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Involvement of the Mature Domain in the in Vitro Maturation of *Bacillus subtilis* Precursor 5S Ribosomal RNA†

Bernd Meyhack and Norman R. Pace*

ABSTRACT: A precursor of 5S ribosomal RNA from *Bacillus subtilis* ($p5_A$ rRNA, 179 nucleotides in length) is cleaved by RNase M5, a specific maturation endonuclease which releases the mature 5S rRNA (m5, 116 nucleotides) and precursor fragments derived from the 5′ (21 nucleotides) and 3′ (42 nucleotides) termini of $p5_A$ rRNA. Previous results (Meyhack, B., et al. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75,* 3045) led to the conclusion that recognition elements in potential RNase M5 substrates mainly reside in the mature moiety of the precursor. Limited digestion of $p5_A$ rRNA with RNase T_1 permitted the isolation of a number of test substrates which contained both precursor-specific segments and were unaltered in the immediate vicinity of the cleavage sites, but which differed in that more or less extensive regions of the mature moiety of the $p5_A$ rRNA were deleted. Tests of the capacity of these partial molecules to serve as substrates for RNase M5 indicate clearly that the enzyme recognizes the overall conformation of potential substrates, neglecting only the double-helical "prokaryotic loop" (Fox, G. E., & Woese, C. R. (1975) *Nature (London) 256,* 505).

The maturation of most RNA molecules, both in prokaryotes and in eukaryotes, includes a series of scissions which reduce the chain lengths of precursor RNA molecules to their mature, functional forms. The nucleases which effect these maturation cleavages are highly selective in their action and therefore allow the exploration of mechanisms involved in specific interactions between proteins and polyribonucleotides. Most known RNA precursors are too large to study in the requisite detail. However, the precursors of 5S ribosomal RNA (rRNA) of *Bacillus subtilis* are relatively simple in structure (Pace et al., 1973) and consequently are reasonably amenable to manipulation and structural analysis. Additionally, the endonuclease responsible for the maturation of these precursors has been isolated in substantial purity (Sogin et al., 1977). We have undertaken to define in detail the interactions of this maturation endonuclease with its precursor RNA substrates.

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One immediate precursor of 5S rRNA of *Bacillus subtilis*, termed $p5_A$,[1] is 179 nucleotides in length (Sogin et al., 1976). The specific maturation endonuclease RNase M5 cleaves precursor-specific sequences from the 3′ (F1, 42 nucleotides) and 5′ (F2, 21 nucleotides) termini of $p5_A$ to yield the mature 5S rRNA (m5, 116 nucleotides) (Sogin et al., 1977). RNase M5 consists of two components, α and β, both of which are required for each of the scissions in $p5_A$. Although distant in nucleotide sequence, the two cleavage sites would be juxtaposed if the primary structure of $p5_A$ is folded into the secondary structure proposed by Fox & Woese (1975) as shown in Figure 1.

Since RNase M5 cleaves $p5_A$ rRNA in vitro with complete fidelity, the enzyme must select and act upon its substrate in a highly specific fashion. We recently have shown that the 5′ precursor specific segment of $p5_A$ rRNA contributes to the information utilized by RNase M5 (Meyhack et al., 1977), but only one or at most a few of the precursor-specific nucleotides immediately adjacent to the substrate phosphodiester bonds are involved in this contribution (Meyhack et al., 1978). Thus, the most important cognitive information must derive from the m5 rRNA sequence within the precursor. In this communication we examine the contribution of various domains of the precursor molecule to the RNase M5 interaction. We have isolated and characterized several partial nuclease digestion products of $p5_A$ rRNA, and where necessary reassembled these into test substrates lacking regions of the m5 rRNA component of the precursor. Although one component of the m5 rRNA sequence is not essential, the susceptibilities of the various test substrates to cleavage by RNase M5 suggest that the enzyme inspects the overall superstructure of the precursor as well as the locale of the cleaved bonds.

