Functional regions in the 5' external transcribed spacer of yeast pre-rRNA

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ABSTRACT

Ribosomal subunits are assembled on a precursor rRNA that includes four spacers in addition to mature rRNA sequences. The 5' external transcribed spacer (5' ETS) is the most prominent one that recruits U3 snoRNA and a plethora of proteins during the early assembly of 90S small subunit preribosomes. Here, we have conducted a comprehensive mutational analysis of 5' ETS by monitoring the processing and assembly of a plasmid-expressed pre-18S rRNA. Remarkably, nearly half of the 5' ETS sequences, when depleted individually, are dispensable for 18S rRNA processing. The dispensable elements largely bind at the surface of the 90S structure. Defective assembly of 5' ETS completely blocks the last stage of 90S formation yet has little effect on the early assembly of 5' and central domains of 18S rRNA. Our study reveals the functional regions of 5' ETS and provides new insight into the assembly hierarchy of 90S preribosomes.

Keywords: 90S preribosome; rRNA processing; ribosome assembly

INTRODUCTION

Biogenesis of yeast ribosome initiates with the transcription of a long 35S precursor rRNA (pre-rRNA) by RNA polymerase I. The pre-rRNA contains the sequences of mature 18S, 5.8S, and 25S rRNAs as well as four external and internal transcribed spacers (ETS and ITS). The spacers are to be removed by exo- and endo-ribonucleases during rRNA processing (Venema and Tollervey 1999; Mullineux and Lafontaine 2012; Fernandez-Pevida et al. 2015; Tomecki et al. 2017). The pre-rRNA undergoes extensive pseudouridylation and 2'-O-methylation catalyzed by box H/ACA and C/D small nucleolar ribonucleoprotein particles (snoRNPs) (Watkins and Bohnsack 2011). In the yeast *Saccharomyces cerevisiae*, more than 200 protein assembly factors (AFs) and four special snoRNAs (U3, U14, snR30, and snR10) are involved in rRNA processing and ribosome assembly (Woelford and Baserga 2013). The pre-rRNA is cotranscriptionally assembled into early precursors of small and large ribosomal subunits (SSU and LSU). The preribosomes progressively mature in a process that involves cleavage of pre-rRNA, protein assembly, structural changes and translocation from the nucleolus, through

the nucleoplasm to the cytoplasm. The structures of many preribosomes have been recently determined by cryo-electron microscopy (cryo-EM), providing major insights into the complicated pathway of ribosome biogenesis (Bassler and Hurt 2019; Klinge and Woelford 2019).

The 90S preribosomes (aka the SSU processome) are the early nucleolar precursors of SSU (Dragon et al. 2002; Grandi et al. 2002). As illustrated in Miller spreads images, the 90S is assembled on the 5' part of the nascent pre-rRNA transcript and dramatically compacts into a large particle seconds after the pre-18S region is completely transcribed (Osheim et al. 2004). Under favorable growth conditions, the pre-rRNA is cotranscriptionally cleaved at the A0 and A1 sites of 5' ETS and at the A2 site of ITS1, releasing a pre-40S ribosome. The pre-40S further develops into the mature SSU in the cytoplasm.

The assembly order of 90S has been dissected by analyzing the proteins and snoRNAs associated with a series of plasmid-expressed pre-18S rRNA fragments of

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increasing length (Chaker-Margot et al. 2015; Zhang et al. 2016). These studies reveal a stepwise and dynamic assembly map for ~70 AFs and the U3, U14, and snR30 snoRNAs. The AFs can be classified as 5' ETS factors, 5' domain factors, central domain factors, 3' major domain factors, and late factors according to their assembly dependence on different pre-rRNA domains. The 5' ETS by itself assembles a plethora of AFs, including three large preassembled subcomplexes UTPA, UTPB and U3 snoRNP (Watkins et al. 2000; Grandi et al. 2002; Gallagher et al. 2004; Krogan et al. 2004; Perez-Fernandez et al. 2007, 2011), into a 5' ETS particle. UTPA is the first assembled complex that binds to the 5' part of 5' ETS (Hunziker et al. 2016; Zhang et al. 2016) and is required for association of many downstream factors (Perez-Fernandez et al. 2007, 2011). The 5' and central domains of 18S rRNA each recruits a specific group of factors, whereas the 3' major domain initially recruits a few factors. When the 18S region is nearly complete, a dozen of late AFs are bound and about ten labile factors, including the U14 and snR30 snoRNAs, which are initially recruited by the 18S region, are released. Following these dramatic events, a mature 90S particle competent in rRNA processing is formed. The last stage of 90S assembly also coincides with the compaction of 90S structure seen in Miller spreads (Osheim et al. 2004). The cryo-EM structures of 90S from *S. cerevisiae* and *Chaetomium thermophilum* have been recently determined in mature and immature states (Kornprobst et al. 2016; Barandun et al. 2017; Chaker-Margot et al. 2017; Cheng et al. 2017, 2019; Sun et al. 2017; Hunziker et al. 2019). The structure of 90S rationalizes many aspects of the assembly map. For example, the late factors bind all over the 90S structure and appear to unite individual ribosomal domains and the 5' ETS particle into an integral structure. The apical part of the 5' domain is invisible in some states of *C. thermophilum* 90S, suggesting that this part is integrated into the 90S structure later than other domains (Cheng et al. 2019).

