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Jonathan D. Dinman
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Biophysical Approaches to Translational Control of Gene Expression

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Editor
Jonathan D. Dinman
Cell Biology and Molecular Genetics
University of Maryland
College Park, MD, USA

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Foreword

Biophysics of Protein Synthesis: An Historical Perspective

Abstract The first evidence of the existence of the particles we now know as ribosomes appeared in the literature about 75 years ago, and ribosomes have been vigorously pursued by molecular biologists, biochemists, and biophysicists for the last 60 of those 75 years. This essay provides a brief history of the field that begins with a description of how the ribosome was discovered in the first place and ends with the announcement of atomic resolution crystal structures of the ribosome in 2000.

Introduction

The goal of the biophysical chemist, like that of every other kind of biologist, is to change the way people think about the living world for the better. Consequently, students must find it hard to understand why in the 1950s so many biophysical chemists concentrated on molecules like bovine serum albumin, myoglobin, hemoglobin, and tobacco mosaic virus. Why work on myoglobin when you could be working on, say, RNA polymerase II? Every biological scientist over the age of 50 knows the answer. Many biophysical techniques consume prodigious amounts of material, especially when they are first being developed, and hence, prior to the invention of methods for over-producing macromolecules, the choice confronted by biophysical chemists was often myoglobin, or nothing.

Biologically, in the 1950s, the ribosome was by far the most important macromolecule that you could prepare in gram quantities. Not surprisingly, therefore, virtually every physical technique that could be used to study ribosomes was used to study them as soon as it was invented, and, in fact, physical observations played an important role in the discovery of the ribosome.

The first observations relevant to the discovery of the ribosome were made in the 1930s by cytologists interested in characterizing biochemically the structures that can be visualized in eukaryotic cells using the light microscope, the most ancient of physical instruments used by biologists. By the early 1940s, it was clear that RNA

is found primarily in the cytoplasms of eukaryotic cells, and that the more RNA there is in a eukaryotic cell, the more active it is likely to be in protein synthesis (Brachet 1941; Caspersson 1941). In that same era, using another physical instrument, the centrifuge, Claude identified a cellular fraction he dubbed "microsomes" (Claude 1941), and a few years later Brachet presciently suggested that the nucleoprotein particles abundant in microsomes might be involved in protein synthesis (Claude 1943; Brachet 1952).

The experiments that confirmed Brachet's hypothesis were carried out in the 1950s. Using two physical instruments that had just become commercialized, the electron microscope and the preparative ultracentrifuge, Porter, Palade, and Siekevitz discovered the endoplasmic reticulum (23–25), and proved that Claude's microsomes are fragments of endoplasmic reticulum and demonstrated that Claude's nucleoprotein particles are found free in the cytoplasms as well as associated with the membranous component microsomes. In 1954, Zamecnik and coworkers published the results of the elegant series of biochemical experiments done with radiolabeled amino acids (another post-war technology) proving that Claude's particles are indeed the sites where proteins are made in the cell (Keller et al. 1954). Experiments done with the analytical ultracentrifuge led to molecular weight estimates for these particles, confirmed that they are found in all cells, and showed that they are all 1:1 complexes of two nonequivalent ribonucleoprotein particles, the larger being about twice the molecular weight of the smaller (Chao and Schachman 1956; Chao 1957; Tissieres and Watson 1958). The name "ribosome" was bestowed on these particles in 1958 (Roberts 1958).

From the mid-1950s until 2000, the contributions made by biophysical chemists to our understanding of protein synthesis and the ribosome were overshadowed by those provided by biochemists and molecular biologists. Prominent among the many highlights of that four decade period were: (a) the discovery of tRNA (Hoagland et al. 1958; Crick 1958), (b) the discovery of messenger RNA (Brenner et al. 1961; Gros et al. 1961), (c) the elucidation of the code (Crick 1966), (d) the discovery that bacterial ribosomes can be reconstituted in vitro (Traub and Nomura 1968), (e) the elucidation of the protein composition of the ribosome (Waller 1964; Traut et al. 1967; Hardy 1975; Wittmann-Liebold 1986), and (f) the sequencing of the large ribosomal RNAs (Brosius et al. 1978; Noller and Woese 1981). [Many interesting accounts of this history have been written: e.g., (Zamecnik 1969; Tissieres 1974; Nomura 1990; Rheinberger 2004). Rheinberger's article is particularly useful because it was written by a professional historian of science, and the accounts it gives of events I know about are both fair and accurate.]

