RNA (2002), *8*:786–797. Cambridge University Press. Printed in the USA.

DOI: 10.1017.S1355838202023063

Dynamic conformational model for the role of ITS2 in pre-rRNA processing in yeast

COLETTE A. CÔTÉ,[1] CHRIS L. GREER,[2] and BRENDA A. PECULIS

[1]Genetics and Biochemistry Branch, National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland 20892-1766, USA
[2]Department of Biological Chemistry, University of California, Irvine, Irvine, California 92697-1700, USA

ABSTRACT

Maturation of the large subunit rRNAs includes a series of cleavages that result in removal of the internal transcribed spacer (ITS2) that separates mature 5.8S and 25/28S rRNAs. Previous work demonstrated that formation of higher order secondary structure within the assembling pre-ribosomal particle is a prerequisite for accurate and efficient pre-rRNA processing. To date, it is not clear which specific sequences or secondary structures are required for processing. Two alternative secondary structure models exist for *Saccharomyces cerevisiae* ITS2. Chemical and enzymatic structure probing and phylogenetic comparisons resulted in one structure (Yeh & Lee, *J Mol Biol*, 1990, *211*:699–712) referred to here as the "hairpin model." More recently, an alternate folded structure was proposed (Joseph et al., *Nucleic Acids Res*, 1999, *27*:4533–4540), called here the "ring model." We have used a functional genetic assay to examine the potential significance of these predicted structures in processing. Our data indicate that elements of both structural models are important in efficient processing. Mutations that prevent formation of ring-specific structures completely blocked production of mature 25S rRNA, whereas those that primarily disrupt hairpin elements resulted in reduced levels of mature product. Based on these results, we propose a dynamic conformational model for the role of ITS2 in processing: Initial formation of the ring structure may be required for essential, early events in processing complex assembly and may be followed by an induced transition to the hairpin structure that facilitates subsequent processing events. In this model, yeast ITS2 elements may provide in *cis* certain of the functions proposed for vertebrate U8 snoRNA acting in *trans*.

Keywords: internal transcribed spacer; ITS2; mutagenesis; pre-rRNA; ribosome; RNA structure; rRNA processing; yeast

INTRODUCTION

Maturation of pre-rRNA requires accurate removal of the internal and external transcribed spacer regions from the precursor as it is concomitantly folded, modified, and assembled (if only transiently) with a number of *trans*-acting factors (see Fig. 1A, reviewed in Kressler et al., 1999; Venema & Tollervey, 1999). Little is known about the structural features of the spacer regions that flank and separate the mature rRNA species in the precursor. ITS2 separates 5.8S and 25/28S rRNA in eukaryotes (Fig. 1A). Removal of this spacer begins with an endonucleolytic cleavage at C2, performed by processing components that have not yet been identified. The resulting 3′ extended 5.8S precursor, known as 7S in yeast, is converted to mature 5.8S by a series of 3′ → 5′ exonucleases, many of which are part of the exosome (Mitchell et al., 1996, 1997; van Hoof et al., 2000). The 5′ end of mature 25S is generated by a 5′ → 3′ exonucleolytic activity that can be performed by either Rat1p or Xrn1p (Geerlings et al., 2000).

The transcribed spacers that are removed in generation of the mature rRNAs are essential and play important roles in the processing events. Deletions or mutations within ITS2, complete omission of ITS2, or replacement of ITS2 in *Saccharomyces cerevisiae* with counterparts from other species results in failure to produce mature 5.8S and 25S rRNA (Musters et al., 1990a, 1990b; van der Sande et al., 1992; van Nues et al., 1995; Cote & Peculis, 2001). These findings demonstrate that rRNA processing requires sequences and/or higher order structures within ITS2. One example may be the extensive folding of the ITS2 sequences (see Fig. 1), bringing into juxtaposition those regions in mature 5.8S and 25S rRNA that must interact but that are at a distance in the primary sequence (Peculis & Greer, 1998; Cote & Peculis, 2001).

Reprint requests to: Brenda A. Peculis, NIH/NIDDK, Genetics and Biochemistry Branch, 10 Center Drive, Room 8N322, Bethesda, Maryland 20892-1766, USA; e-mail: bp51h@nih.gov.

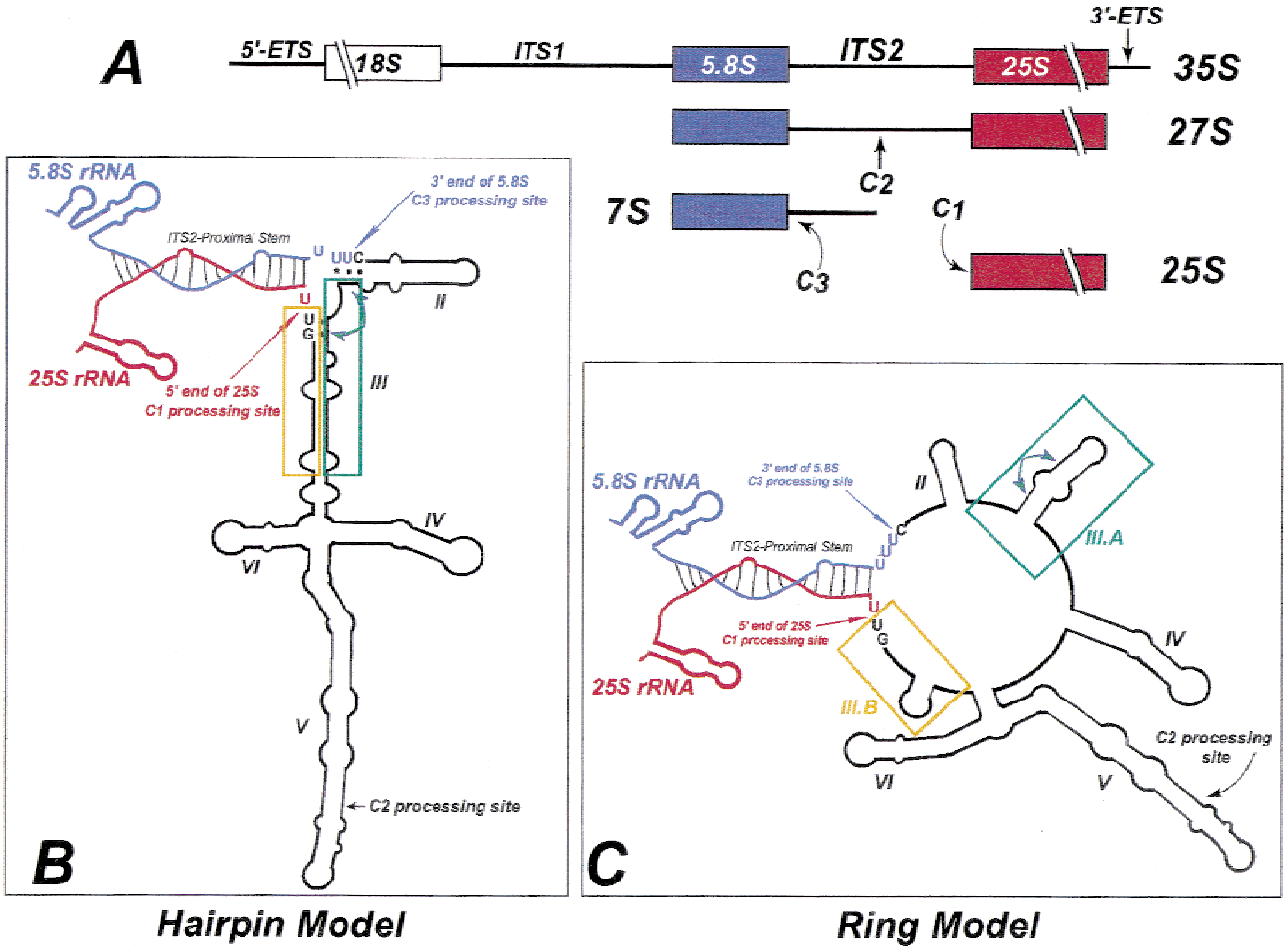

FIGURE 1. Alternative ITS2 structures in *S. cerevisiae*: hairpin versus ring models. **A:** Outline of yeast pre-rRNA structure and processing events relevant to this work. The external transcribed spacers (ETS) and internal transcribed spacers (ITS) must be accurately removed from the 35S precursor and 27S and 7S processing intermediates to yield mature 18S, 5.8S (blue) and 25S (red) rRNA. Within ITS2, the first cleavage occurs at site C2 and is followed by exonucleolytic processing to produce the mature termini at sites C3 and C1. **B:** Diagram of the hairpin ITS2 structural model. Roman numerals identify each of the major structural domains as designated in the model proposed by Yeh and Lee (1990). Orange and green boxes identify the structural elements that provide the focus for this work and that are distinct in the hairpin and ring structural models. The double arrow denotes one site of mutagenesis (mutations C, D, F, G and Y—described in the text) and is used here for orientation purposes. Other symbols are as described in **A**. **C:** Diagram of the ring ITS2 structural model (Joseph et al., 1999). Roman numerals in black indicate structural domains with identical counterparts in the hairpin model. Colored numerals indicate those structural domains that are the focus of this work and unique to the ring model (outlined by orange and green boxes). Other symbols are as described in **A** and **B**.

