Assembly of the 30S ribosomal subunit:

Positioning of ribosomal protein S13 in the S7 assembly branch

by

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CHAPTER 1. INTRODUCTION

General Introduction

Assembly of the 30S ribosomal subunit in *Escherichia coli* (*E. coli*) is a remarkably intricate process involving the association of a 1542 nucleotide 16S ribosomal RNA (r-RNA) with a set of 20 ribosomal proteins (r-proteins). The study of 30S subunit assembly began over three decades ago and is a process that is still not completely understood. In 1968 Traub and Nomura discovered the autonomous *in vitro* association of 16S rRNA with a set of r-proteins yields the formation of a functional 30S ribonucleoprotein (RNP) particle. The investigation of this process led to the development of the 30S subunit assembly map, depicting the assembly dependencies of each of the 20 r-proteins and the 16S rRNA (Mizushima and Nomura 1970; Held et al. 1974). The work presented here represents one of the most significant changes to the assembly map since its conception, the new placement of r-protein S13 in the S7 assembly branch (Grondek and Culver, in press).

Thesis Organization

This thesis contains three chapters. The first chapter is an introduction to the thesis and to 30S subunit assembly, with historical background for the thesis topic. The second chapter contains a paper which presents the data and discussion for the thesis. Finally, the third chapter is a brief conclusion to the work presented here in.

30S Subunit Assembly and the Historical Role of R-protein S13

Most of the biochemical and biophysical data concerning 30S ribosomal subunit assembly agrees with the placements and associations depicted by Nomura's assembly map,

with the exception of S13 (Mizushima and Nomura 1970; Held et al. 1974). The following is a discussion of conflicting data regarding the placement of r-protein S13 within the assembly map, and a depiction of the overwhelming support for an alternate assembly dependency. The first experiments discussed are assessing the assembly dependency of S13 and they yield conflicting data. The Nomura group asserts that S13 requires S20 as its binding precursor (Mizushima and Nomura 1970; Held et al. 1974), where the Kurland group shows S13 being largely dependent on S7 for association (Green and Kurland 1973). Next is a discussion of the relative physical location of S7, S13 and S20 on the 30S subunit as determined by chemical cross-linking (Pohl and Wittmann-Liebold 1988) and various biophysical techniques. The placement of S13 is largely in agreement within the literature, but the placement of S20 is in contention, and this placement had assembly implications (Lake 1975; Capel et al. 1987; Schwedler et al. 1993; Wimberly et al. 2000). Finally, the technique primer extension analysis is introduced (Mozad et al. 1986) and with it a series of reconstitution studies that observe the association of S13 in the presence of all the primary binding proteins including both S7 and S20, supporting S13's location on the subunit, however failing to discern a specific binding precursor for S13 (Powers et al. 1988; Powers and Noller 1995; Heilek and Noller 1996).

The 30S subunit assembly map was constructed by Nomura and co-workers after first discovering a functional 30S subunit could be reconstituted *in vitro* from a mixture of the Total Proteins from the 30S subunit (TP30) and 16S rRNA (Traub and Nomura 1968). In order to further dissect the assembly process, Mizushima and Nomura attempted to isolate and purify each of the r-proteins using HPLC and PAGE (Mizushima and Nomura; 1970). The r-proteins were then individually ^{3}H labeled and reconstituted with different

combinations of natural, non-radiolabeled r-proteins and 16S rRNA (Mizushima and Nomura; 1970). The resulting RNPs were purified using sucrose gradient sedimentation and analyzed with a scintillation counter to determine the amount of labeled protein bound (Mizushima and Nomura 1970). It was found that only a subset of r-proteins could independently bind 16S rRNA (Mizushima and Nomura 1970). After this initial assessment, all of the r-protein combinations were attempted using each protein that bound the naked 16S rRNA to determine the hierarchical binding requirements in small subunit assembly (Mizushima and Nomura 1970; Held et al. 1974). The 30S subunit assembly map resulted from these studies, depicting the binding order and synergistic dependencies of each of the 30S r-proteins with the 16S rRNA (Fig. 1A) (Mizushima and Nomura 1970; Held et al. 1974). The r-proteins were hence organized into three groups based on binding requirements. First are the primary (1°) binding proteins (S4, S7, S8, S15, S17 and S20) defined by their ability to bind naked 16S rRNA, then the secondary (2°) binding proteins (S5, S6, S9, S11, S12, S13, S16, S18, and S19) whose associations are dependent on the prior binding of one r-protein, and finally the tertiary (3°) binding proteins (S2, S3, S10, S14, and S21) whose binding necessitates a 2° protein be prebound (See Fig. 1A) (Mizushima and Nomura 1970; Held et al. 1974).

While the assembly map was being developed, 30S subunit assembly was also being addressed by Kurland and co-workers. They assessed single and multiple r-protein addition experiments with purified ^{3}H labeled r-proteins using a similar reconstitution strategy as Nomura (Green and Kurland 1973; Mizushima and Nomura 1970). They employed gel filtration to determine which r-proteins were associated with 16S rRNA (Green and Kurland 1973). Although most of their data agreed with Nomura's results, one significant difference

was the association of S13, shown to be dependent on S7 and S16 for binding rather than being dependent on S20 as Nomura observed (Green and Kurland 1973). Kurland and Green, however, were only able to isolate S13 as a mixture that consisted of 75% S13 and 25% S12, and therefore it is difficult to fully ascertain the significance of these results (Green and Kurland 1973).

The relative physical locations of r-proteins in the 30S subunit have been studied by a variety of biochemical and biophysical techniques. One important study preformed by Pohl and Wittmann-Liebold sought to determine what 30S subunit proteins were in close proximity by protein cross-linking (Pohl and Wittmann-Liebold 1988). Natural 30S subunits were subjected to the cross-linking agent diepoxybutane (Pohl and Wittmann-Liebold 1988). S13 was found to be cross-linked to S19, an r-protein that has been widely documented to require S7 as its binding precursor (See Fig. 1B) (Pohl and Wittmann-Liebold 1988). These results also suggest that S13 and S19 are within 4Å of one another, the distance required for the diepoxybutane reaction, and that S13 is located in close proximity to the S7 protein family (Pohl and Wittmann-Liebold 1988).

Although the placement of S13 on the 30S subunit is not contested within the literature, the relative location of r-protein S20 has been brought into question. Neutron diffraction studies localized S20 in the 3' major domain, or the 'head', of the 30S subunit adjacent to S13 (Capel et al. 1987), where immunoelectron microscopy (IEM) in contrast had shown S20 to be located some distance away from S13, in the body of the 30S subunit (Lake 1975; Schwedler et al. 1993). The 30S crystal structure was recently published showing the locations of each of the r-proteins (See Fig. 2A) (Wimberly et al. 2000). In agreement with the IEM data, S13 was shown to be located in the head of the 30S subunit and S20 was found

100Å away in the body, near a helical projection known as the 'spur' (Wimberly et al. 2000). The likelihood of an assembly dependency of S13 on S20 at this distance is in doubt, and these structural studies further exemplify the ambiguity of the assembly relationship of S13 and S20 within the literature.

A key structural study was preformed by the Noller group utilizing only a subsection of the 16S rRNA (Samaha et al. 1994). The region of rRNA consisted of the head and the 'penultimate stem', residues 923-1543 (Samaha et al. 1994). The S7 assembly family had been shown to associate with the head region (See Fig. 1B), and Noller theorized that this branch of r-proteins and section rRNA could independently assemble (Samaha et al. 1994). To test this theory the subsection of rRNA was transcribed and combined with TP30 for *in vitro* reconstitution (Samaha et al. 1994). The resulting RNP was then purified using sucrose gradient sedimentation and analyzed for r-protein content using two dimensional polyacrylamide gel electrophoresis (2D-PAGE) (Samaha et al. 1994). The RNP was found to contain r-proteins S2, S3, S4, S7, S9, S10, S13, S14 and S19, and the RNP affinity for spectinomycin was similar to that of wild type 30S subunits, an antibiotic known to bind to the head of the 30S subunit. (See Fig. 2B) (Samaha et al. 1994). These results suggest the S7 assembly branch can be reconstituted independently *in vitro*, and that S13 may indeed belong to the S7 assembly family (Samaha et al. 1994).

Another advancement expanding the study of 30S subunit assembly was the advent of primer extension analysis by Noller and co-workers (Mozad et al. 1986). This technique, similar to radiolabeled DNA sequencing, involves the modification or cleavage of 16S rRNA, followed by extension using specifically designed primers, an α^{32}P-TTP spiked

nucleotide pool and reverse transcriptase (Mozad et al. 1986). The result is nucleotide level resolution for observing changes in 16S rRNA.