It would be incorrect to conclude from what has just been said that no biophysical research of consequence was done on the ribosome between ~1955 and 2000. (For a critical evaluation of much of that work see Moore 2011). For example, starting in the late 1960s, a lot of experiments were done on the ribosome using small angle X-ray (SAXS) and neutron scattering (SANS) techniques that provided a more refined sense of the overall shapes of the ribosome and its two subunits. The most complicated of these experiments were the SANS experiments that ultimately led to the so-called neutron map of the positions of proteins in the small ribosomal

subunit from *Escherichia coli* (Capel et al. 1987). In those same years, an attempt was made to determine the locations of proteins in the ribosome by fluorescence energy transfer (FRET) (Huang et al. 1975), an approach to the study of protein synthesis that has come of age in the last decade or so.

Electron microscopy continued to contribute to our understanding of the ribosome, and of protein synthesis more generally. The first images of negatively stained ribosomes appeared in 1960 (Huxley and Zubay 1960), and by the mid-1970s, an accurate understanding of the shapes of the two ribosomal subunits, and their relationship in the intact ribosome had emerged (Lake 1976), as had a lot of information about the locations of specific proteins (Oakes et al. 1990). A highlight of this work was the discovery of the exit tunnel in 1982 (Bernabeu and Lake 1982; Milligan and Unwin 1986). EM studies of the ribosome done in that era also contributed significantly to the development of single particle reconstruction techniques, which are having a growing impact in all areas of structural biology today. Over the last 15 years, single particle reconstructions done with ribosome specimens maintained at liquid nitrogen temperatures (or below), i.e., cryo-EM, have become an increasingly important source of information about the conformational changes that occur as ribosomes function in protein synthesis (Frank et al. 1995).

Crystallographers too were hard at work. The first ribosome-related crystal structures solved were those of isolated ribosomal proteins (Leijonmarck et al. 1980). Over the years, roughly a dozen crystal structures and NMR structures were obtained for proteins from the bacterial ribosome. In addition, the structures of a number of ribosomal RNA fragments and ribosomal protein/RNA fragments were also determined. It was in this same era that crystallographic investigations of intact ribosomes and ribosomal subunits got underway. The first crystals of ribosomes large enough to work with were prepared in the late 1970s in Wittmann's laboratory in Berlin (Yonath et al. 1980). They diffracted poorly, as did all the other crystals of ribosomes and ribosomal subunits produced for many years thereafter. However, as experience deepened, resolution slowly improved (von Bohlen et al. 1991; Hope et al. 1989). By the early 1990s, the resolution of the diffraction patterns being obtained from ribosome crystals was so high that it was clear that atomic resolution structures of ribosomes would emerge if reliable strategies could be found for phasing them. That last hurdle was overcome in 1998 (Ban et al. 1998), and in the summer of 2000, as everyone knows, the landscape in the protein synthesis field was transformed by the publication of the first, atomic resolution structures of ribosomal subunits (Ban et al. 2000; Wimberly et al. 2000; Schluenzen et al. 2000).

The crystal structures of ribosomes that appeared in 2000 not only transformed the protein synthesis field, they re-energized it. Since 2000, a field that had for decades suffered from a dearth of atomic resolution information has been reveling in an abundance of riches. Crystal structures of 70S ribosomes trapped in different conformational states appear regularly. Cryo-electron microscopy continues to deliver an ever more detailed account of the structural dynamics of the ribosome. This structural information has enabled biochemists and molecular biologists to design and execute powerful experiments that are delivering important mechanistic insights, as well as stimulating renewed activity in the area of kinetics. Finally, single

molecule studies are now underway that are likely to further transform the way we think about protein synthesis. The field is now in a golden age, and the chapters in this book, which of course will fill in much of the background missing from this sketch, will provide its readers with a sense of what the future is likely to bring.