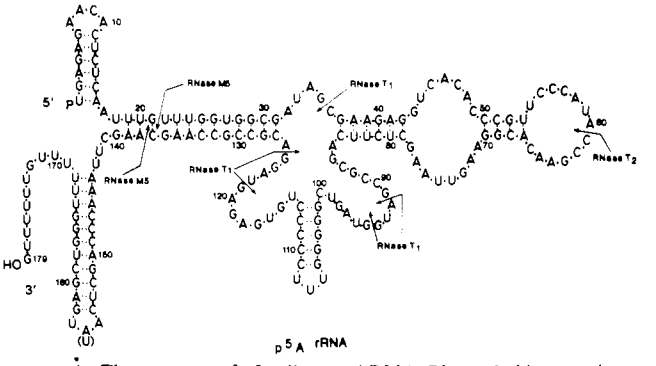

FIGURE 1: The structure of $p5_A$ ribosomal RNA. The probable secondary structure of $p5_A$ (Sogin et al., 1976) is formed by folding complementary bases in the mature part of the molecule as suggested by Fox & Woese (1975). RNase M5 removes the precursor-specific segments F1 and F2 from the 3′ and 5′ termini, respectively; the cleavage sites are indicated by arrows. Partial digestion of $p5_A$ with RNase T_2 results in a single scission at position 60 (Meyhack et al., 1977). Partial digestion with RNase T_1 introduces cuts at positions 35, 91, 94, 121, 125 as indicated (see text).

Materials and Methods

Isolation and Partial Digestion of $p5_A$ rRNA. ^{32}P-labeled $p5_A$ was isolated from chloramphenicol-treated cultures of *B. subtilis* 168 as detailed previously (Sogin et al., 1977). RNase T_1 partial digestion products were generated under conditions similar to those employed by Vigne et al. (1973) with *E. coli* m5 rRNA. ^{32}P-labeled $p5_A$ rRNA in 0.01 M Tris-HCl (pH 7.3), 0.02 M $MgCl_2$, 0.2 M NaCl was incubated at 60 °C for 10 min and cooled slowly (over a 2-h interval) to room temperature. After samples were adjusted to contain 180 μg/mL total *E. coli* tRNA (purified from high molecular weight RNA and 5S rRNA by Sephadex G-100 chromatography), RNase T_1 (Sankyo) was added to a final concentration of 0.9 U/mL and the reaction mixture held at 0 °C for 15 min. Reaction mixtures then were adjusted to contain 0.4% sodium dodecyl sulfate ($NaDodSO_4$), 8 mM Na_2EDTA (pH 7.2) and approximately 10% sucrose, and the products were resolved by electrophoresis through 15% polyacrylamide and 0.15% bisacrylamide in E buffer (Loening, 1967) with a stacking gel of 6% acrylamide and 0.06% bisacrylamide in 25% E buffer. Electrophoresis was carried out in E buffer containing 0.1% $NaDodSO_4$ at 10 V/cm and 4 °C. For denaturing conditions RNA was dissolved in 0.5 mM Na_2EDTA containing 4 M urea and 0.1% $NaDodSO_4$ and resolved on denaturing polyacrylamide slab gels containing 10% acrylamide, 0.5% bisacrylamide, E buffer, and 8 M urea. Gel slices were monitored for ^{32}P content by Cerenkov radiation, and appropriate regions were pooled and eluted by shaking with E buffer containing 0.1% $NaDodSO_4$. RNA was recovered by precipitation from ethanol and dissolved in H_2O.

Fingerprint Analysis of Isolated Digestion Products. Complete digests of RNA by RNase T_1 (Sankyo) were analyzed according to Sanger et al. (1965) with the following modifications. Cellulose-acetate strips (Schleicher and Schuell) saturated with 0.3 M ammonium formate (pH 3.5), 7 M urea, 1 mM Na_2EDTA were run in 0.4 M ammonium formate (pH 3.5), 1 mM Na_2EDTA at 80 V/cm for 30 min. In the second dimension the oligonucleotides were resolved on DEAE paper for 7.5 h at 15 V/cm in 7% formic acid. Oligonucleotides were detected by autoradiography and then excised and monitored for radioactivity.