Among the multitude of assembly studies by the Noller group implementing primer extension analysis, there are two studies of specific relevance that involve S13. The first of these studies examined a subset of r-proteins, S2, S3, S10, S13 and S14 (Powers et al. 1988). Pools of primary binding r-proteins were used as a platform for reconstitution of the target proteins in different combinations, and with the addition of secondary and tertiary binding proteins. Kethoxal and dimethyl sulfate (DMS) were used as modification reagents, modifying guanine alone, and adenine and cytosine, respectively (Powers et al. 1988). Although changes were observed in the 3' major domain, or head, region upon S13 addition, it is difficult to establish a direct binding precursor and specific S13 dependent interactions in the presence of all of the primary binding r-proteins, a pool that includes r-protein S20 (Powers et al. 1988).

The second of these studies looked at 30S reconstitution in its entirety by single and multiple r-protein addition experiments, and implemented hydroxyl radical footprinting and primer extension (Powers and Noller 1995). The resulting 'footprints' represent cleavages of the sugar-phosphate backbone by the hydroxyl radicals, which are generated by Fe^{+2} and EDTA (Powers and Noller 1995). Cleavages in the 3'major domain as a result of S13 binding were observed, and all of the primary binding proteins were again present negating the possibility of determining a specific binding precursor (Powers and Noller 1995). In another study by the Noller group, Fe^{+2} was directly tethered a cysteine of S13. This allowed for the probing of the specific environment surrounding S13 within a fully reconstituted 30S

subunit (Heilek and Noller 1996). Cleavages were again found in the 3' major domain supporting S13 binding to this region of the head (Heilek and Noller 1996).

In addition to primer extension, another recent advancement for the study of 30S subunit assembly was the advent recombinant reconstitution system by Culver and Noller in 1999. Each of the 20 small subunit proteins was individually cloned, over-expressed and shown to functionally reconstitute into a 30S subunit, providing large pools of recombinant r-proteins for reconstitution studies (Culver and Noller 1999). R-proteins expressed by this system were used for the reconstitution studies described below.

There are a multitude of structural and biochemical data placing S13 in the head of the 30S subunit and in close proximity to the S7 assembly group, however its specific assembly dependency has yet to be shown. To determine the assembly requirements of S13 we sought to observe the individual assembly of S7, S13 and S20 onto the 16S rRNA. The S7 r-protein family has been shown to independently reconstitute (Samaha et al. 1994), and S7 is the most likely candidate as a precursor for S13 association due to its close proximity, as previously discussed. The use of primer extension analysis and probing of the individual addition of recombinant ribosomal proteins S7, S13 and S20 to the 16S rRNA, and different combinations there of, should yield specific information regarding the binding requirements of S13, and differentiate between S7 and S20 as candidates for its binding precursor (Grondek and Culver, in press). The systematic addition of other late stage S7 family r-proteins would then further dissect any interdependencies and synergistic binding relationships, and lead to a more accurate depiction of 30S subunit assembly (Grondek and Culver, in press).

CHAPTER 2: ASSEMBLY OF THE 30S RIBOSOMAL SUBUNIT: POSITIONING RIBOSOMAL PROTEIN S13 IN THE S7 ASSEMBLY BRANCH

A paper accepted by *RNA*

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Abstract

Studies of *Escherichia coli* 30S ribosomal subunit assembly have revealed a hierarchical and cooperative association of ribosomal proteins with 16S ribosomal RNA; these results have been used to compile an *in vitro* 30S subunit assembly map. In single protein addition and omission studies, ribosomal protein S13 was shown to be dependent on the prior association of ribosomal protein S20 for binding to the ribonucleoprotein particle. While the overwhelming majority of interactions revealed in the assembly map are consistent with additional data, the dependency of S13 on S20 is not. Structural studies position S13 in the head of the 30S subunit over 100Å away from S20, which resides near the bottom of the body of the 30S subunit. All of the proteins that reside in the head of the 30S subunit, except S13, have been shown to be part of the S7 assembly branch, that is, they all depend on S7 for association with the assembling 30S subunit. Given these observations, the assembly requirements for S13 were investigated using base-specific chemical footprinting and primer extension analysis. These studies reveal that S13 can bind to 16S rRNA in the presence of S7, but not S20. Additionally, polyspecific interaction between S13 and other members of the S7 assembly branch have been observed. These results link S13 to the 3' major domain family of proteins, and the S7 assembly branch, placing S13 in a new location in the 30S subunit assembly map where its position is in accordance with much biochemical and structural data.

Introduction

The *Escherichia coli* (*E. coli*) small (30S) ribosomal subunit is structurally comprised of 20 ribosomal proteins (r-proteins) and a 1542 nucleotide, 16S ribosomal RNA (rRNA). The 30S subunit in conjunction with its larger counterpart the 50S subunit form the 70S ribosome; a ribonucleoprotein particle (RNP) that is responsible for the essential cellular process of protein synthesis. The 30S subunit serves as the condon-anticondon recognition site for the messenger RNA (mRNA), which acts as the template for protein translation, and the appropriate transfer RNA (tRNA), the adaptor that delivers the corresponding amino acid.

Assembly of the 30S subunit is an elaborate process that has been under investigation for over three decades. In early studies it was shown that the small subunit may be reconstituted *in vitro* from its isolated constitutive parts, 16S rRNA and a mixture of the Total Proteins from the 30S subunit (TP30), to form a functional 30S particle (Traub and Nomura 1968). Additionally it has been shown that the individually purified proteins, both natural (Traub and Nomura 1968) and recombinant (Culver and Noller 2000), could support the *in vitro* reconstitution. This system has allowed detailed studies of the role of these components in 30S subunit assembly and function. Single protein addition and omission studies of the *in vitro* 30S subunit reconstitution process led to the elucidation of an ordered assembly map and the definition of distinct assembly branches (Mizushima and Nomura 1970; Held et al. 1974) (Fig. 1A). These studies and others (Schaup et al. 1970; Lutter et al. 1974) revealed that a subset of the small subunit r-proteins bind independently and specifically to 16S rRNA. This subset contains the r-proteins S4, S7, S8, S15, S17 and S20, and is often referred to as the primary (1°) binding proteins. Association of the primary binding proteins with 16S rRNA is a necessary step for the subsequent association of the remaining r-proteins. Thus the primary binding proteins can be used to define assembly branches and dependencies for 30S subunit assembly. For example, the S7 assembly branch (see Fig. 1) contains not only S7 but also r-proteins S9, S19, S14, S10, S3, and S2. Proteins

found in the same assembly branch tend to correlate well with proteins that bind within the same domain of 16S rRNA (Samaha et al. 1994; Agalarov et al. 1998; Agalarov et al. 1999; Agalarov et al. 2000). Again, using the S7 branch as an example, all of these proteins bind the 3' major domain of the 16S rRNA and are found in the head of the 30S subunit (Fig. 2A). Generally, there is good agreement between the dependencies revealed in the *in vitro* 30S subunit assembly map and structural and biochemical studies that reveal interactions and locations of proteins within the subunit. However, there is at least one significant outlier to this correlation. R-protein S13 has been implicated in ribosomal function (Cukaras et al. 2003), yet its assembly requirements have historically remained rather ambiguous. Experiments that led to the formulation of the assembly map (Fig. 1A) suggested that S13 is dependent on S20 for association (Mizushima and Nomura 1970; Held et al. 1974); however, conflicting evidence suggested that S13 might also require S7 as part of its binding precursor (Schaup et al. 1970; Lutter et al. 1974). *In vitro* reconstitution studies using only the 3' domain of 16S rRNA and a complete set of 30S subunit r-proteins supported this latter idea (Samaha et al. 1994). S13, along with r-proteins S2, S3, S4, S7, S9, S10, S14 and S19 were found bound to this 16S rRNA 3'domain fragment, and the resulting RNP was functional as monitored by spectinomycin binding (Samaha et al., 1994). These studies suggest that S13 can stably assemble with a 16S rRNA-containing RNP without the prerequisite stable association of S20.

Studies that have focused on the positions of r-proteins within the mature 30S subunit also suggest that S13 is misplaced in the assembly map. Protein cross-linking studies have shown that S13 can be crosslinked to S19 in natural 30S subunits, revealing a proximal relationship between the two proteins (Pohl and Wittmann-Liebold, 1988). R-protein S19 is unambiguously located in the S7 assembly branch (see Fig. 1B), and it has been localized to the head of the 30S subunit by neutron diffraction (Capel et al. 1987), immunoelectron microscopy (IEM) (Lake 1975), and x-ray crystallography (Wimberly et al. 2000).