New Haven, CT, USA Peter B. Moore

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Contents

Chapter 1
X-Ray Analysis of Prokaryotic and Eukaryotic Ribosomes

Lasse B. Jenner, Adam Ben-Shem, Natalia Demeshkina, Marat Yusupov, and Gulnara Yusupova

1.1 X-Ray Crystallography and the Ribosome

1.1.1 X-Ray Methodology

X-ray crystallography is an experimental technique that takes advantage of the fact that X-rays are scattered by electrons. Using electromagnetic radiation to visualize objects by scattering requires the wavelength of the radiation to be comparable to the smallest features to be resolved. Since the atomic bond lengths most commonly found in biological materials are in the 1–2 Angstrom (Å) range, the X-rays produced by in-house rotating anodes and large-scale facilities such as synchrotrons are well suited for this purpose. While scattering from one molecule is too weak to be measured, diffraction from a crystal containing millions of molecules all oriented in a regularly repeated manner is detectable. The diffraction data acquired by X-ray scattering off the periodic assembly of molecules in the crystal can be used to reconstruct the electron density. Electron distributions observed this way provides the locations of the atomic nuclei. An atomic model is iteratively constructed and refined into the observed electron density leading to a rather accurate molecular structure.

L.B. Jenner • A. Ben-Shem • N. Demeshkina • M. Yusupov (✉) • G. Yusupova (✉)
Département de Biologie et de Génomique Structurales, Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS, UMR7104, University of Strasbourg, Strasbourg, Illkirch 67400, France
e-mail: Marat.Yusupov@igbmc.fr; gula@igbmc.u-strasbg.fr

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1.1.2 Challenges for X-Ray Studies of Ribosomal Complexes

Crystallographic methods can shed light on many structure-related issues, from overall molecular conformations and ternary and quaternary interactions to secondary structure information and details about atomic bonds. In contrast to NMR and Cryo-EM approaches, there is no limitation to the size of molecule or assembly to be studied.

The main bottleneck in crystallographic studies is that a well-diffracting crystal must be found, and thus the information gleaned about the dynamic nature of the molecules to be studied will be very limited from only a single diffraction experiment. In other words, the price to pay for the high accuracy of X-ray crystallographic structures is that the method is very time-consuming.

For the ribosome as a huge complex consisting more than 50 components, it is very important to ensure that the samples are homogenous for crystallization to succeed. In our studies we have achieved this by two different strategies: For the prokaryotic studies we have chosen to work with a thermophilic bacteria because the ribosomes isolated from this organism are more robust and resistant to degradation. For the eukaryotic ribosome very gentle isolation protocols were developed to ensure that all the ribosomal components are intact and present. We exploited the observations that glucose starvation of the growing yeast cells leads to inhibition of initiation and accumulation of very homogenous ribosomes without any ligands (Ashe et al. 2000).

A further complication arises since ribosome crystals, as typically seen in RNA crystallography, diffract only poorly which results in electron density maps that are imprecise and difficult to interpret. Therefore special care has to be taken during post-crystallization treatment to avoid damaging the crystals (i.e., when transferring cryo-protection) and even for the freezing process itself we only use the most robust methods of freezing directly in the gaseous N_2 stream at 100 K rather than plunging into liquid N_2, ethane, or propane as is common practice in X-ray structural projects. A combination of severe radiation decay and generally weak diffracting power limits the amount of data that can be collected from each crystal making it necessary to merge data collected on different crystals to obtain complete datasets which invariantly degrades the data quality.