Reannealing of RNA Sequences from Partial Digestion Products. RNA sequences isolated under denaturing conditions from partial RNase T_1 digestion products were mixed in equimolar amounts in the presence of 3 mM Na_2EDTA (pH 7.3) and 0.43 M NaCl. The mixtures (14 μL) were sealed in 50-μL capillary tubes, held for 5 min at 70 °C, and then allowed to cool slowly to room temperature over a period of 2–3 h.

Maturation Assay. The RNase M5 α and β subunits were purified from *Bacillus subtilis* 168 as detailed previously (Sogin et al., 1977; Meyhack et al., 1977). Standard reaction mixtures (60 μL) contained 10 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 30% (w/v) glycerol, 1 mM dithiothreitol, about 5 μg of RNase M5 subunit β preparation, 0.5 μg of RNase M5 subunit α preparation and substrate. After appropriate incubation at 37 °C, the mixtures or aliquots thereof were chilled on ice and 5 μL of 0.1 M Na_2EDTA, 5% $NaDodSO_4$, and bromphenol blue (as electrophoresis dye marker) were added. The reaction products were separated by electrophoresis through a gel composed of 15% polyacrylamide and 0.15% bisacrylamide in E buffer, overlaid with a stacking gel of 6% acrylamide and 0.06% bisacrylamide in 25% E buffer. Electrophoresis was carried out for 12 h at 8 V/cm at 4 °C to minimize heat accumulation which might dissociate the reannealed products. The slab gels were dried onto Whatman no. 3 MM paper under vacuum at 95 °C. RNA bands were located by autoradiography and, when necessary, excised from the slab for scintillation counting.

[1] Abbreviations used: $p5_A$, precursor 5S ribosomal RNA, species A; m5, mature 5S ribosomal RNA; F1, 42-nucleotide precursor specific fragment released from the 3′ end of $p5_A$ during maturation; F2, 21-nucleotide precursor specific fragment released from the 5′ end of $p5_A$ during maturation. $NaDodSO_4$, sodium dodecyl sulfate.

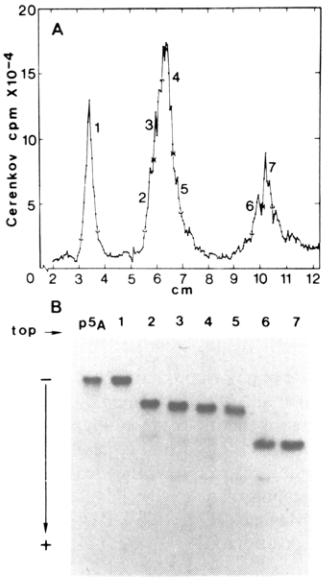

FIGURE 2: Gel electrophoresis of RNase T_1 partial digestion products under nondenaturing conditions. ^{32}P-labeled $p5_A$ was incubated with RNase T_1 as detailed in Materials and Methods. (A) The reaction products were resolved by polyacrylamide gel electrophoresis under nondenaturing conditions. Gel slices were monitored by Cerenkov radiation and extracted in pools as indicated by brackets. (B) The extracted RNA species (1–7) were analyzed on a nondenaturing polyacrylamide gel; their positions are shown on the autoradiogram.