Seemingly, these studies would support an S7 assembly branch assignment for S13. However, S20 was inconsistently positioned in the body by IEM (Schwedler et al. 1993) but in the head by neutron diffraction (Capel et al. 1987). Thus, the question of the proper assembly dependencies for S13 still remains unresolved.

More recently, with the determination of the crystal structure of the *Thermus thermophilus* 30S subunit, the relative positions of S13 and S20 have been more precisely revealed (Fig. 2A); S13 is located in the head of 30S subunit with the 3' major domain of 16S rRNA, and S20 is found at a distal site, binding to the 5' domain of the 16S rRNA adjacent to the spur in the body of the subunit (Wimberly et al. 2000). These data position S13 and S20 100Å apart in the mature subunit. The majority of assembly dependencies depicted in the *in vitro* assembly map are supported by the close proximity of dependent proteins within the 30S subunit (Wimberly et al. 2000; Brodersen et al. 2002). Thus it appears unlikely that the assembly of S13 onto the developing pre-30S subunit would depend on S20, and that S7 is clearly a more likely candidate (Fig. 2A). R-proteins S4 and S8 are the other possible assembly interacting proteins for S13 that were suggested by the *in vitro* assembly map (Mizushima and Nomura 1970; Held et al. 1974). S13 showed very weak dependence on these proteins for binding to 16S rRNA-containing RNPs, but again structural data does not support these relationships (Wimberly et al. 2000). Both S4 and S8 are located in the body of the 30S subunit, not quite as remote as S20, but significantly removed from S13 (Wimberly et al. 2000). The location of S13 in the head of the 30S subunit with all the components of the S7 assembly branch (Wimberly et al. 2000), and solely these components, suggests that S13 might belong in this branch.

While footprinting studies can reveal assembly relationships, previous work has not dissected the requirements for S13 binding. In prior experiments, S13 interactions with 16S rRNA were probed (Heilek and Noller 1996) and footprinted (Powers et al. 1988) in the presence of all the 1° binding proteins. These studies suggest that the binding site for S13 is

more closely allied with that of S7 and not S20 (Fig. 2B) since S13 footprints are localized to the 3' major domain of 16S rRNA adjacent to those of S7. However, from the previous footprinting studies it is not possible to accurately dissect the association requirements for S13 binding since all of the 1° binding proteins (including S4, S7, S8, and S20) were present prior to the addition of S13 (see Fig. 2B). Therefore, to understand which 1° binding protein(s) is required for S13 assembly, more minimal particles containing S13 were prepared. Different RNPs containing 16S rRNA and the individual primary binding proteins S7 or S20, and S13 were formed, subjected to chemical modification and primer extension analysis to determine their protein-16S rRNA footprinting patterns and thus to determine requirements for S13 association. Our data supports a sequential relationship between S7 and S13, not S20 and S13, resulting in a new position in the *in vitro* 30S subunit assembly map for S13. Probing of RNPs containing S7, S13 and other members of the S7 assembly branch also support additional interactions between S13 and other proteins found in this branch, mainly S9 and S19.

Results

Reconstitution of 16S rRNA containing RNPs

In order to determine the requirements for association of S13 with 16S rRNA, different sets of 16S rRNA r-protein containing complexes were compared. Initially, the appropriate recombinant primary binding protein, either S7 or S20, was added to 16S rRNA; after the binary complex was formed, S13 was added to the RNP. To investigate interactions of S13 with other proteins beyond the primary binding proteins, sequential addition of proteins, either those in the S7 assembly branch or the tertiary binding proteins, S2, S3, S10, and S14, were added to S13 containing complexes. In all cases the RNPs were used directly in chemical probing experiments.

Footprinting S13 in the presence of S7 and S20

The reconstituted RNPs were subjected to kethoxal modification, which modifies guanine bases, and to dimethyl sulfate (DMS), which modifies both adenine and cytosine bases. Primer extension analysis was used to map the sites of 16S rRNA modification in various complexes to determine if the addition of S13 has an effect on the modification pattern. Association of S13 with the binary complexes should reveal novel sites of altered reactivity of 16S rRNA as compared to the modification pattern in the absence of S13.

Changes attributable to S13 binding are observed only in the presence of S7. All of the changes that are attributable to S13 footprints in the S7/S13/16S rRNA complexes are found in the 3' major domain (Figs. 3,4), consistent with prior structural and biochemical data (Powers et al. 1988). The data representing relative changes in the modification pattern upon the addition of S13 to S7/16S rRNA have been highlighted on the secondary structure of 16S rRNA (Fig. 5) and on the three dimensional structure of the 30S subunit (Fig. 6). Many changes resulting from S13 binding can be seen in the 930 to 980 region (Fig. 3A). The base of helix 30 is protected at nucleotides 944 and 945 with corresponding protections on the 3' side of the helix and the adjacent loop at nucleotides 1236-1239 (Fig. 3A, lane 7; Fig. 3B, lane 13). An additional protection at nucleotide 951 and a single enhancement at 954 are also found in this helix (Fig. 3A, lane 7). There are weak protections at nucleotides 933 in helix 28, and 942 in helix 29 (Fig. 3A, lane 7). Also a series of medium strength enhancements spanning nucleotides 977-980 in the loop connecting helices 31and 32 (Fig. 3A, lane 13) are observed.

Helix 41 contains two medium enhancements at nucleotides 1278 and 1279 (Fig. 3B, lane 7), and two groups of weak protections, nucleotides 1248-1252 and 1287-1289 (Fig. 3B, lane 13). An additional weak enhancement is seen at nucleotide 1297 near the base of helix 41 (Fig. 3B, lane 7). Two adjacent medium enhancements of nucleotides 1337 and 1338 (Fig. 3B, lane 7) are observed in the loop connecting helix 29 and helix 42. The lone change

observed in helix 43 is a weak enhancement at nucleotide 1355 (Fig. 3B, lane 7). The numerous changes observed upon the addition of S13 to the S7/16S rRNA complex are in marked contrast to the total absence of changes observed upon addition of S13 to the S20/16S rRNA complex (Fig. 3, lanes 5,11).

Since no changes are observed upon addition of S13 to a complex of S20 and 16S rRNA, it is important to verify the binding of S20 in the reconstituted RNP. In the S20/16S rRNA complex, strong kethoxal protections can be seen at nucleotides 251, 265-266, and 281 (Fig. 4A, lanes 4,5). In addition, a weak enhancement may be seen at nucleotide 278 (Fig. 4A, lanes 4,5). These footprints are consistent with previous S20 footprinting studies (Fig. 2B) (Powers et al. 1988) and thus confirm that S20 is indeed bound in these particles and that S13 does not appear to associate with 16S rRNA solely in the presence of S20. These data support the inclusion of S13 in the S7 assembly branch rather than the S20 branch.

The relative position of S13 with regard to the other members of the S7 assembly branch (Fig. 1) also warranted further investigation. RNPs containing various combinations of S7, S13 and other S7-dependent binding proteins were prepared and analyzed (Fig. 4B). Several increased protections are observed in the 930-960 region when S13 is added to complexes containing S7-S9 or S7-S19 particles (Fig. 4B, lanes 6,7). In these regions the addition of S13 appears to augment binding of S9 and S19 since these residues are partially protected in the absence of S13 but become more protected upon S13 addition. These results suggest a synergistic assembly relationship between S7, S13, S9 and S19. Given the complexities of the S7 assembly branch, significant further analysis will be required to dissect the role S13 plays in the association of the 3° binding proteins S2, S3, S10, and S14 that also bind to the head of the 30S subunit.

Discussion

This study was designed to address the assembly relationship of S13 with the primary binding proteins S7 and S20 in the course of 30S subunit assembly. As a result, it has been shown that S13 has an assembly dependence on S7 as its binding predecessor to 16S rRNA, and that S13 binding is not affected by the presence of S20 (Figs. 3,4A). In addition it was shown that S13 influences the binding of two other proteins in the S7 assembly branch, S9 and S19 (Fig. 4B). As a consequence of these studies, S13 has been relocated in the assembly map downstream of S7 (Fig. 6A), localized within the group of proteins that bind to the 3' major domain and form the head of the 30S subunit. Previous base-specific S13 footprinting studies were performed in the presence of additional small subunit r-proteins (Powers et al. 1988) that complicate interpretations of the assembly requirements for S13. These studies assessed the binding of S13 in the presence of 15 additional proteins, including S7 and S20. Thus from these experiments, it is difficult to fully ascertain the binding requirements for and interactions of S13 during the 30S subunit assembly. From these studies the authors suggest that S7, S9 and S19 are necessary and sufficient for S13 binding. Based on the results presented here, it appears that S7 alone is necessary for S13 binding (Fig. 3), thus solidifying S13 as a secondary binding protein. Nevertheless, some synergistic effects between binding of S7, S13, S9 and S19 have been observed (Fig. 4B).