A proposed secondary structure for ITS2 in *S. cerevisiae* was determined experimentally, based primarily upon minimum free energy modeling in combination with structural mapping by chemical and enzymatic methods (Yeh & Lee, 1990). This yielded an extensively base-paired structure consisting of a series of hairpins (Fig. 1B, the hairpin model). This structure is supported by both genetic and phylogenetic analyses; the ITS2 sequences for other yeasts can also be folded into this tightly base-paired "hairpin" structure [*Saccharomyces rosei* and *Hansenuli wingei* (van der Sande et al., 1992), *K. lactis* and *Kluyveromyces lactis marxianus* (van Nues et al., 1995), and *Candida* (Lott et al., 1998)].

Possible ITS2 secondary structures have been proposed for other organisms based on mapping sequences on the secondary structure of yeast or by phylogenetic comparisons with another closely related organism. Examples include structures proposed for trematodes (Morgan & Blair, 1998), nematodes (Chilton et al., 1998; Hung et al., 1999), insects (dipterans; Wesson et al., 1992; Fritz et al., 1994) and plants (angiosperms; Hershkovitz & Zimmer, 1996) and green algae (Bakker et al., 1995). A common theme among these structures is the presence of an extended series of hairpins analogous to that proposed for yeast.

Although the hairpin model was well supported in yeast and a few other species, an examination of ITS2 sequences in other eukaryotes demonstrated that not all ITS2 sequences could form this extensively base-paired structure. Computer modeling and folding based

on conserved ITS2 sequence among diverse organisms was used to propose an alternative structure that appeared to be consistent across mammals, fish, plants, and dipterans (Michot et al., 1983; Hershkovitz & Zimmer, 1996; Chilton et al., 1998; Morgan & Blair, 1998; Hung et al., 1999; Joseph et al., 1999). This structure consisted of a series of extended hairpins (frequently four independent domains) radiating from an open central core or ring (the "ring model"; see Fig. 1C for yeast structure). The proposed ring structure differs from the previous hairpin model primarily in the ITS2 regions adjacent to the mature 5.8S and 25S ends (indicated by green and orange boxes, respectively, in Fig. 1). It was recently shown that yeast ITS2 sequences can be folded into a form more consistent with the evolutionarily conserved ring model (Joseph et al., 1999; Fig. 1C).

Because our previous results suggested that the higher order structure of yeast ITS2 may be critical to pre-rRNA processing, it is imperative to discriminate between the two alternative structural models that exist for this region. Using a functional genetic assay, we have shown that the ability of ITS2 to form the ring structure is required for recognition by the processing machinery, but that formation of the hairpin structure affects the efficiency of processing. These data suggest that ITS2 in *S. cerevisiae* may be a dynamic structure, flexing between two alternative folding patterns. The relevance of this finding to the proposed role for U8 snoRNA in vertebrates is discussed.

RESULTS

Previously we used a functional genetic assay consisting of a tagged rDNA plasmid in yeast to identify elements of sequence or structure essential for pre-rRNA processing (Peculis & Greer, 1998; Cote & Peculis, 2001). The assay has two key features. The first is a plasmid-borne copy of a single yeast rDNA gene (Nogi et al., 1991b) containing a unique sequence tag (Musters et al., 1990a) fused to an inducible RNA polymerase II promoter (GAL7; Nogi et al., 1991a). The second is a yeast strain containing a temperature-sensitive mutation in a subunit of polymerase I (Nogi et al., 1993). This combination allows direct measurement of processing for transcripts unique to the plasmid-borne gene in yeast transformants shifted to the restrictive temperature (37 °C) under inducing conditions (+ galactose). The efficiency of processing of the plasmid-borne rRNA transcripts can be determined by northern blot analysis using a 5′-end-labeled oligonucleotide complementary to the unique tag present in the plasmid-encoded 25S rRNA (Peculis & Greer, 1998; Cote & Peculis, 2001). Using this assay, we demonstrated previously that formation of the ITS2 proximal stem, formed by an interaction between the 3′ end of 5.8S and the 5′ end of 25S, was a prerequisite for efficient processing in vivo (Peculis & Greer, 1998). Further, we identified critical structural elements within the ITS2 proximal stem required for precursor stability and processing (Cote & Peculis, 2001). These studies demonstrate that correct formation of structures within the mature rRNA are prerequisites for pre-rRNA processing and that few if any sequence-specific elements are required near the termini of mature rRNA.

Comparisons of ITS2 mutants in the two structural models

The use of a genetic approach to identify ITS2 elements required for processing is significantly complicated by the existence of two distinct and equally viable models for the structure of this region. This can be illustrated by considering some of the existing ITS2 mutations in light of each model.

One set of ITS2 mutations is part of a collection of constructs produced in our previous studies that were focused on the identification of essential elements within the mature rRNA termini (Cote & Peculis, 2001). Constructs designated C, D, F, and G (see Fig. 2) were designed to test proposed base-pairing interactions between the mature rRNA termini and ITS2 sequences predicted in the hairpin model (the only structure available in yeast at the time these mutants were designed). Constructs C and D alter the sequence and in some cases the structure of the proposed 5.8S interaction, whereas F and G similarly alter the proposed 25S-proximal interaction. Construct Y simultaneously alters both interactions. All of these mutations completely blocked processing (Cote & Peculis, 2001; see Fig. 4, lane 3).

When considered in light of the hairpin model (and in combination with other data; Cote & Peculis, 2001), this result is consistent with a sequence-specific requirement within these ITS2 elements (see Fig. 2). However, a different conclusion would be drawn for the ring model. The two models have many features in common, including the structures of the stems designated II, IV, V, and VI (although the structures at the junctions of these stems differ; see Fig. 1). Although both models are consistent with the results of chemical and enzymatic structure probing (Yeh & Lee, 1990) the two differ extensively in the predicted structure of the segments designated III. In the hairpin model structure, this segment is formed entirely through long-range interactions between 5′ and 3′ termini of ITS2. In the ring model, these long-range interactions are absent and instead, two independent stems (termed III.A and III.B) are formed through local interactions. The segments altered by mutations F and Y described above are part of these long-range interactions in the hairpin model and short-range interactions in the ring model (indicated by blue double arrows in Fig. 1B and C, respectively). The impact of these mutations on stem III.A in the ring model