1.2 Crystal Structures of Prokaryotic Ribosome Complexes

1.2.1 Introduction

Translation of nucleotide sequence information in the form of mRNA codons into amino acids lies at the heart of protein biosynthesis. This process is accomplished by tRNA molecules that act as adaptors between the mRNA codon and the amino acids they code for. For accurate protein synthesis, the ribosome is required to position the

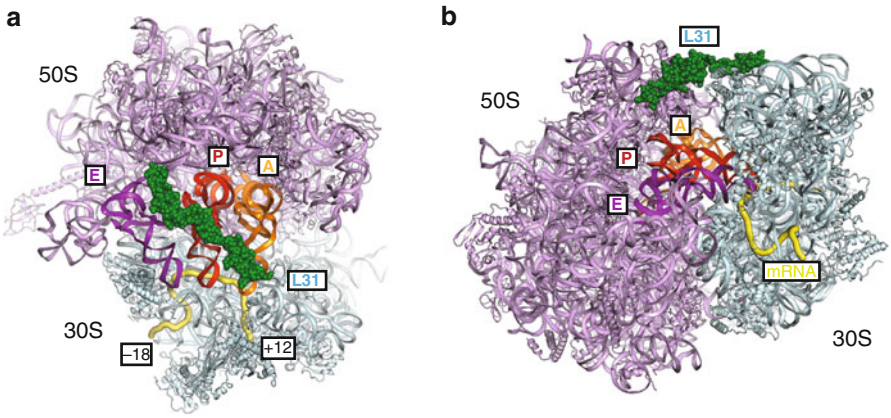

Fig. 1.1 Overall view of the 70S ribosome in elongation state. A, P, and E tRNA are shown in *orange*, *red*, and *magenta*, respectively, and 60-mer mRNA (position −18 to +12 visible) is shown in *gold*. Ribosomal proteins and RNA of the small and large subunits are shown in *light blue* and *violet*, respectively. The new intersubunit bridge formed by the protein L31 is shown in green. (**a**) *Top* view; (**b**) view from the E site

tRNAs in such a way that the reading frame of the mRNA (each codon consists of three consecutive nucleotides) is maintained throughout the translation process. The elongating ribosome contains three binding sites for tRNAs: The aminoacyl (A) site to which a cognate aminoacyl-tRNA is delivered such that it base pairs with the appropriate mRNA codon; The peptidyl (P) site where the tRNA carrying the nascent peptide chain is located. When a cognate aminoacyl-tRNA enters the A site the peptidyl transferase reaction takes place and the peptidyl chain carried by the P tRNA is added to the aminoacyl tRNA essentially adding one amino acid to the growing peptide chain; Last is the exit (E) site from which deacylated tRNA that has completed its role in translation is released.

After each peptide bond reaction, the ribosome must rearrange its contacts with mRNA and tRNA to allow translocation along the mRNA by a single three nucleotides codon. The ribosome controls the positioning of mRNA and tRNAs during the translation process through a number of direct intermolecular contacts. These interactions not only help to stabilize the binding of tRNA to the ribosome but are involved directly in functional processes such as mechanisms for discrimination of aminoacyl-tRNAs to increase the accuracy of tRNA selection; maintenance of the correct reading frame to avoid frame-shifting errors; and translocational movement of the tRNAs and mRNA within the ribosome. We are striving to understand these mechanisms by studying how the main substrates of protein synthesis such as mRNA and tRNA interact with the ribosome.

In order to shed light on these issues we recently determined high-resolution crystal structures of *Thermus thermophilus* 70S ribosomal complexes with different mRNA constructs and naturally modified tRNAs (Fig. 1.1) (Jenner et al. 2010a, b).

Crystals of the ribosome modeling the elongation state were obtained from ribosome complexes prepared with a 60 nucleotides long poly (U) mRNA containing a

Shine–Dalgarno (SD) sequence, UUU (Phe) codons in the A and P sites, and $tRNA^{Phe}$. The structure of this complex was determined at 3.1 Å resolution. Crystals of the ribosome complex modeling the initiation state were prepared with a 27 nucleotide long mRNA comprising the SD sequence with AUG (Met) codon and initiator $tRNA^{fMet}$ in the P site. The structure of this complex was determined at 3.5 Å resolution.

1.2.2 A Novel Intersubunit Bridge Formed by Protein L31 May Regulate Swiveling of the 30S Head

Inspection of the electron density map corresponding to our elongation state revealed well-defined density for a novel element not fully seen in previous high-resolution structures. This element was the ribosomal protein L31 (Figs. 1.1 and 1.2) (Jenner et al. 2010a, b).

