only confirmed that idea, but paved the way for ever more sophisticated experiments to understand the mechanism of translation. Finally, we are now beginning to understand the ribosome as a dynamic machine in which large conformational changes are essential to its function.

The Ribosome as an RNA Machine

Early on, it was thought the many different proteins of the ribosome might be responsible for its various functions, with the RNA as a scaffold to hold the various proteins in place. However, the ribosome poses the classic "chicken or egg" question: If the ribosome consists of both RNA and protein, and is needed to make protein, how did it originate? Crick (1968) rather presciently wrote, "It is tempting to wonder if the primitive ribosome could have been made *entirely* of RNA" (original italics). To my knowledge, this was the first idea that RNA could both carry genetic information and perform catalysis and can be thought of as the origin of the "RNA world hypothesis," which postulates a primordial world consisting of replicating RNA molecules before the advent of proteins.

The first indication that rRNA contributes to translation at all arose from the discovery that complementarity between the 3′ end of 16S rRNA and a sequence on mRNA upstream of the start codon was important for proper initiation of translation (Steitz, 1969; Shine and Dalgarno, 1974). However, base pairing was already known to be a feature of nucleic acids and unrelated to the broader functions of the ribosome such as catalysis, ligand binding, and movement. Similarly, modification of rRNA, but not protein, was shown to affect tRNA binding, but in the absence of any evidence for catalytic properties of rRNA, it was suggested that the binding sites must consist of both RNA and protein (Noller and Chaires, 1972).

The sequencing of 16S and 23S ribosomal RNAs (Brosius et al., 1978; Brosius et al., 1980) enabled the establishment of their secondary structure (Woese et al., 1980; Glotz and Brimacombe, 1980) and had consequences for biology far beyond the ribosome: they paved the way for the discovery of a third branch of life, the archaea (Woese et al., 1990).

In conjunction with the ability to reconstitute the ribosome from purified components (Held et al., 1973; Nierhaus and Dohme, 1974), knowledge of the sequence of rRNA also laid the groundwork for the use of chemical footprinting methods to study its interaction with various ligands (Moazed and Noller, 1986; Moazed and Noller, 1989a). Such studies, as well as complementary crosslinking methods (Döring et al., 1994), identified regions of rRNA that interact with tRNAs in the A, P, and E sites.

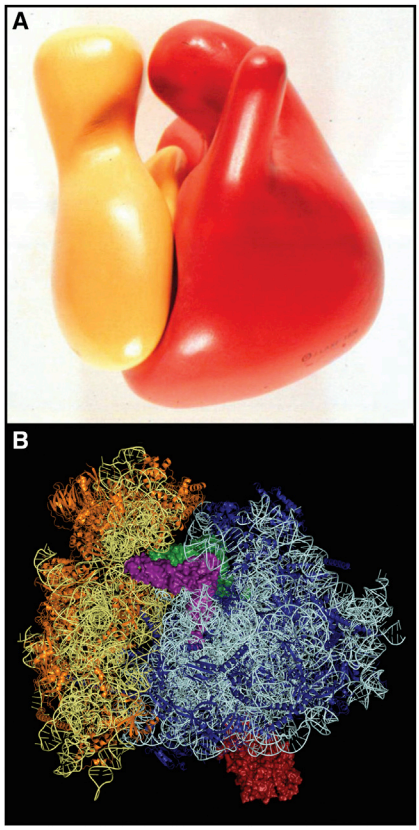

Figure 1. The Ribosome Then and Now
(A) Model of the *E. coli* ribosome 40 years ago, obtained by negative stain electron microscopy (Lake, 1976). The small subunit is shown in yellow and the large subunit in red.
(B) High-resolution structure by single-particle cryoEM of a translating mammalian ribosome (small subunit in yellow and large in blue) bound to the translocon (red) and A- and P-site tRNAs in purple and green respectively (Voorhees et al., 2014).

These studies provided initial physical constraints for building models of the ribosome.