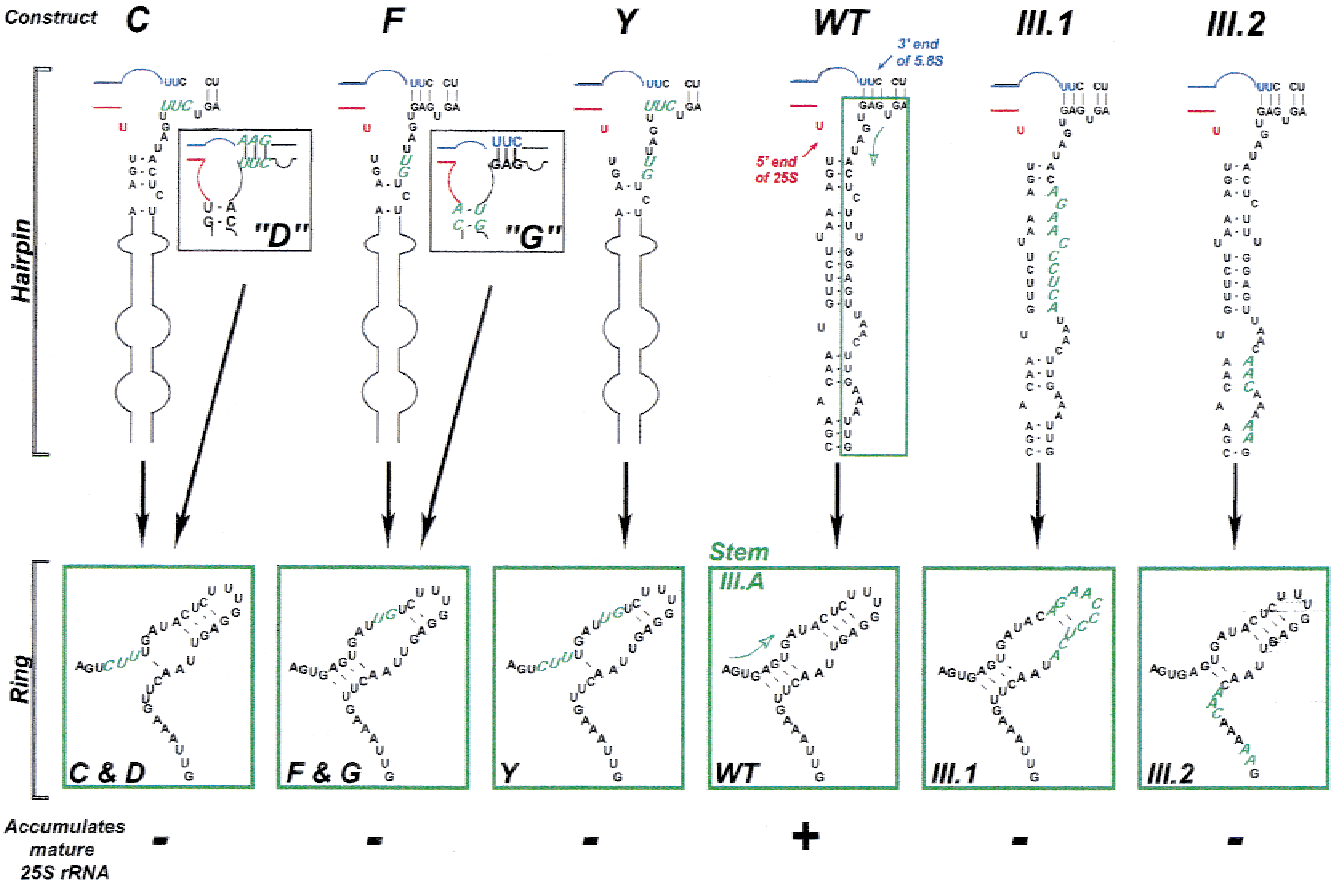

FIGURE 2. Comparison of hairpin and ring structural model contexts for evaluating mutations. Shown are the relevant predicted structures and sequences for wild-type pre-rRNA and several previously described mutations mapped on the structure of stem III of the hairpin model (top) and on stem III.A of the ring structure (bottom). The green arrow shown in the WT diagram (center) indicates the 5′ → 3′ direction and can be used to orient the two structures with respect to one another. For additional orientation see Figure 1. Wild-type sequences are in black text; mutated positions are indicated by green italics. The 3′ end of 5.8S (blue) and 5′ end of 25S (red) are indicated. Results of a series of independent northern hybridization analyses to measure processing are summarized for each of the constructs at the bottom of the figure; + indicates mature 25S rRNA accumulated in this mutant, − indicates little or no detectable accumulation of mature 25S.

is shown at the bottom of Figure 2. In the context of the ring model, these mutations are not sufficient to discriminate between requirements for primary sequence versus secondary structure.

Mutations within ITS2 segment III that block processing (designated III.1 and III.2; see Fig. 2) were described by van Nues et al. (1995). As shown in Figure 2, very different interpretations regarding the structural impact of these mutations would be drawn in the context of these two models.

Thus, although genetic analyses have clearly established a critical role for ITS2 segment III in pre-rRNA processing, the existence of alternative structural models has complicated efforts to identify discrete elements of sequence that are required within this segment. To address this problem, constructs were generated that would distinguish between the structural aspects unique to each model and allow the identification of specific elements required for processing.

Test of hairpin model: Stem III sequence and structure

The first set of mutations were designed to alter the distinctive structures of stem III in the hairpin model while attempting to hold constant the overall structure of the corresponding stem III.A in the ring model (see Fig. 3). Stem III in the hairpin model is formed via a long-range interaction and includes short helices interrupted by three unpaired or bulged segments. Constructs 3A-1 and 3A-3 alter both the sequences of helices and the position and composition of unpaired segments in the context of the hairpin model (see Fig. 3, top). In contrast, these constructs preserve the overall structure while altering only the sequence composition of the corresponding stem (III.A) in the ring model (Fig. 3, bottom). Construct 3A-2 preserves the position and composition of bulges in the hairpin model stem (Fig. 3, top). However, this construct alters the primary se-

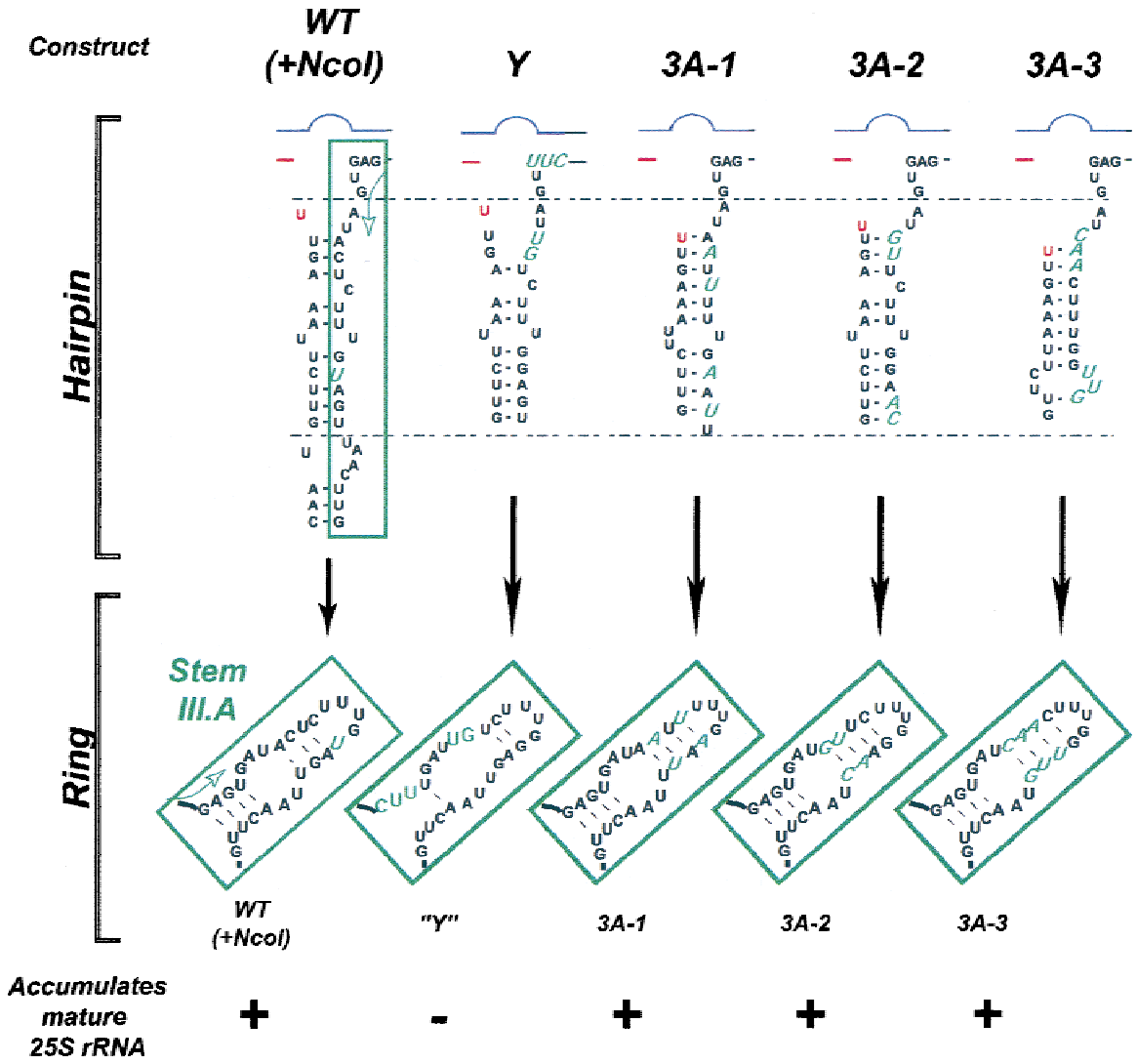

FIGURE 3. Mutations targeted at hairpin stem III sequence and structure. Each construct is shown in the predicted hairpin structure (top half of figure) and ring structure (bottom half) and described in the text. WT(+*Nco*I) is shown on the left for reference. Note this construct contains a point mutation relative to WT at the tip of stem 3A (in green). Wild-type sequences are in black text; mutated positions are indicated by green text. The 3′ end of 5.8S (blue) and 5′ end of 25S (red) are indicated. Results of a series of independent northern hybridization analyses are summarized at the bottom of the figure; + indicates mature 25S rRNA accumulated in this mutant, − indicates little or no accumulation of mature 25S. The green arrow in the WT (+*Nco*I) construct shows the 5′ → 3′ direction and can be used to orient the two structures with respect to one another.

quence of the existing helical segments in both the hairpin and ring model stems (compare Fig. 3, top and bottom). Thus, this latter construct provides a test for sequence-specific requirements within this segment in the context of either model.