Compared to the previous studies discussed above (Powers et al. 1988), additional sites of altered reactivity are observed when S13 is footprinted solely in the presence of S7. This is likely due to the absence of the additional proteins which could mask sites of potential changes. A likely example of this is the data observed in the loop connecting helices 31 and 32. In complexes composed of S7 and 16S rRNA, nucleotides 975-980 showed enhanced reactivity (Fig. 3A, Powers et al. 1988). Upon addition of S19 to this complex (Fig. 3A, Powers et al. 1988), these same nucleotides become protected from reactivity. In our experiments, upon the addition of S13 to the S7/16S rRNA complexes, nucleotides 975-980

become more reactive. Thus it appears that S13 augments the effect of S7, with S19 having the opposite effect. Also these sites are further protected upon the addition of S2 and S3 (Powers et al. 1988). As these proteins are the last of the S7 assembly branch to bind, S13 appears to be involved in a series of concerted and consecutive changes within this branch that aid 30S subunit assembly. Similar patterns are observed at many of the nucleotides whose reactivities are altered in the minimal S7/S13/16S rRNA-containing complexes. Thus, the inclusion of S13 in the S7 assembly branch is consistent with structural data revealing the location and contacts between S13 and 16S rRNA and other ribosomal proteins.

In the presence of S7, minor changes in reactivity in helices 28 and 29 due to the addition of S13 are observed (Fig. 3A). Interestingly, S7 directly contacts these regions in the 30S subunit (Brodersen et al. 2002). G933, which is protected upon S13 addition, makes both base and backbone interactions with S7 in 30S subunits (Brodersen et al. 2002). Also G942 is immediately adjacent to an extensive stretch of nucleotides which interacts with S7, and is also protected upon the binding of S13. Protections by S13 in the loop connecting helix 30 with helix 41 also overlap with and are adjacent to sites of S7/16S rRNA interactions within the mature subunit. An S13-induced enhancement occurs at G1297 (Fig. 3B) where S7 makes a backbone contact in the 30S subunit. These results suggest that S13 association with the S7/16S rRNA containing RNP aids in the full association of S7 with its complete binding site during 30S subunit assembly (Fig. 6A)

R-protein S13 appears to impact the interactions of other members of the S7 assembly branch with 16S rRNA. Previously, it was shown that in the presence of sub-stoichiometric amounts of S7, stoichiometric association of S19 and S13 to 16S rRNA is observed if they are added together but not separately (Dijk et al. 1977). Initial studies suggested that S9 was weakly dependent on S13 for assembly (Mizushima and Nomura 1970). However the authors noted that this effect could be due to the enhanced association of S7 in the presence of S13. Our results are consistent with these observations, as some additional changes are

observed when S13, and either S9 or S19, are bound to the S7/16S rRNA complex (Fig. 4B). These observations, taken together, suggest that S13 belongs not only downstream of S7 but also that co-dependencies between S13, S9 and S19 exist (Fig. 6A).

When S13 footprinting data are mapped on the tertiary structure of the 16S rRNA from 30S subunits (Fig. 6B, Wimberly et al. 2000), it is clear that the majority of changes in reactivity are localized in the area surrounding S7 and S13. Some of the S13 binding interactions observed correspond with both backbone and base-specific interactions between S13 and 16S rRNA observed in the 30S subunit (Brodersen et al. 2002). One such interaction is present in helix 30, the protection of nucleotide G951. The enhanced nucleotide G953 on helix 30 is also a site of backbone and base interactions of S13 within the 30S subunit (Brodersen et al. 2002). This difference could be explained if the interaction of S13 with this site is a late assembly event, occurring only with the addition of other 3' major domain proteins. The nucleotide G1297 at the base of helix 41 also displays enhanced reactivity upon S13 binding to the minimal RNP but has been shown to form an interaction via its backbone with S13 in the full 30S subunit (Brodersen et al. 2002). Again, the association of other r-proteins could influence these interactions or it is possible that interactions with the RNA backbone caused an increased availability of the base to modification. Our data on interactions within minimal S13 containing RNPs are largely consistent with the position of elements within the fully formed 30S subunit, strongly supporting the inclusion of S13 in the S7 assembly branch.

Although a specific functional role for S13 is still unknown, crystallographic data suggest S13 may play a pivotal role in translation (Yusupov et al. 2001). S13 is a component of the only protein-protein bridge between the 30S and 50S subunits within the 70S ribosome (Yusupov et al. 2001). This is the only interaction in the crystal structure between the 3' major domain and the 50S subunit, a domain that is thought to be mobile during the translation process. In addition, the tail of S13 directly interacts with the transfer RNA

(tRNA) at the peptidyl-site (P-site) of the 30S subunit within the 70S complex (Yusupov et al. 2001). Among the 16S rRNA nucleotides that have contacts with the P-site tRNA are 1338 (Moazed and Noller 1990; Yusupov et al. 2001), and in our study nucleotides 1337 and 1338 become enhanced first by the binding of S7 and then further by S13 binding (Fig. 3B, lanes 6,7). Additionally, r-protein cross linking studies of the 30S subunit have also linked these and adjacent nucleotides with S13, signifying they are exposed in wild-type 30S subunits, in the absence of tRNA. These data suggest that S13 might participate in the formation of the P-site within the assembling 30S subunit. These observations, crystallographic data, and other recent experiments (Cukaras et al. 2003) support the hypothesis that S13 plays an essential role in the pretranslocation state of the 70S ribosome. The data presented herein reveal the appropriate assembly dependencies for S13 and allow a better understanding of the functional role of S13 in assembly and functioning of the 30S ribosomal subunit.

Materials and Methods

Natural 16S rRNA was extracted and purified from MRE600 as previously described (Moazed et al. 1986). Recombinant small subunit ribosomal proteins were purified as previously described (Culver and Noller 2000).

Preparation of complexes

The reconstitution was performed with 40 pmols 16S rRNA and 280 pmols of recombinant r-proteins S7, S9, S13, S19 and S20 in appropriate combinations. 16S rRNA was heat activated at 42°C for 15 minutes and cooled on ice for 20 minutes. The reconstitution was performed in Recon A buffer (80 mM K^+-Hepes 7.6, 20 mM $MgCl_2$, 330 mM KCl, and 0.01% Nikkol), with the final KCl concentration of 330 mM at each reconstitution stage. The first stage consists of 16S rRNA and a 7-molar excess of the

appropriate 1° r-protein (S7 or S20) in Recon A buffer and 1 mM β-mercaptoethonal (BME) which was incubated at 42 °C for 20 minutes. Next, a 7-molar excess of the appropriate 2° r-protein(s) (S9, S13 and S19) is added with conditions adjusted to those of Recon A buffer, followed by a 42 °C incubation for 20 minutes. Protein storage buffer (1 M KCl, 20 mM $MgCl_2$, and 80 mM K^+-Hepes 7.6) was added in place of the r-protein to achieve a final KCl concentration of 330mM.

Chemical Probing and Primer Extension Analysis

The resulting RNPs were cooled on ice for 20 minutes, and subjected to kethoxal or dimethyl sulfate (DMS) modification on ice for 2 hours (as described by Moazed et al. 1986). The rRNA was then extracted as previously described (Culver and Noller, 2000). The resulting RNA-modified complexes were used for primer extension analysis with α^{32}P-TTP and primers (Moazed et al. 1986).

CHAPTER 3. CONCLUSION

General Conclusion

The work presented here represents definitive evidence for the placement of r-protein S13 in the S7 assembly branch (Grondek and Culver, in press). This relationship is supported by numerous previously described biochemical and biophysical data that place S13 in the 3' major domain of the 30S subunit. This is in sharp contrast to the originally proposed placement of S13, as being dependent on S20 for association (Mizushima and Nomura 1970; Held et al. 1974), a protein known be located on the opposite end of the 30S subunit 100Å away (Wimberly et al. 2000). We have also shown that there are synergistic assembly relationships between S7 and S13, and between S13 and the secondary binding r-proteins S9 and S19 (Grondek and Culver, in press). There are many similar interactions depicted in other areas of the assembly map (Mizushima and Nomura 1970; Held et al. 1974), and such relationships would be expected in the dynamic process of multiple proteins binding RNA in close proximity. This work in total represents a significant change to the assembly map and a greater understanding of 30S subunit assembly.