Further evidence for functional roles for rRNAs came from studies on antibiotic binding sites in the ribosome. The first antibiotic resistance mutation mapped (to streptomycin) was on ribosomal protein S12 (Traub and Nomura, 1968), but in hindsight this was misleading, since antibiotics have little affinity for ribosomal proteins. Subsequent chemical footprinting showed that antibiotics, including streptomycin, interact with specific and distinct sites on ribosomal RNA (Moazed and Noller, 1987a; Moazed and Noller, 1987b).

Together, these studies suggested that rRNA was likely to be of functional importance. However, the notion of the ribosome as fundamentally an RNA enzyme really gained plausibility only when RNA catalysis was discovered in the context of the group I intron (Zaug and Cech, 1986) and RNase P (Guerrier-Takada et al., 1983). These discoveries led to a resurgence of interest in the ribosome and ribosomal RNA in particular (Moore, 1988).

Unlike the case with the simpler group I intron and RNase P, proving that catalysis in the ribosome was a property of RNA proved difficult. When thermophilic 50S subunits were treated extensively with protease and phenol extraction to remove proteins, they nevertheless retained peptidyl transferase activity, strongly suggesting a catalytic role for RNA (Noller et al., 1992). However, even after extensive protease treatment, several peptide fragments and even some entire proteins resisted digestion and extraction, and the complete removal of proteins resulted in a loss of activity. So, as implicitly acknowledged by the authors in their cautiously worded title, "Unusual resistance of peptidyl transferase to protein extraction procedures," these experiments were not conclusive. The limitations of such experiments were foreseen by Crick, who, when he suggested that primitive ribosomes may have consisted entirely of RNA, also said, "Without a detailed knowledge of the structure of present-day ribosomes it is difficult to make an informed guess" (Crick, 1968). As discussed below, that structure took many decades after the discovery of the ribosome.

Toward the Atomic Structure of the Ribosome

Forty years ago, the gross morphology of the two ribosomal subunits had been determined by conventional negative-stain electron microscopy with the 30S subunit having a "head," a "platform," and a "body" and the large subunit having a central protuberance flanked by two stalks. But strikingly, even essential features such as the number and location of all of the tRNA binding sites and the path of the nascent peptide were established only gradually.

tRNA Binding Sites

Aminoacyl tRNAs are the substrates of the ribosome, responsible for delivering each new amino acid to the growing polypeptide chain. Early on, it was accepted that the ribosome would have two tRNA binding sites, a P site occupied by a tRNA holding the nascent peptide and an A site for the tRNA delivering the new amino acid corresponding to the codon on mRNA. A third site, called the E (exit) site, into which the deacylated tRNA moves prior to being ejected from the ribosome, was first proposed a long time ago (Wettstein and Noll, 1965), but even its existence remained controversial until convincingly demonstrated by Nierhaus and coworkers (Rheinberger et al., 1981). The fact that the E site spans both the small and large subunit became universally accepted only after more detailed structures of the ribosome by cryoEM began to emerge (Agrawal et al., 1996; Stark et al., 1997). These studies also showed that the mRNA snakes around a cleft in the small subunit.

Passage of the Nascent Peptide through a Tunnel in the Large Subunit

Early cartoons of the ribosome and a more recent sculpture at Cold Spring Harbor Laboratory simply show the peptide chain being extruded from the intersubunit space. The existence of a tunnel in the large subunit through which the nascent peptide must emerge was inferred by localizing antibodies to the nascent peptide to a region opposite the intersubunit interface (Bernabeu and Lake, 1982). More direct evidence for an exit tunnel came from electron microscopy of two-dimensional crystalline sheets or sections (Milligan and Unwin, 1986; Yonath et al., 1987), but the tunnel was firmly established only when cryoEM reconstructions of sufficient resolution became available (Frank et al., 1995), and its atomic nature was elucidated by analysis of the crystal structure of the large subunit (Ban et al., 2000; Nissen

et al., 2000). The discovery of the exit tunnel solved a number of mysterious problems, such as how the ribosome ensured proper insertion of membrane proteins into a lipid bilayer by direct docking of the tunnel exit with the translocon, as well as understanding the context in which proteins emerge from the ribosome and begin to fold.