Plasmids bearing these constructs were transformed into yeast, and processing of the corresponding transcripts was examined by northern blot analysis of RNA prepared from cells expressing the plasmid-borne rDNA gene (see Materials and Methods). The results are shown in Figure 4, lanes 4, 5, and 6. Production of mature 25S rRNA for each of these constructs was approximately equivalent to that for the tagged, wild-type gene (WT(+*Nco*I), lane 2). Thus, none of these mutations significantly affected processing. Two conclusions may be drawn from these results. First, there are no apparent requirements for primary sequence within the mutated segments of ITS2. This is consistent with the variation in ITS2 sequence in this region observed among various yeast species (van der Sande et al., 1992; van Nues et al., 1995). Second, there are no apparent requirements for the formation of the specific structure of helices and unpaired segments predicted for stem III in the hairpin model. This is in contrast to other mutations (e.g., construct Y; see Fig. 3 and Fig. 4, lane 3) predicted to disrupt sequence and structure of these segments in both the hairpin and ring models.

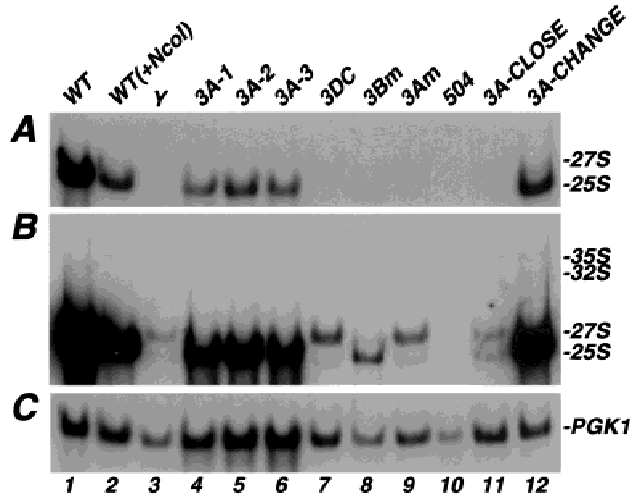

FIGURE 4. Northern hybridization analysis of processing for ITS2 mutations. RNA samples from yeast transformants uniquely expressing plasmid-borne copies of WT and mutated rDNA constructs (indicated at top edge) were prepared for northern hybridization analysis as described in Materials and Methods. The blot was probed with a 5′-end-labeled oligonucleotide complementary to either the unique tag in 25S rRNA (**A** and **B**), or to *PGK1* (**C**), used as a control for sample loading. **B** is an extended exposure of the same filter **A** to allow visualization of products present in low yield. The positions of mature 25S rRNA and its precursors are noted on the right. 504 is the untransformed parental yeast strain that contains no plasmid and represents background hybridization for the tag probe. Constructs are described in the text and shown in Figures 3 and 5.

Although the results for constructs 3A-1, 3A-2, and 3A-3 are consistent with the possibility that formation of the specific structure predicted by the hairpin model is not required for processing, it remained possible that more extensive disruption of the hairpin model stem might block processing. This possibility was examined by constructing a series of mutations (3Bm, 3Am, and 3DC; see Fig. 5) designed to extensively alter the long-range interactions constituting stem III in the hairpin model. The first construct, 3Bm, extensively altered both the sequence and secondary structure predicted in the hairpin model (Fig. 5, top). In the context of the ring model, this construct preserved the overall structure but altered only the primary sequence of stem III.B (Fig. 5, middle) and did not affect stem III.A (Fig. 5, bottom). The second construct, 3Am, is analogous to the first except that the extensive sequence alterations were targeted to the complementary strand in the hairpin model stem III interaction (Fig. 5, top). This second construct left the ring model stem III.B intact (Fig. 5, middle) but altered both the structure and primary sequence of stem III.A (Fig. 5, bottom). The third construct, 3DC, was a double, compensatory mutation in the hairpin structure. This mutation combined the sequence alterations in both 3Am and 3Bm so that, in the hairpin model context, the base-pairing potential of stem III is restored while the primary sequence is extensively altered for both strands (Fig. 5, top). In the ring model context, this double, compensatory mutation preserves the secondary structure of III.B while altering the primary sequence of this stem and both sequence and structure of stem III.A (Fig. 5, middle and bottom, respectively).

Processing of transcripts for each of these three constructs was analyzed by northern hybridization as described above and the results are shown in Figure 4. Although the 3Bm construct extensively disrupts both primary sequence and secondary structure of hairpin model stem III, mature rRNA did accumulate, albeit at levels reduced in comparison to the wild-type-tagged construct (Fig. 4, compare lane 8 and lane 2). This result demonstrated that accurate processing did not require the formation of the stem III structure predicted by the hairpin model. The reduced level of accumulation for this construct raised the possibility that either formation of the hairpin stem III or primary sequence constraints in the ring model stem III.B may be required for efficient processing. Mutations in both the 3Am and 3DC constructs almost completely blocked the formation of mature 25S rRNA and resulted in the accumulation of a small amount of 27S precursor (Fig. 4B, lanes 7 and 9). In light of the results for each of the constructs described above, the defect in processing of these constructs is almost certainly not due to any effect on the proposed hairpin stem III structure. Instead, these constructs (unlike those described above) have in common a disruption of the sequence and structure of ring model stem III.A. Thus, these results are consistent with a requirement for formation of stem III.A, unique to the ring model, for processing.

Test of ring model: Stem III.A sequence and structure

Two further constructs were generated to test the role of stem III.A by altering the predicted structure of this segment in the context of the ring model without altering the structure predicted for hairpin stem III. The first, designated 3A-close, altered the sequence of the unpaired bulge in the center of the ring model III.A segment (Fig. 5, bottom). The sequence replacement was designed to allow pairing across the "bulge," thus, eliminating the unpaired region and creating a continuous, extended helical segment. In the context of the hairpin model, this sequence alteration inserted 1 nt and altered the sequence of an existing unpaired region (Fig. 5, top). The second construct, designated 3A-change, contains five nucleotide changes that altered the sequence of both strands in the unpaired central segment of ring model stem III.A (Fig. 5, bottom). In the hairpin model context, this construct altered the sequence of two separate single-stranded regions (Fig. 5, top).

Processing of transcripts for these two constructs was analyzed by northern blots and the results are shown in Figure 4. Processing for the 3A-change con-