Protein L31 displays a considerable degree of similarity among bacteria (Fig. 1.2a), and is comprised of a three β-sheets Zn-binding domain followed by a loop area and an α-helix at its C-terminal. Interestingly, L31 crosses the intersubunit space yoking together the central protuberance of the 50S subunit and the head domain of the 30S subunit. At the 30S subunit head, L31 interacts with the two highly conserved proteins S13 and S19 that are known to form a loose hetero-dimer (Brodersen et al. 2002) (Fig. 1.2b).

Protein S13 is part of the B1a and B1b intersubunit bridges and has a C-terminal that approaches the P site (Yusupov et al. 2001). The central part of L31 (amino acids 32–52) interacts directly with S13 mostly through electrostatic interactions (Fig. 1.2c) while the interaction surface between S19 and L31 is not only of polar but also hydrophobic nature (Fig. 1.2d). The majority of the interacting residues of proteins S13, S19, and L31 are conserved. Overall, protein L31 clips together the globular N-domains of S13 and S19, presumably tightening their association.

The biological relevance of this intersubunit bridge composed of protein L31 may lie in regulating and safeguarding the swiveling of the 30S subunit head domain. It might function as a safety belt, delimiting the extent of 30S head rotation, in the ratchet-like motion supposed to happen during translocation (Spahn et al. 2004a; Frank and Agrawal 2000; Gao et al. 2003).

1.2.3 The Path of Messenger RNA Through the Ribosome

In the initial high-resolution (2.8–3.6 Å) structures of 70S ribosomal complexes, the mRNA was visualized only from positions −4 to +7 (Selmer et al. 2006; Weixlbaumer et al. 2007a, b; Korostelev et al. 2008; Laurberg et al. 2008) although earlier medium resolution structures (4.5–5.5 Å) had previously indicated the entire mRNA path (Jenner et al. 2007, 2005; Yusupova et al. 2006, 2001). In our recent high-resolution