The 5' ETS is the most prominent spacer in pre-rRNA and contains 700 nucleotides (nt) in *S. cerevisiae*. In the 90S structure, the 5' ETS folds into a highly branched structure and associates with U3 snoRNA and numerous proteins. Previous functional studies of 5' ETS are limited, involving the U3 snoRNA-binding sites, the A1 and A0 sites and a few large regions (Musters et al. 1990; Beltrame et al. 1994; Beltrame and Tollervey 1995; van Nues et al. 1995; Venema et al. 1995; Dutca et al. 2011; Marmier-Gourrier et al. 2011). The functional importance of many other individual structural elements of the 5' ETS is not known. Here, we conduct a systematic mutational analysis of 5' ETS on a plasmid-expressed pre-18S RNA and examine how the rRNA processing and 90S assembly are affected. Our study reveals the functional regions of 5' ETS and provide new insights into the assembly hierarchy of 90S.

RESULTS

Mutational analysis of 5' ETS

To determine the functional element of 5' ETS, we mutated a pre-18S RNA fragment that encompasses 5' ETS, 18S and part of ITS1 (Fig. 1). The pre-18S RNA is transcribed by RNA polymerase II from a plasmid and can be processed into 18S rRNA and assembled into functional 90S preribosomes (Nogi et al. 1991; Liang and Fournier 1997; Zhang et al. 2016). The pre-18S RNA additionally contains a tandem MS2-coat protein binding sequence (MS2-tag) at the 5'-end, a neutral sequence at the 18S region (18S-tag) and a tobramycin-binding aptamer (TOB) sequence at ITS1. These sequences allow for distinguishing plasmid-derived rRNAs from chromosome-derived rRNAs by Northern hybridization.

The secondary structure model of *S. cerevisiae* 5' ETS was initially proposed based on prediction and structure probing data (Yeh and Lee 1992). The current model of 5' ETS is based on cryo-EM structures of 90S (Barandun et al. 2017; Chaker-Margot et al. 2017; Sun et al. 2017) and contains ten helices, termed as H1 to H10 here, and two hybrid helices with U3 snoRNA (Ha and Hb) (Fig. 2). The A0 site and H10 are not visible in cryo-EM structures of 90S.

FIGURE 1. Construct and processing pathway of pre-18S RNA. The pre-18S RNA construct spans the transcription start site to position 239 of ITS1 and is expressed under a GAL7 promoter from plasmid pLM104. The location of hybridization probes are indicated for three plasmid-specific sequences: a MS2 coat protein binding motif at the 5' end of 5' ETS, a neutral sequence (18S-tag) inserted between positions 232 and 233 of 18S, and a tobramycin-binding aptamer (TOB) between positions 152 and 153 of ITS1. By analogy to processing of 23S pre-rRNA, the pre-18S RNA is expected to be sequentially cleaved at the A0, A1, A2, and D sites, yielding 22S-, 21S-, and 20S-like intermediates.

FIGURE 2. Secondary structure model and functional map of 5' ETS. The model is based on cryo-EM structure of 90S and secondary structure prediction. The ten helices of 5' ETS are termed H1 to H10. The four intermolecular helices between pre-18S and U3 snoRNA are termed Ha to Hd. The A0 and A1 sites are marked. Parts of 18S RNA (blue) and U3 snoRNA (red) are displayed. The sequence of 5' ETS is color-shaped according to their functional importance for 18S rRNA processing. 18S/pre-18S > 16, gray; 8–16, yellow; 3–8, orange; < 3 red; blue, minimal length required.

We made a series of deletions that cover almost the entire region of 5' ETS and assessed the processing of plasmid-derived 18S rRNA by northern blot analysis (Fig. 3). If deletion of one region affected 18S rRNA processing, smaller segments were often subsequently analyzed to narrow down the responsible region. The deletions were originally designed on a working secondary structure model of 5' ETS, but the results will be discussed in the latest model.

The 35S pre-rRNA is normally processed at A0, A1, and A2 sites to yield a 20S pre-rRNA. When cleavage at the A0 site is inhibited, the 35S pre-rRNA could be cleaved at the A3 site without prior cleavage at the A0–A2 sites, generating a 23S pre-rRNA. The pre-18S RNA terminates between the A2 and A3 sites and is similar in structure with the 23S pre-rRNA. The pre-18S RNA is expected to be processed at the A0–A2 sites, generating intermediates equivalent to 22S, 21S, and 20S pre-rRNAs (Fig. 1). As cleavages at the A1 and A2 sites are generally coupled, the 21S pre-rRNA is a minor species. Northern blot analysis showed that the wild-type pre-18S RNA was processed to 18S rRNA, as expected (Fig. 3A, lanes 1,14,27,39,45). In addition, a processing intermediate was detected by the TOB probe that hybridizes to a site between the D and A2 sites and likely corresponds to the 20S pre-rRNA, which is the most abundant intermediate during normal 18S rRNA processing. Notably, the intermediate was present at much

lower levels (~30%) compared to the primary transcript and even invisible for some samples. This suggests that the A0 cleavage is rate-limiting for processing of the plasmid-derived pre-18S RNA. In contrast, the 20S pre-rRNA is the most abundant intermediate during the processing of chromosome-derived 18S rRNA and cleavage at the D site is rate-limiting in this case.