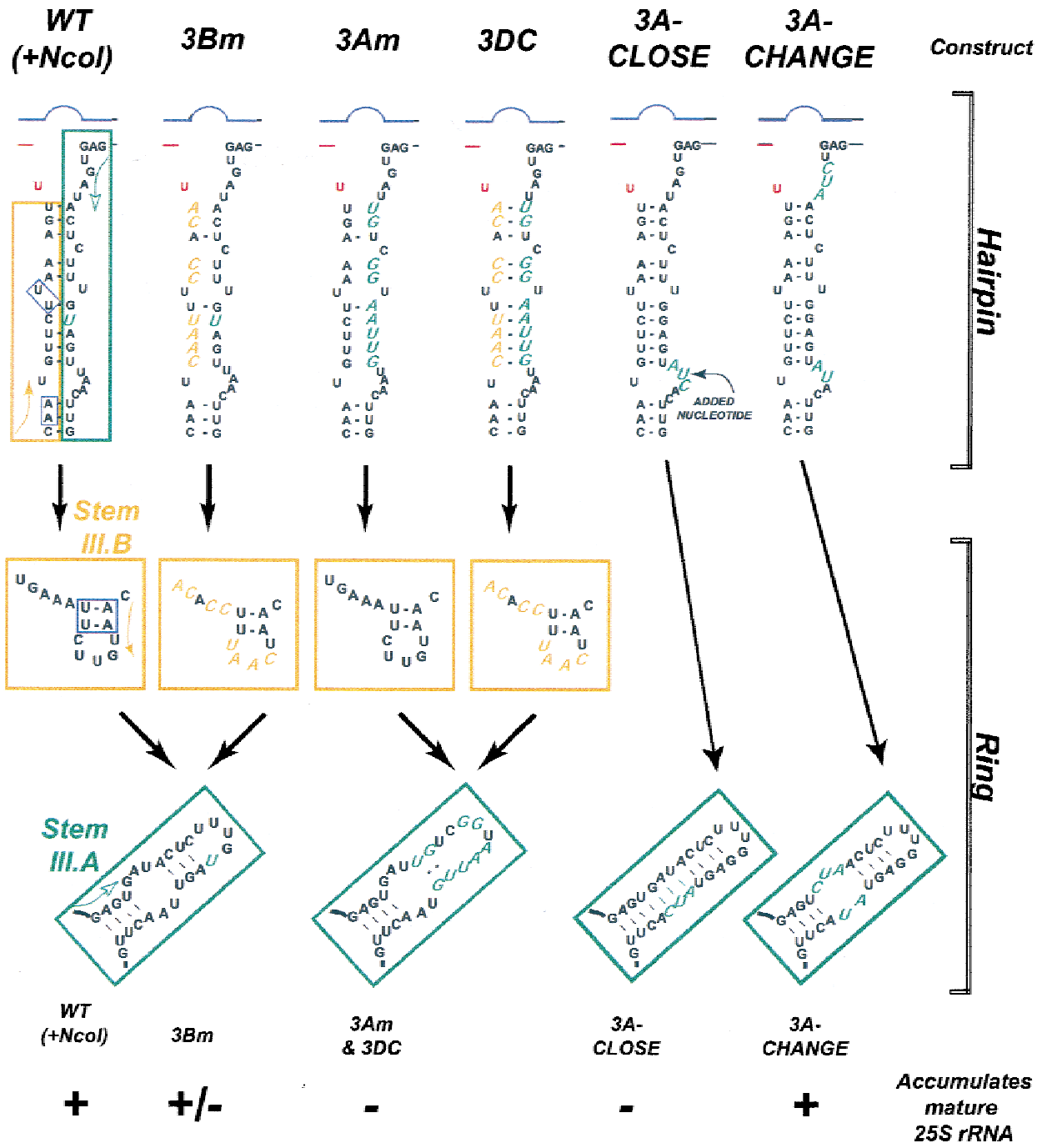

FIGURE 5. Mutations designed to differentiate between hairpin and stem models. Each of the relevant constructs is shown in the predicted hairpin structure (top half of figure) and ring model stem III.A (bottom half) and stem III.B (middle). WT(+*Nco*I) is shown on the left for reference. Wild-type sequences are in black text; mutated positions are indicated by green (in stem III.A) or orange (stem III.B) text. The 3′ end of 5.8S (blue) and 5′ end of 25S (red) are indicated. The results of a series of independent northern hybridization analyses are summarized at the bottom of the figure; + indicates mature 25S rRNA accumulated in this mutant, − indicates little or no accumulation of mature 25S, +/− indicates mutants that did accumulate mature 25S but at a markedly lower efficiency. The green and orange arrows in the WT (+*Nco*I) construct shows the 5′ → 3′ direction; with the blue boxes, these can be used to orient the two structures with respect to one another.

struct was similar to that for the wild-type reference (Fig. 4, compare lanes 12 and 2). This result demonstrates that the primary sequence of this segment, in either the ring or hairpin context, is not required for normal processing. In the 3A-close construct, processing was dramatically reduced relative to the wild-type template with accumulation of only small amounts of mature 25S rRNA (Fig. 4, lane 11). This result is consistent with a requirement for formation of the secondary structure of stem III.A present in the ring model.

Note that the observed differences in processing for the 3A-close and 3A-change constructs cannot be readily accommodated by the predictions of the hairpin model.

Test of ring model: Stem III.B sequence and structure

The processing efficiency of construct 3Bm was quite reduced compared to WT(+*Nco*I). Because both constructs could form complete and intact stem III.A regions, there were two possible explanations for the reduction in processing efficiency. Either there were structural requirements for efficient processing (formation of either a hairpin stem or stem III.B in the ring model) or there were sequence-specific requirements at the 3′ end of ITS2. To distinguish between these various possibilities we generated three additional constructs. Construct 3B3 altered the sequence at the 3′ end of ITS2 in a manner such that the hairpin structure was unaltered. However, in the ring model, the sequence changes in 3B3 prevented the formation of stem III.B (Fig. 6, top and bottom). Construct 3B2 altered four additional nucleotides in this region. Like construct 3B3, 3B2 could not form stem III.B in the ring model. However, the sequence changes in 3B2 prevented much of the base-pairing potential in stem III of the hairpin model. Construct 3B1 altered the same number of nu-

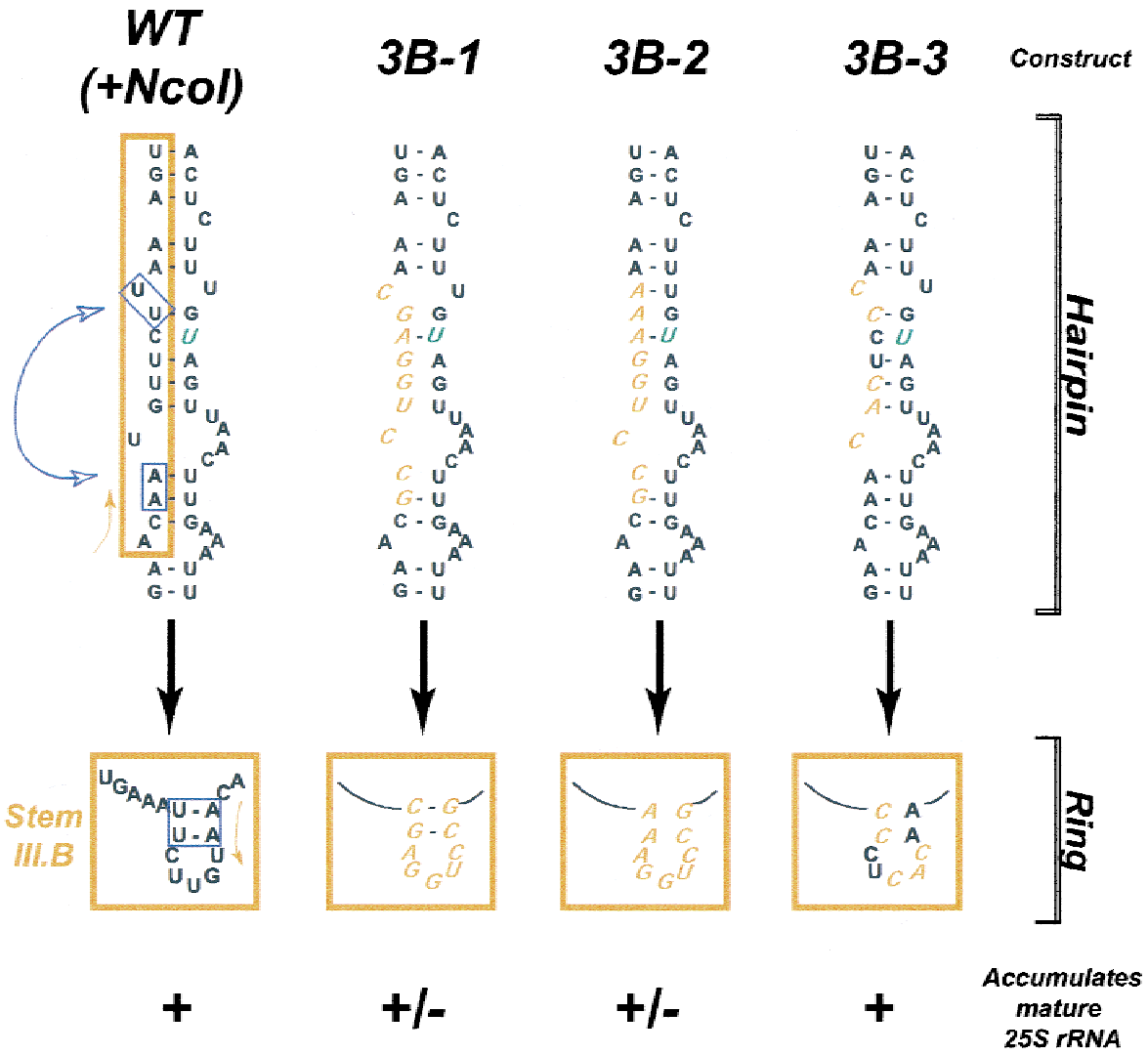

FIGURE 6. Mutations targeted at hairpin stem III versus ring stem III.B requirements. Each construct is shown in the predicted hairpin structure (top half of the figure) and ring model stem III.B (bottom half). WT(+*Nco*I) is shown on the left for reference; note there is one point mutation (shown in green) in this construct relative to WT. Wild-type sequences are in black text; mutated positions are indicated by orange text. The results of a series of independent northern hybridization analyses are summarized at the bottom of the figure; + indicates mature 25S rRNA accumulated in this mutant, +/− indicates inefficient processing of 25S rRNA. The orange arrow in the WT (+*Nco*I) construct shows the 5′ → 3′ direction, and the blue boxes and arrow can be used to orient the two structures with respect to one another

cleotides as in 3B2 and was about equally deficient in its ability to form the hairpin structure. However, in 3B1 the sequence, changes were designed to allow formation of the structure of stem III.B in the ring model.