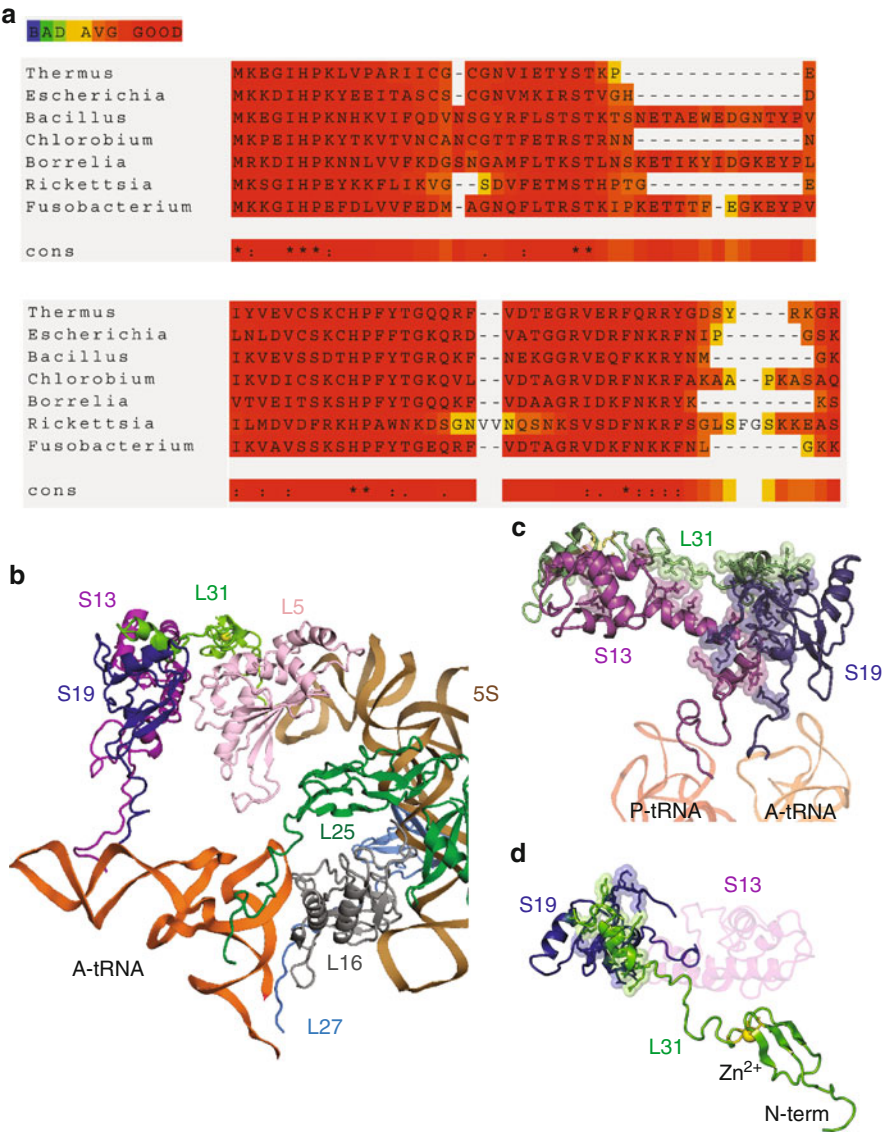

Fig. 1.2 Novel intersubunit bridge formed by protein L31. (**a**) Sequence alignment for L31. The degree of similarity among the major bacterial classes is color coded and specified in the *top* bar. The *bottom* color line indicates the conserved residues. (**b**) Protein L31 closes the circle of interactions around A-site tRNA. (**c**) Interactions between L31, S13, and S19. (**d**) Close-up view of the interactions between L31 and S19. The Zn^{2+} atom is shown as a *yellow sphere*

structure (3.1 Å) of a ribosomal complex in the elongation state we were again able to confidently model the mRNA from positions −18 to +12, which delimit the ribosome boundaries (Fig. 1.3a).