The reduction of 18S rRNA correlated with the decrease of the intermediate in most cases, suggesting that the 5' ETS mutants primarily affected 5' ETS processing at the A0 and A1 sites. In a few mutants (lanes 34,36), 18S rRNA was greatly reduced, yet the intermediate was accumulated, suggesting that the steps downstream 5' ETS processing are also affected. In a mutant of H6 (lane 34), a new species migrated between pre-18S and 20S and likely represented a degradation product of pre-18S.

Effect of 5' ETS mutation on 18S rRNA processing

H1–H2

The truncation of H1 and its linker to H2 completely blocked the production of 18S rRNA (Fig. 3A, lane 2). To further map the functional element of this region, part of H1 (lanes 3–5) or the entire H1 (lane 6) were deleted, which caused no apparent processing defect. In contrast, deletion of the linker between H1 and H2 (lane 7) abolished

H2 is essential. The deletion of various parts of H2 moderately affected the 18S production (lanes 9–13), indicating that H2 is largely dispensable.

Removal of the upper stem of H3 did not affect the level of 18S rRNA (lane 15), whereas the deletion of the 5' (lanes 16,17) or 3' arm (lane 18) of the lower stem of H3 greatly reduced the production of 18S rRNA. These data indicate that the lower stem of H3 is critical. Consistently, 18S rRNA was significantly reduced when the entire H3 and part of H2 (lane 19) or H2 and the 5' lower part of H3 (lane 20) were deleted.

Removal of the 3' lower part of H3 alone yielded a medium level of 18S rRNA (lane 18) and additional deletion of the entire H4 further reduced the level of 18S rRNA (lane 21), suggesting that H4 is important for 18S processing. To examine whether the helical structure or sequence of H4 is important, 9 nt in the 5' arm of H4 were mutated to complementary sequences (Fig. 3C). The mutation significantly decreased the level of 18S rRNA (lane 22). The processing defect could be fully rescued by the compensatory mutation at the 3' arm of H4 that restored the base-pairing interaction (lane 23). Thus, the helical structure of H4 is important for 18S processing.

The 5' ETS forms two intermolecular helices Ha and Hb with U3 snoRNA, which are critical for 18S rRNA processing (Beltrame et al. 1994; Beltrame and Tollervay 1995; Dutca et al. 2011; Marmier-Gourrier

et al. 2011; Clerget et al. 2020). Consistently with the previous studies, the 18S rRNA production was abolished when the helical structure of Ha was disrupted by substitution of the first U3 binding site of 5' ETS to complementary sequences (lane 24). A residual amount of 18S rRNA persisted upon mutation of the second U3 binding site on

the 5' ETS (lane 25). In contrast, no 18S rRNA was produced from the previously analyzed Hb mutants (Beltrame and Tollervey 1995). The discrepancy may be related to different pre-rRNA constructs (pre-18S vs. 35S RNA) and introduced sequences. In our Hb mutant, nucleotides 469–471 (CAG) of the mutated 5' ETS have the potential to pair with nucleotides 45–47 (UUG) of U3 (Fig. 3C). Partial disruption of Hb helix was not lethal, but caused a cold-sensitive growth phenotype (Clerget et al. 2020). Nucleotides 461–466, which form an extension of Hb helix with U3 snoRNA (Barandun et al. 2017; Sun et al. 2017), could be replaced with complementary sequences without affecting much the 18S rRNA production (lane 26), indicating that the helix structure of Hb extension is not essential. Consistently, the U3 sequence forming the Hb extension is also not required for growth and pre-rRNA processing (Clerget et al. 2020).

H5

Removal of the sequence between H4 and H6 stopped the 18S rRNA processing (lane 28). Additional deletion analyses showed that the 5' half (nt 293–309) of this region is essential (lane 29), but the 3' half (nt 310–339) is not required (lane 30). Moreover, mutation of nt 293–300 also eliminated the production of 18S rRNA (lane 31), suggesting that these sequences are critical for contacting 90S and/or maintaining the short helical structure of H5.

H6

Deletion of H6 prevented the processing of 18S rRNA (lanes 32,33). Substitution of seven nucleotides in the 5' arm of H6 to complementary sequences greatly reduced the level of 18S rRNA and the processing defect was rescued by compensatory mutations in the 3' arm of H6 that restored the base-pairing interactions (lanes 34, 35). These data indicate that the helical structure of H6 is required for 18S rRNA processing.

H7

Deletion of the major part of H7 greatly decreased the level of 18S rRNA (lane 36). Additional deletion of the 3' lower part of H6 eliminated the 18S rRNA (lanes 37,38). These indicate that the junction between H6 and H7 is also critical.