RNA from each of these constructs was analyzed by northern blot hybridization and the results are shown in Figure 7. Processing for construct 3B-3 was equivalent to that of wild type demonstrating that whereas stem III.A formation is absolutely required (Figs. 2–4), formation of the structure of stem III.B in the ring model is not necessary for processing (Figs. 6 and 7). Processing for constructs 3B2 and 3B1 was equally inefficient. This is a crucial result. Formation of stem III.A in the ring model is absolutely required for processing. Of those constructs which have stem III.A intact, all of the mutations that *specifically destabilize* the secondary structure of the predicted hairpin stem (i.e., constructs 3B2, 3B1, and 3Bm) resulted in reduced but detectable levels of mature 25S rRNA. All of the mutations that *specifically retain* stable base-pairing potential in the predicted hairpin stem, regardless of changes to primary sequence or position and content of paired and unpaired segments (i.e., constructs 3B-3, 3A-change, 3A-1, 3A-2, and 3A-3) resulted in levels of mature 25S comparable to the wild-type construct. These results cannot be readily accommodated by the predictions of the ring model. Instead, the data suggest that the ability to form any stable, paired structure annealing these long-range interactions (comparable to that predicted by the hairpin model) is required for efficient processing. Collectively, our results suggest that both the ring and hairpin model structures play important, albeit distinct, roles in achieving normal levels of processing.

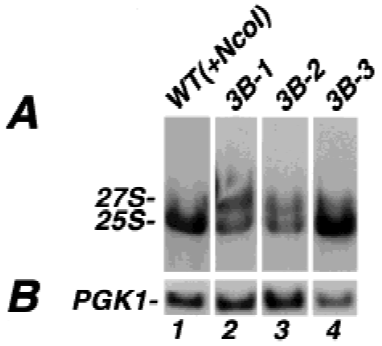

FIGURE 7. Northern hybridization analysis of processing for stem III.B mutations. RNA samples from yeast transformants uniquely expressing plasmid-borne copies of WT and mutated rDNA constructs (indicated at top edge) were prepared for northern hybridization analysis as described in Materials and Methods. The blot was probed with a 5′-end-labeled oligonucleotide complementary to either the unique tag in 25S rRNA (**A**), or to *PGK1* (**B**), used as a control for loading. The positions of mature 25S rRNA and its precursors are noted at the edges of panels. 504 is the untransformed parental yeast strain that contains no plasmid and provides a control for background hybridization by the tag probe. Mutants are described in the text and shown in Figure 6.

DISCUSSION

We have used a functional genetic assay in yeast to identify elements of ITS2 structure recognized by the processing apparatus in *S. cerevisiae*. For this purpose, a series of constructs was generated by site-directed mutagenesis and specifically designed to distinguish between critical structural differences in the two models previously proposed for yeast ITS2. Analyses of these constructs using the functional assay allowed us to differentiate between the alternative structural models and identify those elements that played a role in processing.

Distinct roles for each of the two structural models

We have demonstrated that there is an apparent absolute requirement for formation of stem III.A structure as predicted by the ring model (and precluded by the hairpin model) for processing in vivo by the yeast machinery. Those constructs that preserved intact the structure of stem III.A in the ring model (WT(+*Nco*I), 3A-1, 3A-2, 3A-3, 3Bm, 3A-change, 3B-1, 3B-2, and 3B-3) were processed. All constructs that disrupted or significantly altered stem III.A were not processed.

The results also indicated that formation of the hairpin structure is important for achieving normal levels of processing. Constructs that can form a correct stem III.A in the ring model *and* that can base pair to form the long range interactions comprising stem III in the hairpin model (WT(+*Nco*I), 3A-1, 3A-2, 3A-3, 3A-change, and 3B-3) process efficiently. Constructs that cannot form stem III in the hairpin model can still be processed, but at a lower efficiency; this is evidenced by either an unusually large accumulation of 27S precursor (3B-1, 3B-2) or a dramatically lower level of mature 25S accumulation and unstable precursor (3Bm).

ITS2 structural requirements: A model for processing

The finding that mutually exclusive structural elements of the ring and hairpin models may play distinct roles in processing suggests that a transition between the respective conformations may be important in processing. A dynamic conformational model that can account for all of our current and previous results is outlined in Figure 8A and can be summarized as follows. Initial folding of the pre-rRNA during transcription may favor short-range interactions. Thus, the initial structure assumed by ITS2 upon transcription may be that predicted by the ring model in which short range interactions predominate. Formation of this initial ring structure may promote the association of proteins (perhaps components of the processing machinery) that further stabilize these short-range interactions and initiate formation

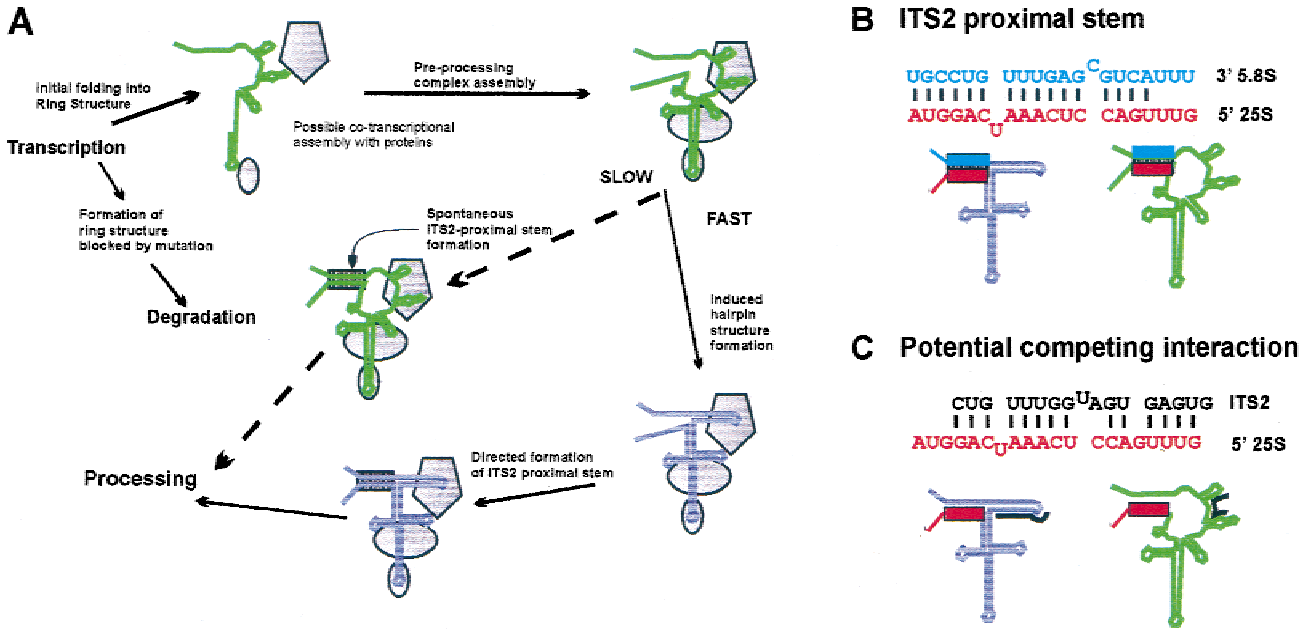

FIGURE 8. Dynamic conformational model for the role of yeast ITS2 in rRNA processing. **A:** Diagram of the dynamic conformational model. Proposed events in the pathway are summarized in clockwise order beginning with transcription at the top left and ending with processing (cleavage at C2 and exonucleolytic degradation to sites C1 and C3) at the lower left. ITS2 in the ring configuration is indicated in green and in the hairpin configuration in purple. Dashed arrows indicate slower steps in the pathway relative to the solid (faster) steps. Gray symbols are intended to indicate possible interacting proteins or protein assemblies (the identities, number, and interaction sites for such components are not known and the symbols shown here are for conceptual clarity only). See text for complete description. **B,C:** The base-paired sequences (top of each panel) and location in the context of the ring (green) and hairpin (purple) structural models (lower portion of each panel) for the ITS2-proximal stem (**B**) and a proposed competing interaction (**C**) are shown for comparison. Bars in the structural figures indicate the positions of each of the sequence segments shown at the top of each panel and are color coded for identification (5.8S, blue; 25S, red; ITS2, black).

of the preprocessing complex. Failure to form the ring structure (e.g., due to mutations that destabilize stem III.A) may prevent further progress in the processing pathway and, instead, result in rapid degradation of the transcript.