Future Work

We have observed some late stage interactions between S13 and a mixture of S2, S3, S9, S10, S14 and S19 (unpublished results), suggesting additional interactions with tertiary members of the S7 family. We have yet to dissect the specific interaction between these tertiary binding proteins and S9, S13, and S19. These single and multiple addition experiments, of an increasingly complex nature, would be the next logical step in studying the assembly role of S13. The result would most likely be a detailed network of synergistic relationships representing late stage binding interactions of the tertiary r-proteins with the 16S rRNA 3' major domain.

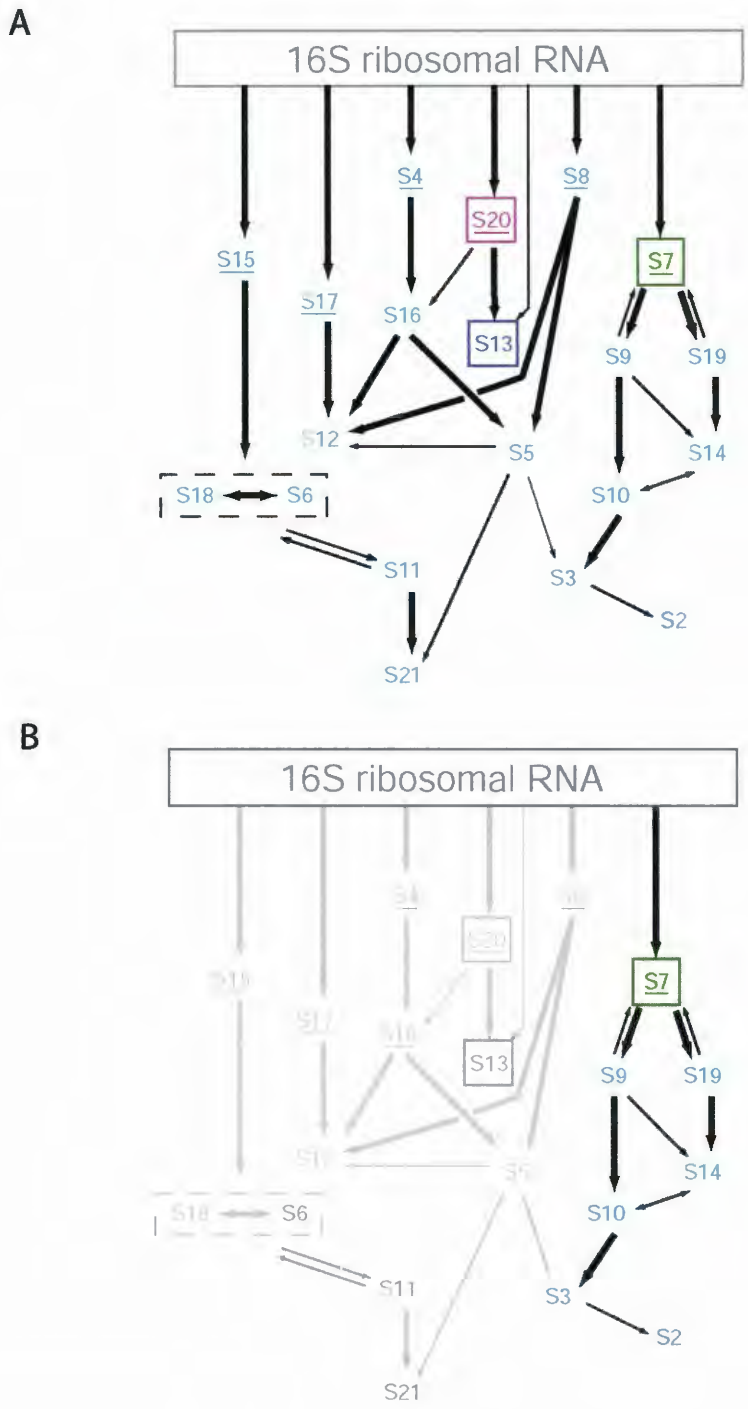

Figure 1. The in vitro 30S subunit assembly map.

A) Simplified in vitro 30S subunit assembly map (Mizushima and Nomura 1970; Held et al. 1974) highlighting the relative position of r-proteins S7 (green), S13 (blue) and S20 (magenta). The remaining r-proteins are colored aqua. The primary binding proteins have been underlined and the 16S rRNA is designated as a rectangle.

B) The in vitro 30S subunit assembly map with the S7 assembly branch highlighted.

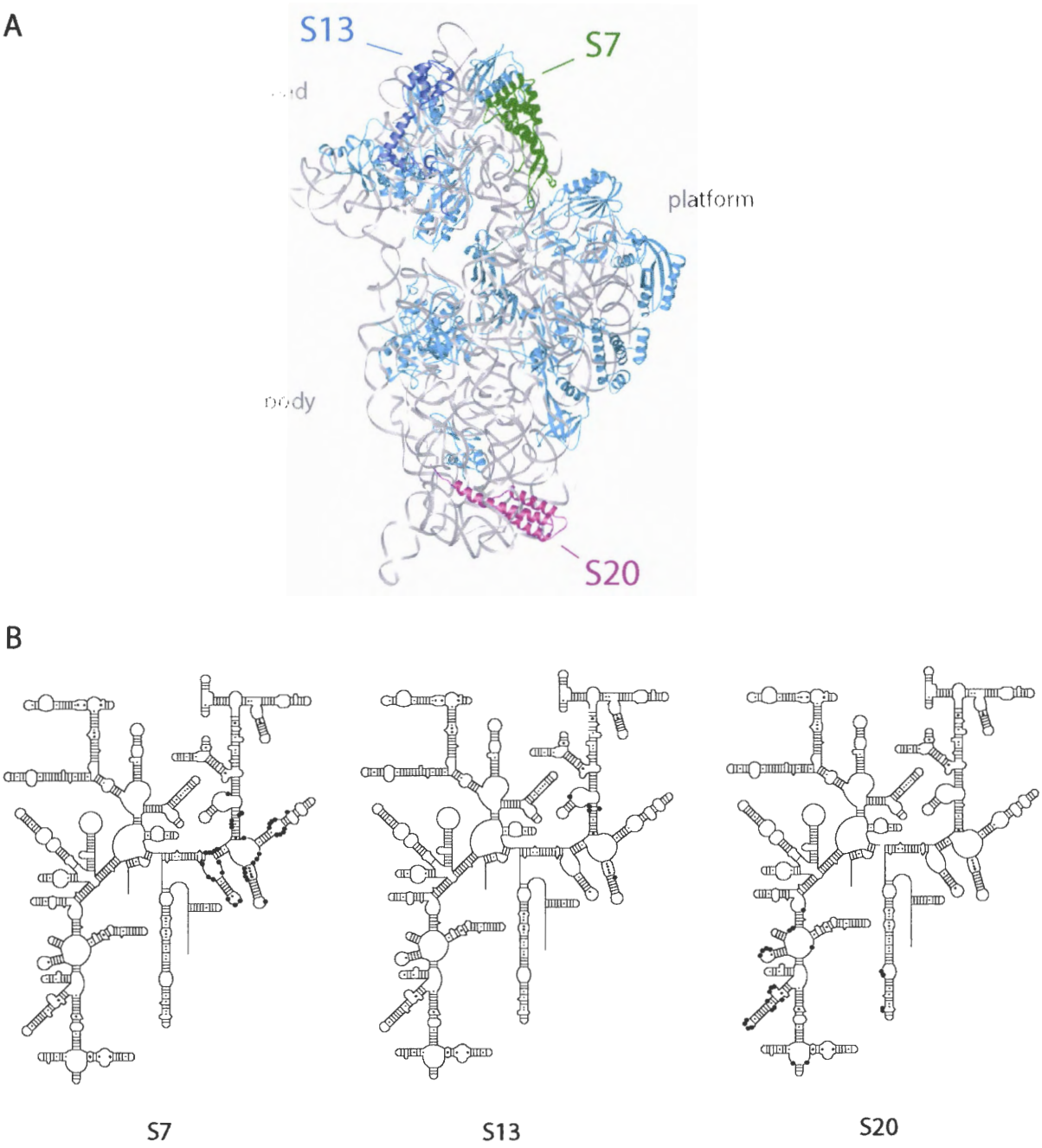

Figure 2. Positions of r-proteins S7, S13 and S20.