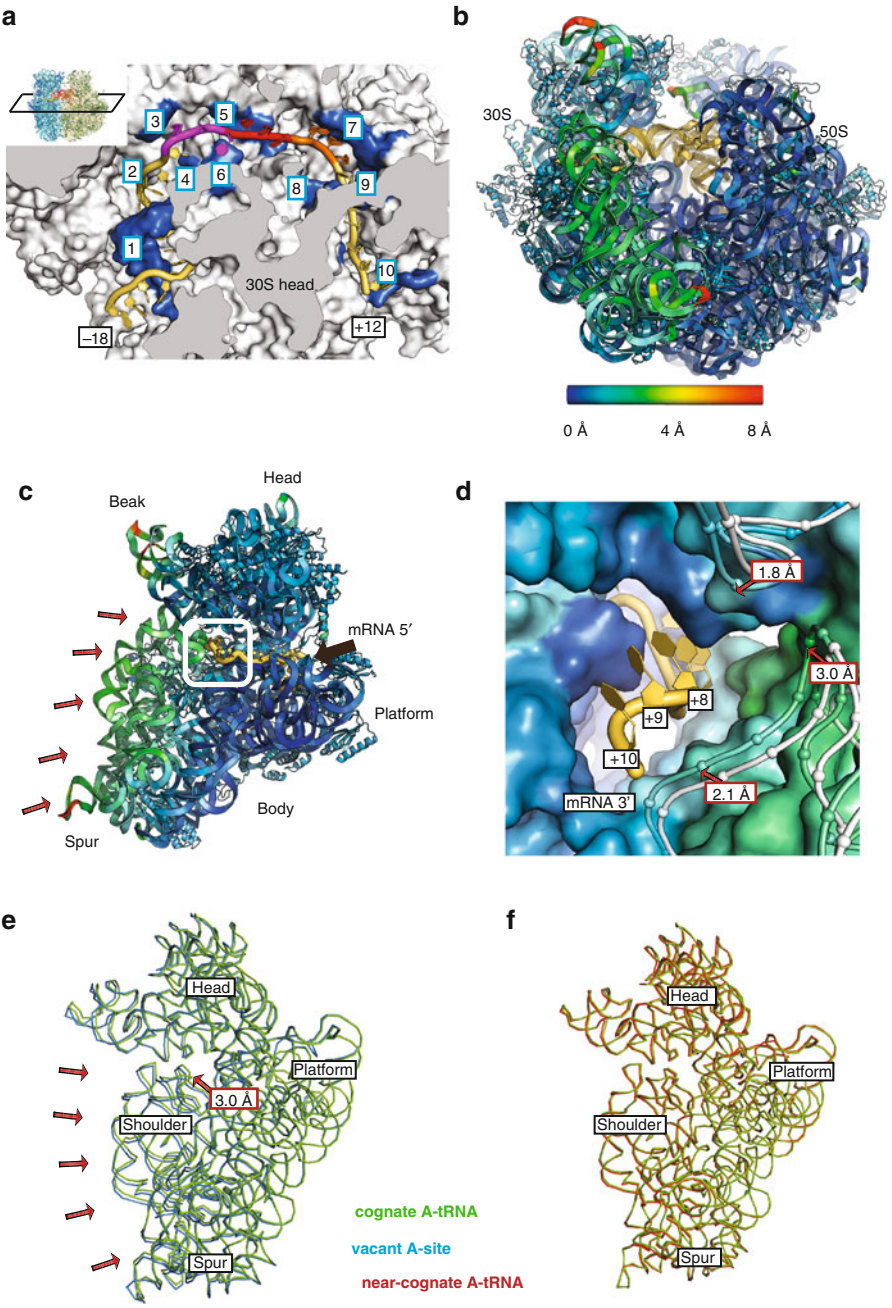

Fig. 1.3 View of mRNA and tRNA interactions with the ribosome. (**a**) Cross-section of the ribosome at the level of mRNA showing interactions between mRNA and the following ribosomal elements: (1) Shine–Dalgarno sequence of the 3′ of 16S rRNA; (2) ribosomal proteins S11 and S18; (3) loop of helix 23b (16S rRNA); (4) A1507 of 16S rRNA; (5) interaction with modified

The 3′ end of the mRNA enters the ribosome through a tunnel formed by ribosomal proteins S3, S4, and S5 (mRNA nucleotides +10 to +12) after which the mRNA passes a layer formed by 16S rRNA elements that are capable of contracting around the mRNA (mRNA nucleotides +7 to +9). The A- (+4 to +6), P- (+1 to +3), and E-site (−3 to −1) codons interact with the respective tRNAs on the interface between the ribosomal subunits. Finally the mRNA emerges on the platform of the 16S subunit where the 5′ end of the mRNA upstream of the E-site codon along with 3′-terminal tail of 16S rRNA forms the SD duplex (Shine and Dalgarno 1974).

1.2.4 Domain Closure

Comparison of two crystal structures of the ribosome modeling the initiation and elongation states reveals that, upon transition from initiation to elongation, the 30S subunit undergoes a conformational change whereupon helices 15–18 from the body of the 30S subunit contract towards the 30S neck (Fig. 1.3b, c) (Jenner et al. 2010b).

The remaining part of the body and most of the head of the 30S subunit remain immobile. This domain closure results in a contraction by 1–2 Å of the mRNA tunnel immediately downstream of the A-site codon causing it to grip the template more tightly in the elongation state than in the initiation state (Fig. 1.3d). A similar conformational change was seen in studies of the isolated 30S subunit with an anticodon stem-loop bound in the A site (Ogle et al. 2002). From those studies it was hypothesized that domain closure occurs only when a cognate tRNA is bound in order to signal correct decoding. Unexpectedly, our results with the full functional