Once successfully assembled, the initial, or preprocessing complex, may induce a conformational transition in ITS2 structure (perhaps by destabilizing interactions within stem III.A) resulting in formation of the long-range interactions comprising stem III of the hairpin model. This "zipping up" of the hairpin stem structure may have the effect of bringing into proximity the 5′ end of 25S and the 3′ end of 5.8S rRNA, facilitating formation of the ITS2 proximal stem. This step is critical because formation of the ITS2 proximal stem is a prerequisite for maturation of both 5.8S and 25S rRNAs (Peculis & Greer, 1998). Thus, the model includes initial recognition of the ring structure in assembly of a preprocessing complex, providing a possible quality-control step, followed by an induced conformational change that speeds formation of the essential ITS2-proximal stem. The model also predicts that requirements within the hairpin model stem III may be only loosely constrained; limited to base-pairing potential, regardless of primary sequence or details of secondary structure. In contrast, features of the ring model stem III.A and of common structural elements (e.g., stems II, IV, V, and VI) may be more highly constrained, as they may be recognized directly by the processing apparatus.

Our data indicate that ITS2 sequences that can form a ring structure but are incapable of forming the hairpin can undergo processing, although at greatly reduced apparent efficiency. Because formation of the ITS2 proximal stem is a prerequisite for processing, this suggests there may be a low frequency of spontaneous formation of the ITS2 proximal stem in the ring structure (indicated by dashed arrows in Fig. 8A). This slower rate of ITS2 proximal stem formation would explain the lower efficiency of processing seen in the constructs that cannot form stem III in the hairpin model.

In vivo functions of hairpin structure

In vivo, the hairpin structure may potentially serve two different functions, the net result of which is to facilitate the efficiency of processing. The first is described above and consists of facilitating the formation of the ITS2 proximal stem by bringing the appropriate interacting segments in close proximity. The second may be to prevent the formation of a competing interaction that

could inhibit the formation of the correct structure (illustrated in Fig. 8B,C).

In *S. cerevisiae,* formation of the ITS2 proximal stem involves a long-range base-pairing interaction between the 5′ end of 25S and the 3′ end of 5.8S, shown in Figure 8B. This interaction, composed of mature rRNA sequences, could be formed in the context of either the ring or hairpin model structures (Fig. 8B). However, there is a potential competing base-pairing interaction between the 5′ end of 25S and a sequence within ITS2. Figure 8C shows that this alternative base-pairing interaction between 25S and ITS2 is not quite as strong as the 25S and 5.8S interaction, but nonetheless includes extensive complementarity over nearly the same nucleotides that are required to form the ITS2 proximal stem. The lower panel in Figure 8C shows that the sequence with the potential for forming this competing interaction is located in very different positions within the ring and hairpin model structures. In the ring structure, the competing segment is contained within partially unpaired structure between stems II and III.A and is available for base-pairing interactions. In contrast, in the hairpin model, the competing segment is tied up within a relatively stable, base-paired structure that may preclude the nonproductive or detrimental interaction with the 5′ end of 25S. Notably, the sequence of this region in ITS2 is well conserved across five species of yeast (Joseph et al., 1999) so the possibility of similar nonproductive 25S:ITS2 interactions in other species that also form hairpin structures is also likely.

Comparison of processing in yeast and in vertebrates

A direct comparison can be made between the functions proposed here for yeast ITS2 hairpin stem III acting in *cis* and a proposed functional role for vertebrate U8 snoRNA acting in *trans*. Specifically, it has been proposed that U8 snoRNA in vertebrates may facilitate formation of the ITS2 proximal stem and inhibit the formation of competing structures by forming a transient base-paired interaction (Peculis, 1997). Thus, it is possible that yeast ITS2 stem III and U8 snoRNA have analogous functional roles.

Two types of observations are consistent with this possibility. First, despite extensive database searches, no homolog of vertebrate U8 snoRNA (i.e., an snoRNA with complementarity to the 5′ end of 25S rRNA) has yet been found in yeast. The possibility that U8 function is provided in *cis* by yeast ITS2 stem III could explain this observation.

A second type of observation concerns the differences in potential secondary structure among the fungal and vertebrate ITS2 sequences. The fungal sequences have in common the ability to adopt either the ring or hairpin model structures (Joseph et al., 1999). Notably, the vertebrate sequences examined thus far can adopt the ring structure but appear unable to form a structure analogous to hairpin stem III (Joseph et al., 1999). Thus, the presence of U8 in vertebrates may compensate for the loss of base-pairing potential within ITS2. Alternatively, yeast may have lost a requirement for U8 base pairing by acquiring the potential to form the ITS2 proximal stem.

The evolutionary implications of the presence of U8 snoRNA versus the ability to form the hairpin model structure for ITS2 are intriguing. Our identification of the ring structure as being essential for in vivo recognition or assembly of the processing machinery will facilitate studies identifying the binding sites for some of the 100+ nonribosomal protein factors involved in ribosome biogenesis (reviewed in Kressler et al., 1999; Venema & Tollervey, 1999). In addition, our evidence supporting a conformational transition as a key step in the processing pathway may help differentiate between components functioning early versus later in this process. Overall, this work provides an important framework in which to study the distinct interactions and multiple steps that appear to make up this complex and important processing pathway.

MATERIALS AND METHODS

Plasmid construction

The tagged WT, WT(+*Nco*I), C, and F constructs have been described elsewhere (Peculis & Greer, 1998; Cote & Peculis, 2001). All additional mutations were created by PCR-mediated mutagenesis and cloned essentially as described for WT(+*Nco*I) (Cote & Peculis, 2001) using the WT(+*Nco*I) construct as template and the primer pairs noted below. Construct Y was amplified using primers *4Y* (5′-AAAACCATGG TGTTTGAGCGTCATTTCCTTCTCAAACATTCTGTTTGGTA GTCTTTGATTGTCTTTGGAGTTAAC-3′) and *2a* (5′-GAGA GATCCATGGTGATTTGAGGTCAAACTTTAAG-3′); construct 3A-1 from primers *Z1s* (5′-AAAACCATGGTGTTTGAGCGT CATTTCCTTCTCAAACATTCTGTTTGGTAGTGAGTGATAA TTTTTGAATTTAACTTGAAATTGC-3′) and *2a*; construct 3A-2 from primers *Z2s* (5′-AAAACCATGGTGTTTGAGCGT CATTTCCTTCTCAAACATTCTGTTTGGTAGTGAGTGATG TTCTTTGGAACTAACTTGAAATTGC-3′) and *2a*; construct 3A-3 from primers *Z3s* (5′-AAAACCATGGTGTTTGAGCGT CATTTCCTTCTCAAACATTCTGTTTGGTAGTGAGTGATCA ACTTTGGTTGTAACTTGAAATTGC-3′) and *2a*; construct 3DC from primers *3As* (5′-AAAACCATGGTGTTTGAGCGT CATTTCCTTCTCAAACATTCTGTTTGGTAGTGAGTGATT GTCGGTAATTGTAACTTGAAATTGC-3′) and *3Ba* (5′-AAA ACCATGGTGATTTGAGGTCAATGTGGAAATTGATTGTTC GCC-3′); construct 3Am from primers *3As* and *2a*; 3Bm from primers *3Ba* and *1s* (5′-GGGGGCCATGGTGTTTGAGCGT CATTTCCTTCTC-3′); construct 3A-close from primers *close(s)* (5′-AAAACCATGGTGTTTGAGCGTCATTTCCTTC TCAAACATTCTGTTTGGTAGTGAGTGATACTCTTTGGAG TATCACTTGAAATTGC-3′) and *2a*; and construct 3A-change from primers *change(s)* (5′-AAAACCATGGTGTTTGAGCGT CATTTCCTTCTCAAACATTCTGTTTGGTAGTGAGTCTAAC TCTTTGGAGTATACTTGAAATTGC-3′) and *2a*.