A) The three-dimensional structure of the 30S subunit with S7 (green), S13 (blue) and S20 (magenta) highlighted. The 16S rRNA is shown in grey and the remaining r-proteins are colored aqua. This figure was produced with Ribbons (Carson 1997) using PDB 1J5E (Wimberly et al. 2000; Brodersen et al. 2002).

B) Base-specific footprinting data for the r-proteins S7, S13, or S20 (Powers et al. 1988) mapped on the 16S rRNA secondary structure (Cannone 2002). S7 and S20 were individually footprinted on 16S rRNA while S13 was footprinted in the presence of all the primary binding proteins, including S7 and S20.

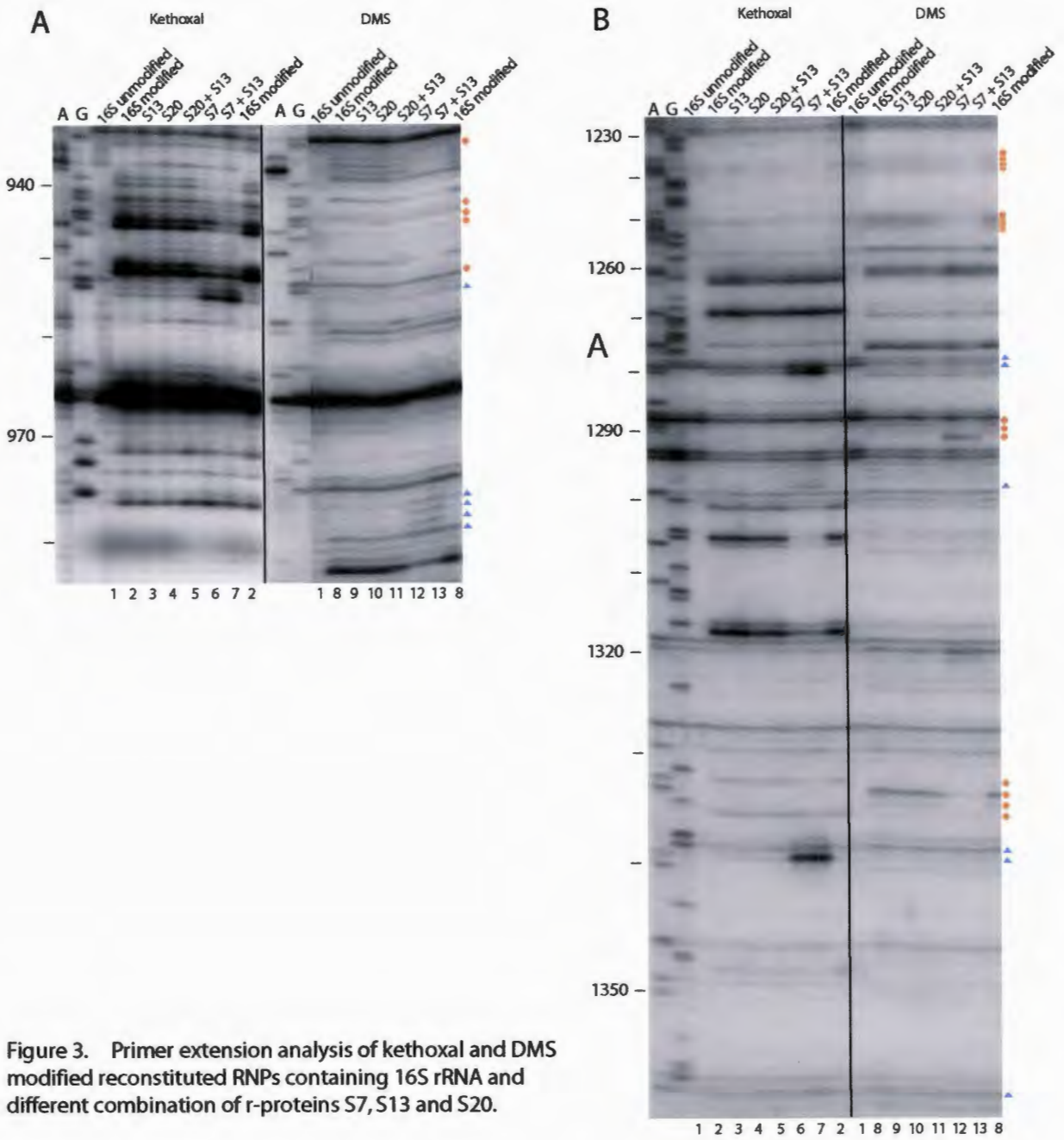

Figure 3. Primer extension analysis of kethoxal and DMS modified reconstituted RNPs containing 16S rRNA and different combination of r-proteins S7, S13 and S20.

Lanes A and G are dideoxy sequencing lanes. Lane 1, 16S unmodified, naked, unreacted 16S rRNA; lane 2, 16S modified, naked 16S rRNA treated with kethoxal, or lane 8, naked 16S rRNA treated with DMS. Lanes 3-7 and 9-13 are RNPs that havebeen treated with kethoxal or DMS respectively, and consist of a combination of naked 16S rRNA and the r-protein(s) S7, S13 and/or S20, as indicated at the top of the gel. Lane 3 & 9, S13 alone; lane 4 & 10, S20 alone; lane 5 & 11, S20 + S13; lane 6 & 12, S7 alone; lane 7 & 13, S7 + S13. The bases that have an increased reactivity, or enhancement, with the addition of S13 onto an RNP, with S7 pre-bound, are noted with blue triangles. The bases with a decreased reactivity, or protection, with the addition of S13 onto an RNP, with S7 pre-bound, are noted with orange circles.

A) Primer extension analysis using primer 945.

B) Primer extension analysis using primer 1391.

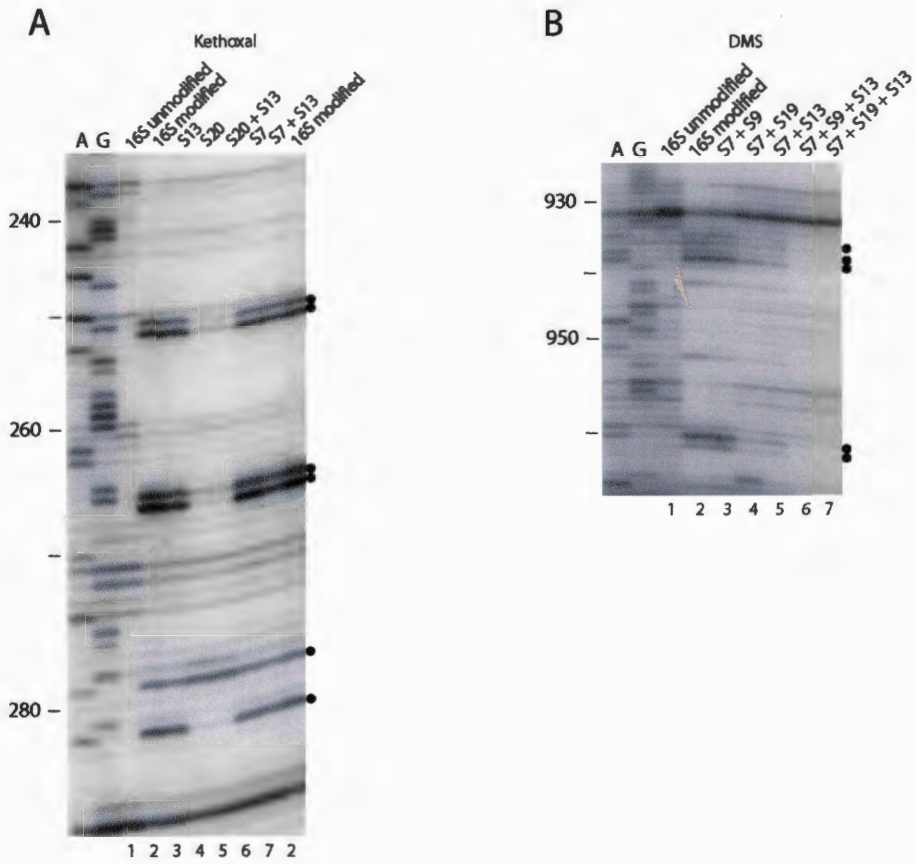

Figure 4. Primer extension analysis of kethoxal and DMS modified reconstituted RNPs containing 16S rRNA and different combination of r-proteins S7, S13 and S20, or S7, S9, S13 and S19.

Lanes A and G are dideoxy sequencing lanes. Lane 1, 16S unmodified, naked, unreacted 16S rRNA; lane 2, 16S modified, naked 16S rRNA treated with either A) kethoxal or B) DMS. The data are highlighted with black circles.