Fig. 1.3 (continued) nucleotide 37 of the P-site tRNA through $Mg(H_2O)_6^{2+}$ and stabilization of the mRNA kink between the P- and A-site codons via interactions with nucleotides from h44 (16S rRNA); (6) stacking of the base of mRNA position −1 with G926 from h28 (16S rRNA); (7) the mRNA A codon interactions with nucleotides G530, A1492, and A1493 (16S rRNA); (8) C1397 from 16S RNA; (9) aromatic stacking network between mRNA and U1196 and C1054 (16S rRNA); (10) ribosomal proteins S3, S4, and S5. (**b**, **c**) Conformational changes of the 70S ribosome. The 30S structure is colored according to the difference between phosphate and C_α positions in the initiation and elongation complexes, ranging from *blue* (0 Å difference) to *red* (8 Å difference). *Arrows* indicate the direction of movement of the domain closure during transition from the initiation to elongation state. The downstream mRNA tunnel has been marked with a white outline. From the superposition it is clear that only the shoulder of the 30S subunit moves, whereas the other parts of the 30S subunit remain immobile, and that the resulting movement leads to a contraction of the mRNA tunnel downstream of the A-site codon. (**d**) Detailed view of the RNA-layer part of the downstream mRNA tunnel seen from the solvent side of the 30S subunit. The RNA chains with the largest movements are shown in *white* (initiation) and color (elongation) with difference vectors marking the changes in position. The contraction of the downstream mRNA tunnel leads to a narrowing of the tunnel diameter by 1–3 Å, tightening the ribosome grip on the mRNA. (**e**) Superpostion of cognate A-tRNA (*green*) and vacant A-site (*blue*) states. The *black arrows* indicate the general movement of the 30S subunit domain towards the neck region of the subunit. (**f**) Superposition of cognate A-tRNA (*green*) and near-cognate A-tRNA (*red*) states

70S ribosome demonstrated the exact same domain closure happening upon binding of near-cognate tRNAs to the A site (Fig. 1.3e, f) [(Jenner et al. 2010a) and unpublished data]. The dissimilarities with the previous studies have led us to conclude that domain closure happens upon binding of any substrate in the A site and does not play an active role in decoding.

Examination of mRNA shows that the third nucleotide of the A-site codon interacts with the nucleotide base of C1397 from the neck region of 16S rRNA (h28). C1397 which nearly intercalates between the mRNA bases at positions +6 and +7 in the initiation complex (Fig. 1.4a) (Jenner et al. 2010b). Nucleotide C1397 protrudes from the side of the mRNA tunnel and seems to be able to adopt different conformations, depending on the state of the ribosome and the presence or absence of tRNA in the A site (Fig. 1.4a, b). Furthermore, in the initiation complex, the sugar moiety of mRNA nucleotide +9 forms a hydrogen bond with Gln162 of protein S3. However, in the elongation complex contraction of the downstream mRNA tunnel triggers the formation of an intricate network of interactions between 16S RNA, protein S3, and the mRNA adjacent to the A-site codon (Fig. 1.4c, d). Nucleotides +8 and +9 of the mRNA are held in place by a combination of hydrogen bonding and continuous aromatic base stacking with Gln162 of protein S3 and nucleotides U1196 and C1054 from helix 34 of 16S rRNA. Finally, C1054 interacts with G34 of A-tRNA as seen previously (Ogle et al. 2002).

This network of interactions between mRNA and the head of the 30S may align the mRNA immediately downstream of the A-site codon before its movement into the A site, such that the codon approaching the decoding center is pre-oriented for the interaction with the tRNA. We suggest that the ribosome preserves this network of interactions during translocation in order to strongly and accurately safeguard the mRNA. Thus, the mRNA reading frame is maintained not only by codon–anticodon interactions but also by this network in the downstream tunnel during swiveling of the 30S head in the course of the ratchet-like movement of the small ribosomal subunit relative to the large ribosomal subunit that accompanies translocation (Fig. 1.4e). After translocation, the mRNA interactions with h34 of 16S rRNA and protein S3 must be disrupted, and the 30S subunit head returns to its initial position.

Fig. 1.4 (continued) position +8 and U1196 of 16S rRNA further stabilizing the network. Finally nucleotide C1054 interacts with the "wobble" nucleotide G34 of the A-site tRNA. (**e**) Interface view of part of the 30S subunit, with mRNA colored according to codon: *magenta* (E), *red* (P), *orange* (A), and *yellow* (downstream of A codon). tRNAs and 50S subunit have been removed for clarity. Ribosomal elements of the 30S subunit head interacting (hydrogen bond or hydrophobic or electrostatic interactions) with either mRNA or tRNA have been colored correspondingly. We propose that when the head of the 30S subunit swivels (indicated by the *arrow*) in the course of the ratchet-like movement of the small ribosomal subunit relative to the large ribosomal subunit, the ribosome translocates the mRNA by maintaining the shown interactions