Yeast transformations and growth conditions

The yeast strain NOY504 *MATα rrn4::LEU2 ade2-101 trp1-1 leu2-3,112 his3-11 can1-100* (Nogi et al., 1993) was a generous gift of Dr. M. Nomura. Cells were initially grown at 25 °C in YPD. Yeast cells were made competent essentially as described in Adams et al. (1997). The tagged plasmids were transformed into the NOY504 cells and were grown on SD + glucose plates (Adams et al., 1997) supplemented with the essential amino acids, then transferred to and maintained on SD + galactose plus essential amino acids. Cells were grown in liquid media (SD + galactose) at 25 °C to a density of 0.1 OD_{600}. The cultures were then shifted to 37 °C for 6 h. While at this elevated temperature, the cells continued to divide. Cells were harvested at the end of the 6-h period and total RNA was isolated essentially as previously described (Peculis & Greer, 1998).

RNA preparation

Cells were harvested and total RNA was isolated for northern blot analysis as described (Cote & Peculis, 2001). Pre-rRNA processing (accumulation of 25S rRNA) was detected using a 5′-end-labeled oligonucleotide complementary to the unique tag sequence in the plasmid-encoded 25S rRNA (*TagB*; 5′-ACTCGAGAGCTTCAGTAC-3′). The same blots were probed with *S. cerevisiae* 3-phosphoglycerate kinase (*PGK1*; SGDID #S0000605; 5′-CGAAGGCATCGTTGATGTAAACATCAGCC-3′) as a control for loading.

ACKNOWLEDGMENTS

We are grateful to George Poy, NIH/NIDDK, for deoxyoligonucleotide synthesis and sequencing, and to Masayasu Nomura for the kind gifts of plasmid pNOY102 and yeast strain NOY504. We also thank Vera Nikodem and past and present members of the Peculis lab for helpful discussions and critical reading of the manuscript. This work was supported in part by NIH grant GM35955 (CLG).

Received February 5, 2002; returned for revision March 6, 2002; revised manuscript received March 11, 2002

REFERENCES

Adams A, Gottschling DE, Kaiser CA, Stearns T. 1997. *Methods in Yeast Genetics*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Bakker FT, Olsen JL, Stam WT. 1995. Evolution of nuclear rDNA ITS sequences in the *Cladophora albida/sericea* clade (Chlorophyta). *J Mol Evol 40*:640–651.

Chilton NB, Hoste H, Newton LA, Beveridge I, Gasser RB. 1998. Common secondary structures for the second internal transcribed spacer pre-rRNA of two subfamilies of trichostrongylid nematodes. *Int J Parasitol 28*:1765–1773.

Cote CA, Peculis BA. 2001. Role of the ITS2-proximal stem and evidence for indirect recognition of processing sites in pre-rRNA processing in yeast. *Nucleic Acids Res 29*:2106–2116.

Fritz GN, Conn J, Cockburn A, Seawright J. 1994. Sequence analysis of the ribosomal DNA internal transcribed spacer 2 from populations of *Anopheles nuneztovari* (Diptera: Culicidae). *Mol Biol Evol 11*:406–416.

Geerlings TH, Vos JC, Raue HA. 2000. The final step in the formation of 25S rRNA in *Saccharomyces cerevisiae* is performed by 5′ → 3′ exonucleases. *RNA 6*:1698–1703.

Hershkovitz MA, Zimmer EA. 1996. Conservation patterns in angiosperm rDNA ITS2 sequences. *Nucleic Acids Res 24*:2857–2867.

Hung GC, Chilton NB, Beveridge I, Gasser RB. 1999. Secondary structure model for the ITS-2 precursor rRNA of strongyloid nematodes of equids: Implications for phylogenetic inference. *Int J Parasitol 29*:1949–1964.

Joseph N, Krauskopf E, Vera MI, Michot B. 1999. Ribosomal internal transcribed spacer 2 (ITS2) exhibits a common core of secondary structure in vertebrates and yeast. *Nucleic Acids Res 27*:4533–4540.

Kressler D, Linder P, de La Cruz J. 1999. Protein *trans*-acting factors involved in ribosome biogenesis in *Saccharomyces cerevisiae*. *Mol Cell Biol 19*:7897–7912.

Lott TJ, Burns BM, Zancope-Oliveira R, Elie CM, Reiss E. 1998. Sequence analysis of the internal transcribed spacer 2 (ITS2) from yeast species within the genus Candida. *Curr Microbiol 36*:63–69.

Michot B, Bachellerie JP, Raynal F. 1983. Structure of mouse rRNA precursors. Complete sequence and potential folding of the spacer regions between 18S and 28S rRNA. *Nucleic Acids Res 11*:3375–3391.

Mitchell P, Petfalski E, Shevchenko A, Mann M, Tollervey D. 1997. The exosome: A conserved eukaryotic RNA processing complex containing multiple 3′ → 5′ exoribonucleases. *Cell 91*:457–466.

Mitchell P, Petfalski E, Tollervey D. 1996. The 3′ end of yeast 5.8S rRNA is generated by an exonuclease processing mechanism. *Genes & Dev 10*:502–513.

Morgan JA, Blair D. 1998. Trematode and monogenean rRNA ITS2 secondary structures support a four-domain model. *J Mol Evol 47*:406–419.

Musters W, Boon K, van der Sande CA, van Heerikhuizen H, Planta RJ. 1990a. Functional analysis of transcribed spacers of yeast ribosomal DNA. *EMBO J 9*:3989–3996.

Musters W, Planta RJ, van Heerikhuizen H, Raue HA. 1990b. Functional analysis of the transcribed spacers of *Saccharomyces cerevisiae* ribosomal DNA: It takes a precursor to form a ribosome. In: Hill ADW, Garrett RA, Moore PB, Schlessinger D, Warner JR, eds. *The ribosome: Structure, function and evolution*. Washington, DC: American Society for Microbiology. pp 435–442.

Nogi Y, Vu L, Nomura M. 1991a. An approach for isolation of mutants defective in 35S ribosomal RNA synthesis in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA 88*:7026–7030.

Nogi Y, Yano R, Dodd J, Carles C, Nomura M. 1993. Gene RRN4 in *Saccharomyces cerevisiae* encodes the A12.2 subunit of RNA polymerase I and is essential only at high temperatures. *Mol Cell Biol 13*:114–122.

Nogi Y, Yano R, Nomura M. 1991b. Synthesis of large rRNAs by RNA polymerase II in mutants of *Saccharomyces cerevisiae* defective in RNA polymerase I. *Proc Natl Acad Sci USA 88*:3962–3966.

Peculis BA. 1997. The sequence of the 5′ end of the U8 small nucleolar RNA is critical for 5.8S and 28S rRNA maturation. *Mol Cell Biol 17*:3702–3713.

Peculis BA, Greer CL. 1998. The structure of the ITS2-proximal stem is required for pre-rRNA processing in yeast. *RNA 4*:1610–1622.

van der Sande CA, Kwa M, van Nues RW, van Heerikhuizen H, Raue HA, Planta RJ. 1992. Functional analysis of internal transcribed spacer 2 of *Saccharomyces cerevisiae* ribosomal DNA. *J Mol Biol 223*:899–910.

van Hoof A, Lennertz P, Parker R. 2000. Three conserved members of the RNase D family have unique and overlapping functions in the processing of 5S, 5.8S, U4, U5, RNase MRP and RNase P RNAs in yeast. *EMBO J 19*:1357–1365.

van Nues RW, Rientjes JM, Morre SA, Mollee E, Planta RJ, Venema J, Raue HA. 1995. Evolutionarily conserved structural elements are critical for processing of Internal Transcribed Spacer 2 from *Saccharomyces cerevisiae* precursor ribosomal RNA. *J Mol Biol 250*:24–36.

Venema J, Tollervey D. 1999. Ribosome synthesis in *Saccharomyces cerevisiae*. *Annu Rev Genet 33*:261–311.

Wesson DM, Porter CH, Collins FH. 1992. Sequence and secondary structure comparisons of ITS rDNA in mosquitoes (Diptera: Culicidae). *Mol Phylogenet Evol 1*:253–269.

Yeh LC, Lee JC. 1990. Structural analysis of the internal transcribed spacer 2 of the precursor ribosomal RNA from *Saccharomyces cerevisiae*. *J Mol Biol 211*:699–712.