A) Footprinting data from kethoxal modified reconstituted RNPs containing 16S rRNA and different combinations of S7, S13 and/or S20 (lanes 3-7) as indicated at the top of the gel. Lane 3, S13 alone; lane 4, S20 alone; lane 5 S20 + S13; lane 6, S7 alone; lane 7, S7 + S13. Primer 323 was used for this extension.

B) Footprinting data from DMS modified reconstituted RNPs containing 16S rRNA and different combinations of S7, S9, S13 and/or S19 (lanes 3-7) as described at the top of the gel. Lane 3, S7 + S9; lane 4, S7 + S19; lane 5 S7 + S13; lane 6, S7 + S9 and S13; lane 7, S7 + S19 and S13. Primer 945 was used for this extension.

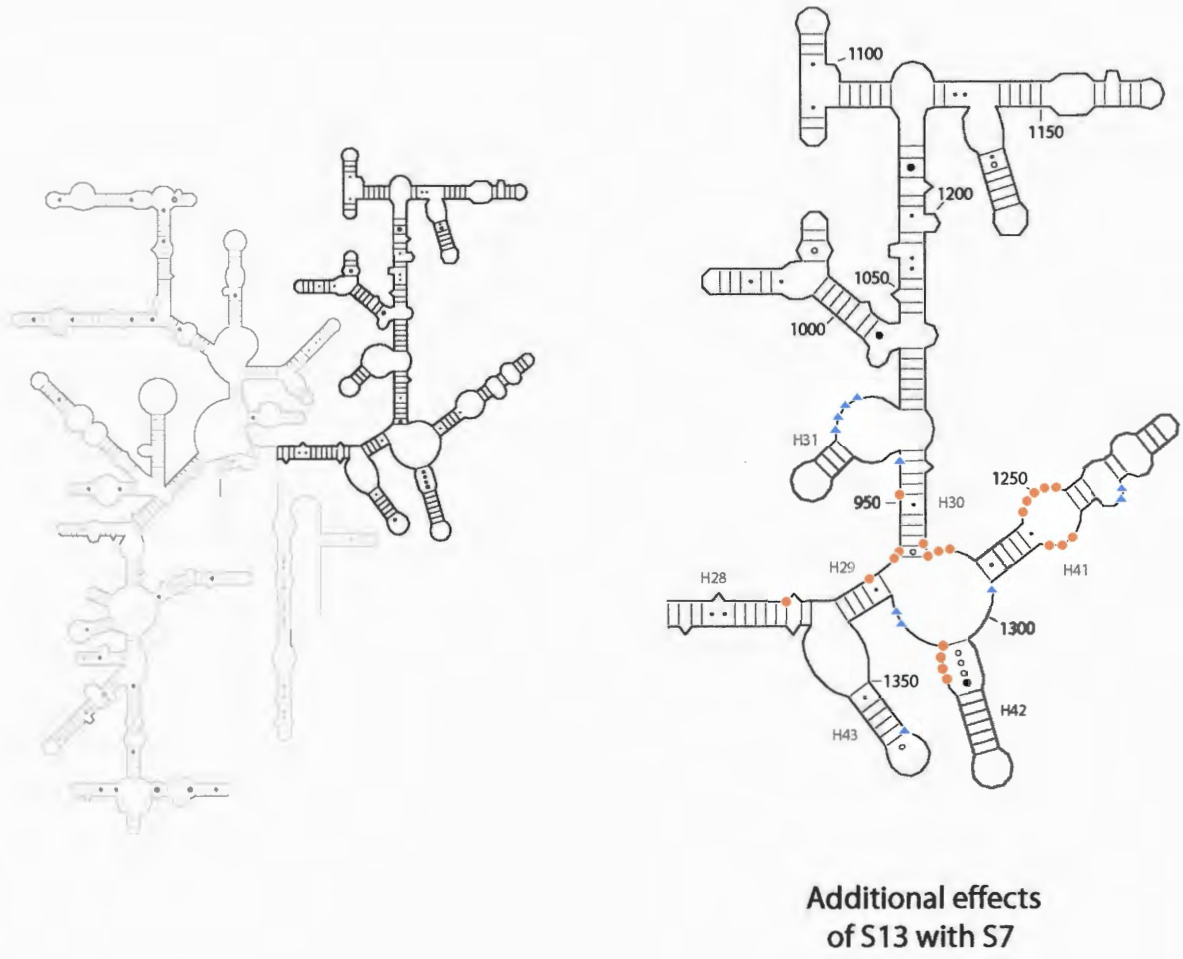

Figure 5. S13 footprinting data mapped on the secondary structure of 16S rRNA.

The 16S rRNA secondary structure (Cannone 2002) with the 3' major domain highlighted is shown and the S13 footprinting data is mapped on this expanded domain. The bases that have an increased reactivity, or enhancement, upon the addition of S13 to the S7/16S rRNA, are noted with blue triangles and the bases with a decreased reactivity, or protection, are noted with orange circles (in accordance with the footprinting data in Figure 3).

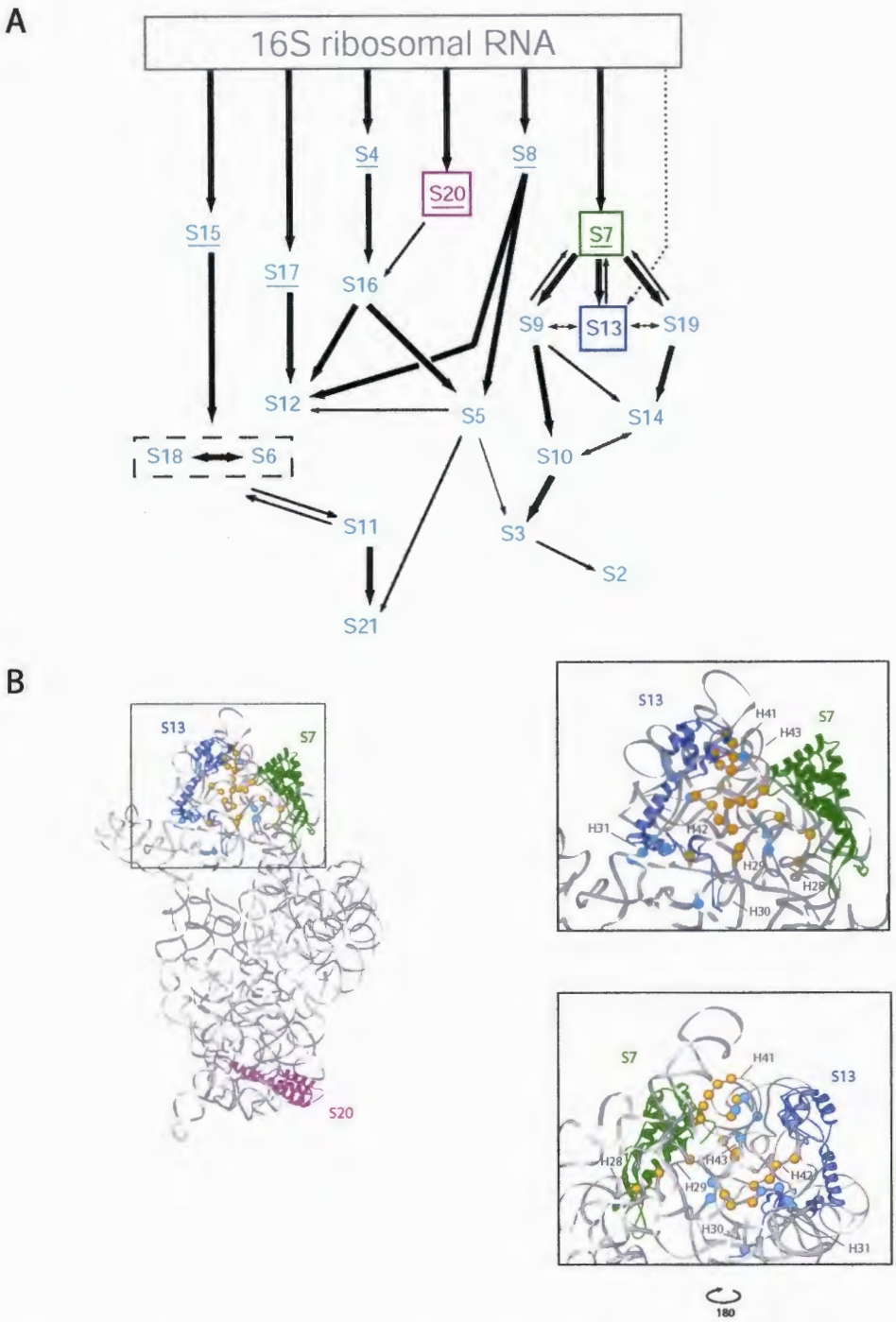

Figure 6. Positioning S13 in the S7 assembly branch of the in vitro 30S ribosomal subunit assembly map.