H8–H10

The level of 18S rRNA was hardly affected when individual or both hairpins of H8 and H9 were deleted (lanes 40–42). The base of H8 and the linker between H8 and Hb were also previously found to be dispensable for 18S rRNA processing (Beltrame et al. 1994). Deletion of 27 nt across the A0 site completely blocked the 18S rRNA processing, indicating that the sequences around the A0 site are important (lane 43). The A0 and A1 sites are separated by a long hair-

pin H10. Deletion of the entire H10 abolished the 18S rRNA production (lane 44). Interestingly, deletions of any of three consecutive regions of H10 were well tolerated (lanes 46–48). Larger deletions with only 16 nt left at either the 5' or 3' arm of H10 were also tolerated (lanes 49,50). Consistently, previous deletion of 18 nt in the tip of H10 did not affect the 18S rRNA production (Venema et al. 1995). We conclude that the hairpin structure of H10 is dispensable, but a certain length of sequence (<16 nt) is required at the position of H10.

Effect of 5' ETS mutations on 90S assembly

The 5' ETS is a key constituent of 90S structure and extensively interacts with U3 snoRNA and proteins. To examine how 90S assembly was affected by the 5' ETS mutations, the pre-18S RNA was expressed in a strain where Utp9, a component of UTPA, was fused to a tandem-affinity-purification (TAP) tag. The 90S preribosome assembled on plasmid-derived pre-18S RNA was affinity purified via the MS2-tag of pre-18S RNA and the TAP-tag of Utp9 protein and first examined for the presence of Utp9, Krr1, and Enp1 with western blot (Fig. 4). The three proteins are bound at different times during the stepwise assembly of 90S (Chaker-Margot et al. 2015; Zhang et al. 2016). As a component of the UTPA complex, Utp9 is one of the first proteins bound to the 5' ETS. Krr1 is recruited by the central domain of 18S and Enp1 assembles at the last stage of 90S formation. Some interesting samples were also analyzed by semi-quantitative mass spectrometry to obtain a comprehensive view of 90S composition (Fig. 5).

The wild-type pre-18S RNA was associated with Utp9, Krr1 and Enp1, as expected (Fig. 4, lane 2). The mutants deleted of H1 or part of H10 can be processed into 18S rRNA (Fig. 3) and also bound normally to the three proteins (Fig. 4, lanes 4,20), suggesting that they form functional 90S preribosomes.

Seven mutants that cannot produce 18S rRNA were also analyzed (Fig. 4, lanes 5–18). The binding of Utp9 was abolished by deletion of the linker between H1 and H2 (lane 6) or part of H3 and the entire H4 (lane 10), and greatly reduced by deletion of H2 and H3 (lane 8). As Utp9 was the bait protein used in the second step of purification, whether Krr1 and Enp1 bound these pre-18S mutants was unclear from these experiments. Actually, purification of 90S preribosome by another bait protein Rrp5 showed that the pre-18S mutant deleted of the H1–H2 linker (Δ 81–95) still bound Krr1, but not Enp1 (Fig. 5, lane 3). These mutants apparently disrupt the association of UTPA since the H1–H4 region of 5' ETS recruits the UTPA complex at the beginning of 90S assembly (Hunziker et al. 2016; Zhang et al. 2016).

Deletion of H6, the A0 site or the entire H10 all blocked the production of 18S rRNA (Fig. 3). However, these

FIGURE 4. Western blot analysis of proteins bound to mutant pre-18S RNAs. Wild-type (WT) or mutant pre-18S RNAs were expressed in Utp9-TAP strain. The RNPs assembled on plasmid-derived pre-18S RNAs were purified via the MS2-tag on RNA and the TAP-tag in Utp9. The input (IN) and immunoprecipitated (IP) proteins were resolved in SDS-PAGE and immunoblotted with antibodies against TAP-tag, Krr1, and Enp1. The 18S processing phenotype and the mutated region are indicated for each mutant.

mutations did not disrupt the association of Utp9, Krr1, and Enp1 (Fig. 4, lanes 14,16,18), nor significantly changed the protein profile of 90S (Fig. 5, lanes 8–10). Notably, Enp1 was more abundant in the defective 90S particles compared with the active ones that can process 18S rRNA (Fig. 4, lanes 14,16,18 vs. 2,4,20; Fig. 5, lanes 8–10 vs. 11). As the bait protein Utp9 is one of the earliest assembled proteins, it would copurify both early partially assembled 90S particles that lack Enp1 and fully assembled 90S particles. The 90S particles assembled on the inactive pre-18S mutants cannot progress further and are mostly trapped in fully assembled states, which would increase the relative level of Enp1 in the samples.

The binding of some individual proteins appeared to be affected. Faf1 was missing in the H6 mutant particle (Fig. 5, lane 8). As Faf1 directly contacts H6, among other factors, in the 90S structure (Fig. 6C), its association to 90S may be weakened by deletion of H6. The level of the RNA helicase Rok1 was reduced upon deletion of the A0 site or H10 (Fig. 5, lanes 9,10). Rok1, the A0 site, and H10 are all invisible in the determined 90S structures. The data suggest that Rok1 may be located near the A0 site and H10. However, the previous RNA–protein UV crosslinking study did not reveal any interaction of Rok1 with the 5' ETS (Martin et al. 2014). Rok1 mainly crosslinks the extension segment 6 of 18S rRNA, which correlates its role in snR30 release (Bohnsack et al. 2008). The three 5' ETS mutants appear to disturb the assembly of individual proteins without overly disrupting the 90S structure.