Beyond Overall Morphology

Despite the characterization of essential features, going beyond the overall morphology of the ribosome proved to be difficult. Various approaches to chipping away at the problem resulted in a mass of data on protein-RNA interactions (Stern et al., 1989; Powers and Noller, 1995; Greuer et al., 1987; Gulle et al., 1988) and on the approximate spatial location of proteins by neutron scattering (Capel et al., 1987). The hope was that in conjunction with emerging structures of individual ribosomal proteins (Ramakrishnan and White, 1998) and improving cryoEM reconstructions, this information could be combined to generate a molecular model for the ribosome. There appeared to be no realistic alternative on the horizon. However, the low-resolution of the information and lack of an overall three-dimensional context made it difficult to obtain a model of sufficient accuracy and resolution to make deductions about its mechanism.

Crystallography of the Ribosome

Ever since the 1950s, crystallography has been used to determine the structure of macromolecules. Beginning in the 1960s (Byers, 1966), the ribosome itself had been shown to form two-dimensional crystalline sheets, and such sheets were used to obtain structures by electron microscopy (Unwin, 1977). These early studies showed that the ribosome had a defined structure that might be capable of producing three-dimensional crystals. Moreover, such crystals had been obtained for large viruses, the nucleosome core particle and F1 ATPase. Against this backdrop, it seemed reasonable to attempt the crystallization of the ribosome. Although the structure of some viruses had been determined to high resolution, they represented a special case of high symmetry. In contrast, even if suitable crystals could be obtained, solving the structure of such a large asymmetric structure as the ribosome seemed a pipe dream because the technology and methods to solve it did not exist.

Nevertheless, crystals of the 50S subunit were obtained from a thermophile, *Bacillus stearothermophilus* (Yonath et al., 1980), and were followed a few years later by crystals of the 30S subunit and the entire 70S ribosome from *Thermus thermophilus* (Trakhanov et al., 1987). These early crystals did not diffract well. Almost a decade later, the discovery of crystals of the 50S subunit from the archaeon *Haloarcula marismortuii* that could diffract to 3 Å resolution (von Böhlen et al., 1991) meant that at least in principle it was possible to determine an atomic resolution structure. Cryocrystallography, in which crystals are cooled to 100 K to minimize radiation damage, was another essential step that allowed data collection (Hope, 1988). Nevertheless, for many years, the prospect of solving the ribosome's structure or even producing maps with recognizable features seemed out of reach.

Ultimately, it took technical advances in synchrotron X-ray sources and detectors, computing, as well as the participation of several research groups who brought in new ideas for structure determination, to eventually obtain complete atomic structures of the 50S and 30S subunits (Ban et al., 2000; Wimberly et al., 2000). These structures were used to model a 5.5 Å structure of the entire ribosome with mRNA and tRNAs the following year (Yusupov et al., 2001). A few years later, high-resolution structures were obtained of both the empty ribosome (Schuwirth et al., 2005) and that of a complex with mRNA and tRNAs (Selmer et al., 2006). Currently there are crystal structures of the bacterial ribosome in many functional states (reviewed in Voorhees and Ramakrishnan, 2013). Moreover, there are now high-resolution structures of the entire eukaryotic ribosome (Ben-Shem et al., 2011) and the isolated 40S and 60S subunits (Rabl et al., 2011; Klinge et al., 2011).

These structures have transformed our understanding of the ribosome. The peptidyl transferase and decoding centers consist almost entirely of rRNA, showing that the ribosome is fundamentally an RNA-based enzyme. The structures have enabled sophisticated biochemical and genetic experiments to elucidate both basic mechanisms and the regulation of ribosome function. Moreover, the structures have enabled the direct visualization of antibiotics bound to the ribosome, enabling a better understanding of their mode of action and the potential for design of new, improved antibiotics (reviewed in Wilson, 2014).

The Ribosome in the 21st Century

How Does the Ribosome Work?

Whereas biochemical experiments designed to obtain structural information, like footprinting and crosslinking, have been almost completely superseded by direct methods such as crystallography or cryoEM, those designed to probe mechanisms are thriving. In particular, the development of pre-steady-state kinetics that use a combination of fluorescent reporters and quench flow methods has greatly helped dissect the various steps along the translation pathway (e.g., Rodnina and Wintermeyer, 2001). These methods have been further extended by the development of single-molecule fluorescence techniques, which can probe the rate and sequence of specific conformational changes in the ribosome during translation (Blanchard et al., 2004). Complementing such studies are the direct measurement of force (and thus work) required during translation (Liu et al., 2014).