A) Revised in vitro 30S subunit assembly map color coded as in Figure 1. S13 has been re-positioned to show dependency on S7 and other interactions with r-proteins in this branch.

B) S13 footprinting data mapped on the tertiary structure of the 30S subunit. The enhancements are colored in blue and protections are colored in orange (in accordance with Figures 3 and 5). S13 is colored blue and S7 is colored green. This figure was produced with Ribbons (Carson 1997) using PDB 1J5E (Wimberly et al. 2000; Brodersen et al. 2002).

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APPENDIX

Assembly of the *E coli* 30S ribosomal subunit is a rapid cellular process hypothesized to initiate during transcription of the S16 rRNA. This supposition implies that as secondary structural features of the transcribing 16S rRNA begin to form, the primary r-proteins nucleate assembly by binding to the growing rRNA with 5' to 3' directionality. During *in vitro* 30S reconstitution there is a heat dependent conformational change required for assembly to go to completion. This heat dependent conformational change may consist of a set of discrete changes within the 16S rRNA representative of a 5' to 3' organization of the assembly process.

For complete *in vitro* 30S subunit assembly the reconstituting particles must be incubated at 42°C. At low temperatures, incubation of Total Proteins from the 30S (TP30) subunit with 16S rRNA results in formation of a Reconstitution Intermediate (RI) that sediments at 21S (Traub and Nomura, 1968). This particle contains only a subset of r-proteins, consisting of the primary and secondary r-proteins (Held and Nomura, 1973). When the RI particle is then heated to 42°C it undergoes a transformation to a particle that sediments at 26S (designated RI* and is read RI star), and this particle is then competent to assemble to a complete 30S subunit (Traub and Nomura, 1968). This process is called the RI to RI* transition and represents the only thermodynamic barrier during the 30S subunit assembly process (Held and Nomura, 1973). This transition has been mapped extensively using primer extension analysis (Holmes and Culver, 2004). It has also been shown that extra ribosomal assembly factors, specifically the DnaK chaperone system (Maki and Culver, 2002), can facilitate *in vitro* 30S assembly at low temperatures.

Temperature dependent rearrangement of 16S rRNA was observed at nucleotide resolution in the environment surrounding the primary binding protein S4 (Powers and Noller, 1995). R-protein S4 was reconstituted with 16S rRNA and probed with kethoxal, DMS and hydroxal radicals at 0°C and 42°C (Powers and Noller, 1995). Primer extension

analysis of the ribonucleoprotein particle (RNP) revealed a concerted set of heat dependent changes to relatively small a region of the 5' domain (Powers and Noller, 1995). This was the first direct evidence of local heat dependent changes during assembly, changes that may be linked to initial stages of the RI to RI* transition.

We sought to implement a similar experimental strategy to determine if other primary binding r-proteins would show both localized and distant heat dependent conformational changes to their 16S rRNA binding environments. To test this, we observed the individual reconstitution of r-proteins S8 and S17 with 16S rRNA at 0°C, 42°C, and 0°C to 42°C shift. S8 has previously been shown to bind two very distant areas of the central domain of the 16S rRNA, in both the 600 and 860 regions, where S17 has been shown to bind a small, localized region of the 5' domain (Power and Noller, 1995). We probed the reconstituted RNPs with kethoxal to modify rRNA guanine residues and preformed primer extension analysis.

We observed S17 binding to the 250-280 region of S16S rRNA, consistent with previous data (Power and Noller, 1988), at both low and high temperatures with no significant changes attributed to a heat dependent transition (data not shown). In contrast, our data demonstrates that S8 binding leads to a series of unique heat dependent changes. One protection, G575, which has been previously shown to be an S8 footprint (Powers and Noller, 1998), is weekly protected at low temperatures and then becomes more protected at both 42°C and the 0°C to 42°C shift, where G521 appears to become enhanced at low temperatures and then further enhanced at both 42°C and the 0°C to 42°C shift, (Fig. 1A; lanes 6-8). Nucleotides G540 and G524 both exhibit enhanced reactivity only at high temperatures (Fig. 1A; lanes 6-8). Protections observed at G846, G858 and G859 all represent down stream heat dependent changes (Fig. 1B; lanes 6-8), and are evident only at high temperatures. These 500-575 region low and high temperature data in combination with unique temperature dependent protections in the 850 region support a 5' to 3' organization of S8 assembly.

These S8 data suggest heat dependent conformational changes associated with the binding of primary r-proteins and may represent the first stages of discrete longer range assembly events, exhibiting a 5' to 3' directionality to the 30S subunit assembly process. Because S17 has been shown to bind only a small region of 16S rRNA (Powers and Noller, 1995) changes were not expected to be seen in other distant rRNA regions, consistent with our data. We also have preliminary data suggesting primary r-protein S15 follows a similar binding pattern as described here for S8. R-proteins S7 and S20 should be evaluated in an analogous manor to determine if this behavior is consistent with all 30S primary binding proteins.

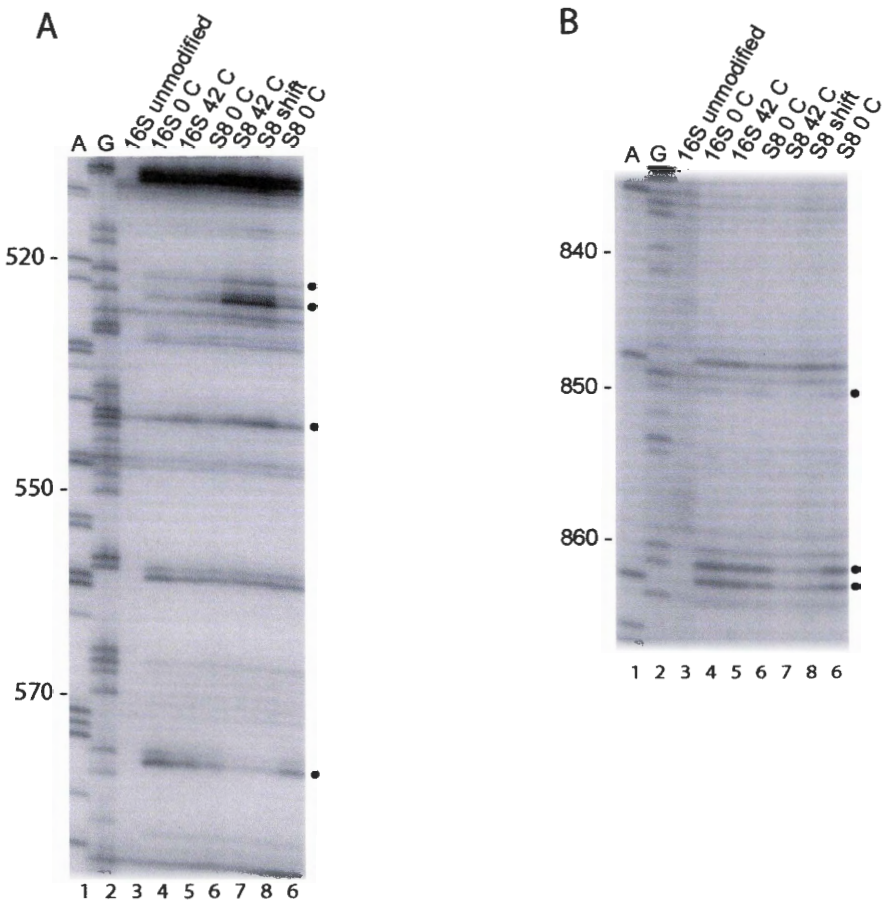

Figure 1. Primer extension analysis of kethoxal modified reconstituted RNPs containing 16S rRNA and ribosomal protein S8.

Lanes A and G are dideoxy sequencing lanes. Lane 1, 16S at 0°C unmodified, naked 16S rRNA; lane 2, 16S at 0°C modified, naked 16S rRNA treated with kethoxal; lane 3, 16S at 42°C modified, naked 16S rRNA treated with kethoxal. Lanes 4-8 are RNPs that have been reconstituted at 0°C, 42°C or 'shifted' from 0°C to 42°C during reconstitution. All of the RNPs were treated with kethoxal. Black circles indicate nucleotides of interest.

A) Primer extension analysis using primer 683.

B) Primer extension analysis using primer 939.

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