Interestingly, deletion of H5 did not affect the binding of Utp9 and Krr1, but abolished the association of Enp1 (Fig. 4, lane 12). Mass spectrometry analysis of the H5 mutant 90S particle revealed severe assembly defects (Fig. 5, lane 5). Among the 5' ETS factors, UTPA, UTPB, U3 snoRNP, Bud21, and Utp11 were bound at normal levels, whereas Utp7, Mpp10, Imp3, Imp4, Sas10, Sof1, Fcf2, and Fcf1/Utp24 were absent. Most of the 5' domain factors

and central domain factors were present, although Lcp5 and Bfr2 had low abundance. The labile factors that are normally released in the mature 90S (Fig. 5, lanes 12,13) were more accumulated in the H5 mutant 90S. Remarkably, all the late factors and Emg1 were totally missing. Emg1 was considered as a 3' major domain factor (Zhang et al. 2016), but should be better assigned as a late factor since its initial association to the 18S-1634 fragment is rather weak (Zhang et al. 2016) and its binding is synchronized with that of the late factors in various 90S particles (see further evidence below). The profile of the proteins recruited by the 18S region was quite similar for the H5 mutant 90S and a previously characterized immature 90S particle assembled on an incomplete pre-18S RNA (18S-1634) (Fig. 5, lane 7). Overall, the H5 mutant 90S is defective in the 5' ETS assembly and arrested in an immature state before the last stage of 90S assembly.

We also analyzed the protein composition of 90S upon disruption the Ha or Hb helix formed between 5' ETS and U3 snoRNA. In the Ha mutant (Fig. 5, lane 4), only the UTPA and UTPB complexes were associated to the 5' ETS. The Ha mutant blocked the binding of U3 snoRNP since the U3-specific protein Rrp9 and the U3 snoRNA were absent (Fig. 5, lane 4; Supplemental Fig. S1). The small amounts of Nop1, Nop56, and Nop58 likely originated from other box C/D snoRNPs involved in modification. Utp10, a component of UTPA complex, was missing, suggesting that it is weakly associated to UTPA when the 5' ETS particle is incompletely assembled. Utp10 is also substoichiometric when the UTPA complex alone associates with short fragments of 5' ETS (Zhang et al. 2016). Disruption of Hb did not affect the binding of UTPA, UTPB, U3 snoRNP, Bud21, Utp11, and Utp7, yet considerably reduced the levels of Mpp10, Imp3, Imp4, Sof1, Fcf2, and Fcf1 (Fig. 5, lane 6; Supplemental Fig. S1). Despite different assembly degrees at the 5' ETS, the Ha and Hb mutants, like the H5 mutant, showed a

FIGURE 5. Heatmap of AFs in 90S preribosome. 90S preribosomes assembled on WT or mutant pre-18S RNAs were affinity purified via the MS2-tag in RNA and the TAP-tag in a bait protein Utp9, Noc4, Rrp5, or Efg1. These samples are ordered roughly by their assembly degree. The samples in lanes 7, 12, and 13 were previously reported and included for comparison (Zhang et al. 2016). The sample in lane 7 (18S-1634) was derived from a pre-18S fragment ending at position 1634 of 18S. The sample in lane 12 was purified via MS2-tag and Noc4-TAP and stands for a mature and unprocessed 90S particle with most liable factors released. The sample in lane 13 was chromosome-derived 90S purified via Noc4-TAP. The relative abundance of each protein is color-coded by normalized RSAF value. Sample 1 is normalized to Efg1, samples 2 and 3 to Krr1, and the other samples to UTPB proteins. Proteins in magenta are the labile factors that are weakly associated to the mature 90S.

similar protein profile at the 18S region: The 5' domain and central domain factors were bound, whereas the late factors were totally absent.

Analysis of the Ha, Hb, and H5 mutants showed that the 5' domain and central domain can assemble even when the 5' ETS particle is incompletely assembled. However, these data cannot assess the contribution of UTPA and UTPB to the assembly of 18S domains as both complexes are still bound in the three mutants. To further study the assembly dependence of individual 18S domains, we purified a particle formed on the pre-18S mutant deleted of the linker between H1 and H2 (Δ 81–95) by using Rrp5 as the bait protein, which binds at the central domain (Fig. 5, lane 3). The deletion of H1–H2 linker is expected to disrupt the binding of UTPA to the 5' ETS (Fig. 4, lane 6). The particle was devoid of all 5' ETS factors and late factors, yet contained most AFs bound to the 5' and central domains and a Nop6 protein transitively bound to the 3' major domain. This finding indicates that the initial binding of UTPA is crucial for the association of all 5' ETS factors, and the 5' domain and central domain can assemble independently of 5' ETS assembly.

We further purified a particle assembled on a 5' domain sequence (residues 1–435 of 18S) using Efg1 as the bait protein (Fig. 5, lane 1), which transitively associates with the 5' domain (Shu and Ye 2018). Efg1, Bud22 and C/D snoRNP proteins were enriched, yet the other 5' domain factors were missing, suggesting that the 5' domain sequence was only marginally assembled. In contrast, in the presence of an intact 5' ETS, the 5' domain sequence is capable of recruiting all 5' domain factors (Zhang et al. 2016). This indicates that the 5' ETS particle helps assembly of 5' domain. Analysis of the isolated central domain using Rrp5 as the bait protein identified most AFs bound to the central domain (Fig. 5, lane 2), suggesting that the isolated central domain can assemble independently.