Complementing the biochemistry, molecular dynamics, which has the potential to calculate both reaction mechanisms (Aqvist et al., 2012) and rates as well as trajectories in large-scale changes (Sanbonmatsu et al., 2005), is likely to play an increasing role in complementing experimental methods to probe ribosome function. These diverse methods are providing a detailed understanding of the mechanisms of translation.

In addition to understanding the internal mechanism of ribosomes, we are now getting insight into broader questions of translation in the cell. A particularly exciting advance is the method of ribosome profiling, which provides a genome-wide in vivo snapshot at nucleotide resolution of ribosomes along mRNA (Ingolia et al., 2009). This approach has already led to many major findings about gene expression in the cell, including translational pausing and the effect of various regulators of translation.

The Ribosome Is a Dynamic Rather Than Static Structure

That the ribosome cannot be static was understood right from the outset because during translocation after peptidyl transfer, the tRNAs and mRNA must move by precisely one codon with respect to the ribosome in order to allow a new cycle of elongation. Bretscher had proposed long ago that tRNAs move in two steps (Bretscher, 1968), first with respect to one subunit to form "hybrid" states and then with respect to the other, possibly coupled by a movement of the two subunits relative to each other. This hypothesis also provided a rationale for the existence of two subunits of ribosomes in all species. However, it languished for almost two decades until it was shown decisively that tRNAs move first with respect to the large subunit and then to the small (Moazed and Noller, 1989b).

Translocation could involve not just a movement of tRNAs but also a coupled rotation of the two subunits. Such a rotation was indeed observed by cryoEM (Frank and Agrawal, 2000). Moreover, even steps that were previously thought to be localized, such as decoding, which involves selection of a cognate tRNA corresponding to a codon, were shown to involve an induced conformational change (Pape et al., 1999). It is now known that major conformational changes are associated with virtually every step of translation including decoding and peptidyl transfer. Moreover, beginning with a study on tRNA associated with EF-Tu (Valle et al., 2002), studies have shown that the tRNAs themselves not only move, but distort considerably as they go through the ribosome.

Recent Advances in CryoEM Are Revolutionizing Structural Studies on the Ribosome

The development of cryoEM for asymmetric structures made a major impact right from the first structures of a ribosomal functional complex (Frank et al., 1995). Initially, the resolutions were not sufficient to derive molecular details without prior information, but they were nevertheless valuable to obtain structures of many states of the ribosome that could not be crystallized and also to analyze conformational changes. Recently however, as a result of direct electron detectors and improved software, it is possible to obtain near-atomic structures of the ribosome from only ~30,000 particles (Bai et al., 2013). Moreover, a complete atomic structure of the yeast mitochondrial large subunit has been determined de novo by cryoEM even when there were no known structural homologs for about half of the constituent proteins (Amunts et al., 2014). CryoEM is revolutionizing the structural biology of large complexes. Apart from eliminating the need for crystals, the method requires orders of magnitude less material, and most importantly can computationally sort both biochemical and conformational heterogeneity in a sample. It is thus ideal for studying complexes that are unstable or dynamic. For translation, this method will help solve more complex problems such as the structures of eukaryotic initiation complexes, of ribosomal complexes with membrane-bound receptors as well as with factors involved in quality control. It is safe to predict that for large complexes, cryoEM will largely supersede crystallography.

Conclusions

Those of us who began studying translation several decades ago could not have dreamed of the state of our understanding today or the dizzying array of technologies that allow us to tackle harder problems in greater detail. We are now in position not only to understand the mechanism of the ribosome itself, but also how it is made, assembled, and regulated. We can visualize its interaction with quality control factors in the cell, see how viruses hijack it, and understand whether ribosomes are specialized for various functions. It is possible that 40 years hence our understanding of translation and its regulation will be as amazing to us now as today's knowledge would have been 40 years ago.

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