DISCUSSION

We have investigated the mutational effect of 5' ETS on 18S rRNA processing and 90S assembly. The analyzed pre-18S RNA is a mini-reporter construct transcribed by Pol II. Transcription of pre-rRNA by Pol II could disturb the morphology of the nucleolus and obviate the requirement of an AF (Nogi et al. 1991; Lafontaine et al. 1998). Because the assembly pathways of the SSU and LSU are largely independent, the pre-18S can be processed into functional 18S rRNA and the 90S particle assembled from pre-18S possesses a similar composition with chromosome-derived 90S (Liang and Fournier 1997; Zhang et al. 2016), the mini-reporter system likely recapitulates the major aspects of 18S rRNA processing. Nevertheless, we found that the 5' ETS is processed very slowly for the pre-18S, leading to over-accumulation of the primary transcript to other processing intermediates. A complete 35S

FIGURE 6. 5' ETS structure in 90S preribosome. (A) Structure of 5' ETS in 90S. The most to least important regions are colored red, orange, yellow, and gray. The Cα atom of residue at position -7 to the A1 site is displayed as a magenta sphere. U3 snoRNA is colored in green. The A0 site and H10 are not modeled in 90S structure and shown as a dotted line connecting H9 and the A1_-7 site. The other components of 90S are shown as semitransparent background. The model is combined from two 90S structures (PDB codes 5WLC and 5WYJ). (B) 90S structure illustrating the interaction between H1-H3 and UTPA proteins. (C) 90S structure illustrating the assembly of H5, H6, H8, and H9. The unmodeled A0 site and H10 are shown as a dotted line. UTPB proteins are marked as B.

pre-rRNA transcribed by Pol II can be processed with normal kinetics with 20S pre-rRNA as the most abundant intermediate (Henry et al. 1994). It appears that artificial splitting of ITS1 in pre-18S reduces the rate of 5' ETS processing. Due to the reduced kinetics of processing, the effect of 5' ETS mutation might be more severe to the 18S miniconstruct than a complete rDNA unit. The assembly pathways of the SSU and LSU may still have some degree of coupling. A few proteins, like Rrp5 (Eppens et al. 1999; Lebaron et al. 2013), involved in assembly of both subunits, may play a role in the coupling.

Mutations around the A1 site have been shown to cause substantial heterogeneity of 5' end of 18S rRNA (Sharma et al. 1999). We mapped the 5' end of plasmid-derived 18S rRNA with primer extension (Supplemental Fig. S2). The products of wild-type pre-18S and a few non-essential mutants contained a homogenous and authentic 5' end, indicating accurate A1 cleavage. Whether all 18S rRNAs derived from the 5' ETS mutants are correctly assembled and functional were not addressed in this study.

Functional regions of 5' ETS

The functionally important regions of the 5' ETS have been mapped through systematic mutagenesis analysis. Remarkably, about half (53%) of the 5' ETS sequence, when analyzed individually, is not required for 18S rRNA processing. However, the combination of the neutral mutations may have a synthetic effect. Structurally, the dispensable elements are bound at the surface of 90S structure (H1, H8, H9 and the linker between H5 and H6) or exposed (H2, the top stem of H3, major part of H10) (Fig. 6A). The central part of 5' ETS that encompasses the lower stem of H3 to Hb constitutes the most important functional core. In addition, the linker between H1 and H2 and the sequences around the A0 site are essential. These essential elements, except for the A0 site, are mostly buried inside the 90S structure and hence play more important structural roles than the dispensable elements.

The H1 to H3 region extensively contacts the UTPA proteins in the 90S structure (Fig. 6B). We show that only a

small part of the region, including the linker between H1 and H2 and the lower stem of H3, are critical for 18S rRNA processing. The H1–H2 linker likely makes sequence-specific interactions with its primary binding protein Utp17, as alteration of its sequence identities blocks the 18S rRNA processing. The linker extensively crosslinks with multiple UTPA proteins (Hunziker et al. 2016). The critical role of the H1–H2 linker and dispensability of H2 are consistent with the previously analyzed mutants covering this region (van Nues et al. 1995).

The Ha and Hb helices formed between the 5' ETS and U3 snoRNA have been found to be required for 18S rRNA processing (Beltrame et al. 1994; Beltrame and Tollervey 1995; Dutca et al. 2011; Marmier-Gourrier et al. 2011; Clerget et al. 2020). Our data are consistent with the essential role of the two helices, but also show that disruption of Hb still allows the production of a residual amount of 18S rRNA in the experimental system that we used. The Hb mutant appears to greatly reduce the stability and activity of 90S. Notably, the equivalent Hb helix is not required for 18S rRNA processing in *Xenopus* (Borovjagin and Gerbi 2004). Our data confirm that Ha, but not Hb, is required for U3 snoRNA to associate 5' ETS (Dutca et al. 2011) and reveal different roles of the two helices on 5' ETS and 90S assembly.

We find that the short H5 is critical for 5' ETS assembly. Deletion of H5 prevents the association of a subset of 5' ETS factors. In the 90S structure, H5 fits closely into a space enclosed by Utp7, Utp21 and Utp1 (Fig. 6C). Deletion of H5 may prevent binding of Utp7 to the UTPB complex, which subsequently affects the binding of other 5' ETS factors. There is a network of interactions among the unbound proteins. For instance, Utp7 directly contacts Sof1 and Imp3 in the 90S structure. Mpp10 interacts with Imp3, Imp4, and Sas10 (Lee and Baserga 1999; Sa-Moura et al. 2017). Utp11 and Bud11 are still retained in the H5 mutant 90S because of their interactions with already bound components: Utp11 interacts extensively with Nop1 and U3 snoRNA and Bud21 binds Nop1, Utp11, and Utp4 (a UTPA protein).

The A0 site and helix H10 are not visible in the current 90S structures. We show that the sequences around the A0 site, including a small part of H10, are needed for 18S rRNA processing. Our data do not necessarily imply that A0 cleavage is important because cleavage at both A0 and A1 sites are blocked in the A0 deletion mutant. In fact, A0 cleavage is considered to be dispensable (Venema and Tollervey 1999). The major evidence comes from the identification of a mutant on the A1 site that strongly inhibits A0 processing but has little effect on A1 processing and 18S rRNA production (Venema et al. 1995). The structural and functional role of the A0 site need further study.

Alignment of the 5' ETS sequences from 34 yeasts reveals that the sequence conservation generally correlates

with the functional importance of 5' ETS elements (Supplemental Fig. S3). In the H1–H3 region, the most conserved are the linker sequences connecting H1, H2, H3, and H4, underscoring their important role in binding 90S proteins. The H4–Hb region is well aligned and also important in function. Many base paired nucleotides in H4, H5, H6, and H7 display a pattern of covariation, indicating the conservation of duplex structure. Except for the A0 site, the H8–H10 region is highly divergent and also largely dispensable.

Assembly hierarchy of 90S

The assembly order of 90S has been derived by examining the proteins and snoRNAs associated with a series of pre-18S fragments (Chaker-Margot et al. 2015; Zhang et al. 2016). We previously dissected the assembly of 5' ETS into three steps. The first step involves the association of the UTPA complex to the H1–H4 region of 5' ETS. Next, the sequence between Ha and Hb induces binding of a large number of AFs, including UTPB, U3 snoRNP, Bud21, Utp11, Utp7, Mpp10, Imp3, Imp4, Mpp10, Sas10, and Sof1. Fcf2 and Utp24/Fcf1 are recruited at the last step when the 5' ETS is complete. However, the assembly order of a large number of AFs bound at the second step is not resolved. In this study, we identify three mutants of 5' ETS (Ha, H5, and Hb) that exhibit different levels of assembly at the 5' ETS region, providing additional insight into the assembly order of 5' ETS. As shown in the Ha mutant, UTPB is assembled in the absence of U3 snoRNP and other 5' ETS factors, suggesting that UTPB is first assembled at the second step. The protein profiles of the H5 and Hb mutants suggest that U3 snoRNP, Bud21, and Utp11 are next assembled and Utp7, Mpp10, Imp3, Imp4, Mpp10, Sas10, and Sof1 are lastly recruited. The assembly order of UTPB and U3 snoRNP is consistent with the previous study showing that Pwp2 (a UTPB protein) is required for U3 snoRNA to bind the pre-rRNA (Dosil and Bustelo 2004; Perez-Fernandez et al. 2007).

The last stage of 90S assembly involves the binding of late factors and release of labile factors and leads to compaction of 90S structure and acquirement of pre-rRNA processing competence. Although the Ha, H5, and Hb mutants disrupt the assembly of 5' ETS to various degrees, they all block the last stage of 90S assembly. Our data show that a properly assembled structure of 5' ETS is critical for the last stage assembly. The complete loss of all late factors in the 5' ETS mutants further suggests that the last stage of 90S assembly is a highly cooperative event. In contrast to the complete loss of late factors, assembly of the 5' domain and central domain factors are rather independent of 5' ETS assembly. Moreover, the central domain can assemble by itself, consistent with a recent study (Hunziker et al. 2019). We show that the isolated 5' domain is rather incompletely assembled and depends

on the 5' ETS particle or the central domain for stable assembly. A more completely assembled 5' domain was purified by using a different bait protein Esf1 (Hunziker et al. 2019). Esf1 may bind more tightly with the 5' domain fragment than the bait protein Efg1 used here. Recent cryo-EM analysis of *C. thermophilum* 90S suggests that the apical part of 5' domain, which is missing in two determined structures, is stably incorporated into the 90S at a late stage (Cheng et al. 2019). The apical part of 5' domain is likely assembled, but too flexible to be visualized in density. Overall, our data support the modular assembly pathway of 90S. The 5' ETS, the 5' and central domains of 18S rRNA are initially assembled as individual structural domains. At the last stage of assembly, the binding of late factors induces the assembly of 3' major domain and unites individual domains into a compact structure in a highly cooperative manner.

MATERIALS AND METHODS

Strains and plasmids

The UTP9-TAP strain (*Mat a*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*, *UTP9-TAP::HIS3MX6*) was purchased from Open Biosystems. Yeast was manipulated according to standard protocol. Yeast cells were grown in YPDA (1% yeast extract, 2% peptone, 0.003% adenine, and 2% dextrose), YPGA (1% yeast extract, 2% peptone, 0.003% adenine, 2% galactose), Synthetic Complete (SC) medium, and SC dropout medium.

pWL184 is a *URA3* multicopy plasmid that encodes a rDNA fragment gene terminating at position 293 of ITS1 between the *GAL7* promoter and terminator elements (Liang and Fournier 1997). The plasmid contains a unique 24-nt sequence (18S-tag) after position 232 of 18S rRNA, which can be used to distinguish plasmid- and genome-derived 18S. To create pLM104, two copies of MS2 coat protein binding sequence (MS2-tag) were inserted into pWL184 at the 5' end of 5' ETS and a tobramycin aptamer sequence (TOB-tag) was inserted after position 152 of the ITS1 with Transfer-PCR (Hoareau-Aveilla et al. 2011). Mutations were introduced into pLM104 with the QuikChange method. All constructs were confirmed with DNA sequencing. pLM104 differs from our previously constructed plasmids where both the MS2-tag and TOB-tag are placed at the 5' end of pre-18S RNA (Zhang et al. 2016). The TOB-tag at the ITS1 can be used to detect all processing intermediates of plasmid-derived pre-rRNA by Northern hybridization. The isolated 5' domain (1–435) and central domain (609–1154) RNAs were derived from the 18S-435 and 18S-1154 plasmids (Zhang et al. 2016), respectively, by removing the 5' sequences.

Purification and mass spectrometry analysis of plasmid-derived 90S

Plasmid-derived 90S preribosomes were purified as previously described (Zhang et al. 2016). Strains with a TAP-tagged AF (Utp9, Rrp5, or Efg1) transformed with an rDNA plasmid were propagated in Ura-deficient SC medium with 2% galactose as

the sole carbon source and then cultured in 6 L of YPGA medium to A₆₀₀ = 1 at 30°C. The cells were collected, resuspended in 40 mL of MS200 buffer (100 mM HEPES at pH 7.9, 200 mM KCl, 1 mM EDTA, 1 mM DTT, 10 mM β-mercaptoethanol, 0.02% NP-40), frozen in liquid nitrogen and broken with a tissue lyser (Shanghai Jinxin). The lysate was clarified with centrifugation at 20,000g for 40 min. Amylose beads (New England Biolabs) were charged with maltose-binding protein-fused MS2 coat protein (MBP-MS2). The supernatant was incubated with 500 μL of charged amylose beads for 1 h. The beads were washed with 100 mL of MS200 buffer and eluted with 1 mL of MSE buffer (20 mM HEPES-K pH8.0, 110 mM KOAc, 40 mM NaCl and 10 mM maltose). The eluate was incubated with 25 mg IgG-coated Dynabeads (Invitrogen) in binding buffer containing 20 mM HEPES-K (pH 8.0), 110 mM KOAc, and 40 mM NaCl for 30 min. The beads were washed three times with binding buffer and incubated with TEV protease in cleavage buffer (20 mM HEPES-K, pH 8.0, 100 mM KOAc and 5 mM DTT) at 4°C overnight.

Mass spectrometry analysis was conducted as previously described (Chen et al. 2017). The total spectral counts per 100 residues (SCPHR) were calculated for each identified protein and further normalized against reference proteins, yielding the relative spectral abundance factor (RSAF) (Supplemental Dataset 1; Zhang et al. 2016).

Northern blot

Northern blotting was performed as previously described (Zhang et al. 2016). The UTP9-TAP strain harboring an rDNA plasmid was grown at 30°C in YPGA medium to OD₆₀₀ of 0.8–1.0. Total RNA was extracted with hot phenol. RNA (15 μg) was separated in 1.2% agarose-formaldehyde gels and transferred to Hybond N membranes (Amersham). The following probes were used for hybridization: MS2-tag (5'-CGTACCCTGATGGTGTACGCC-3'), 18S-tag (5'-CGCCGAGGATCCAAGTACGGGGCT-3') and TOB-tag (5'-GGCTTAGTATAGCGAGGTTTAGCTACACTCGTGCTGAGCC-3'). Hybridized membranes were exposed to phosphor screen or X-ray films. Hybridization signals were quantified with Quantity One (Bio-Rad). Three data points with invisible pre-18S were not used in the calculation of 18S/pre-18S ratio.

Primer extension

The 5' end of plasmid-derived 18S rRNA was mapped with primer extension as previously described (Chen et al. 2017). Total RNA (5 μg) was mixed with 2 pmol of 5'-³²P-labeled 18S-tag primer. cDNA was transcribed by Superscript III Reverse Transcriptase (Invitrogen). Sequencing ladders were generated with the Sanger dideoxy method on plasmid pLM104. DNA was resolved in a 6% polyacrylamide/6M urea sequencing gel and visualized by phosphor screen.

Western blot

Western blotting was conducted following standard protocol. The TAP tag was detected with peroxidase-anti-peroxidase antibody (1:5000, Sigma). Anti-Krr1 and Anti-Enp1 polyclonal antibodies were raised in rabbit with recombinant proteins.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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