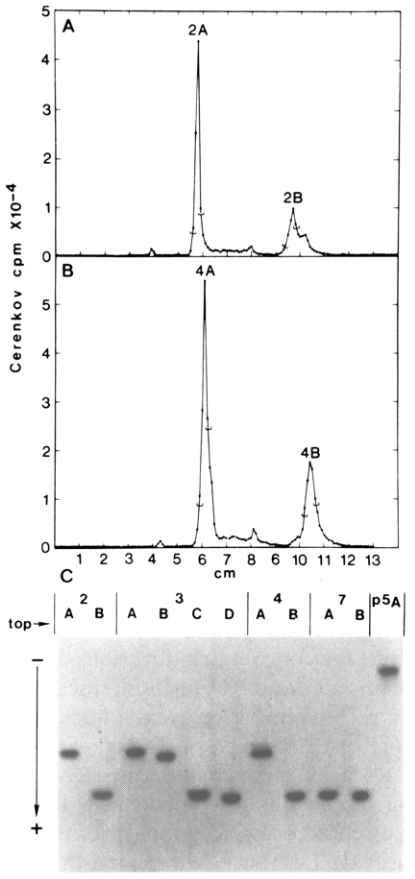

FIGURE 3: Gel electrophoresis of RNase T_1 partial digestion products under denaturing conditions. The noncovalently associated, constituent fragments of the RNA species derived by limited digestion of $p5_A$ were separated by electrophoresis through denaturing polyacrylamide gels containing 8 M urea. Gel slices were monitored by Cerenkov radiation and extracted in pools as indicated by brackets. As representatives, preparative gels for species 2 RNA (panel A) and species 4 RNA (panel B) are shown; the fragments resulting from the preparative denaturation are designated with letters. Panel C shows an autoradiogram of a denaturing, analytical gel to inspect the homogeneity of the various fragments used.

Results

Isolation and Analysis of $p5_A$ Fragments. Partial digestion of $p5_A$ rRNA with RNase T_1 generates fragments suitable for exploring the involvement of certain sequences within the mature (m5) domain of the precursor in the RNase M5–$p5_A$ interaction. As detailed in Materials and Methods, ^{32}P-labeled $p5_A$ was heated to 60 °C in the presence of Mg^{2+} and then slowly cooled, to allow the molecules to assume their most stable conformation. After limited RNase T_1 digestion at 0 °C and in the presence of 0.2 M NaCl to stabilize resident secondary and tertiary structure, the digestion products were separated by polyacrylamide gel electrophoresis under nondenaturing conditions. Figure 2A shows a preparative electropherogram of the products of the digestion and indicates by brackets the gel slices which were pooled for extraction of the various RNA species. The positions of these RNase T_1 partial digestion products upon subsequent gel electrophoresis, again under nondenaturing conditions, are shown in Figure 2B. It is evident that species 1 RNA is of similar size to intact $p5_A$ RNA (179 nucleotides), although it may contain internal nicks which are not revealed under the nondenaturing conditions used during the isolation. RNA species 2–5 are clearly of smaller size than $p5_A$ rRNA, each containing about 150 nucleotides, and species 6 and 7 consist of only about 90 nucleotides. The homogeniety of the RNA bands observed in Figure 2B demonstrates that the isolated species are stable to manipulation, even though they are not covalently intact (see below).

Preparative quantities of RNA species 2, 3, 4, and 7 also were subjected to denaturing polyacrylamide gels, containing 8 M urea; illustrative electropherograms, indicating the pooled regions of the gel, are shown in Figures 3A and 3B. The resulting RNA fragments are designated according to their sizes and species of origin. Figure 3C is an autoradiogram of a subsequent analytical gel, displaying all of the fragments selected for experimental use. RNA species 2, 4, and 7 each release two useful fragments upon denaturation. Species 3 RNA dissociates into four fragments, equivalent in size to those derived from species 2 and 4, and subsequent characterization of these fragments showed species 3 RNA in fact to be a mixture of species 2 and 4.

The exact structures of the various $p5_A$-derived RNA species and their constituent fragments were determined by oligonucleotide fingerprint analysis. As detailed in Materials and Methods, aliquots of the various molecules were digested to completion with RNase T_1 and the product oligonucleotides were resolved by two-dimensional electrophoresis. Representative fingerprints are shown in Figure 4. The molar yields of the RNase T_1 oligonucleotides for all molecules of interest are collected in Table I. We previously have reported the nucleotide sequence analysis of $p5_A$ rRNA from *B. subtilis* (Sogin et al., 1976), so the nucleotide sequences of the various molecules were evident from the fingerprints and molar yields of the oligonucleotides. The regions of the intact $p5_A$ rRNA (Figure 1) which were recovered in the different RNA species are identified in Table II. RNase T_1 primarily cleaves $p5_A$ rRNA at G residues 35, 91, 94, 121 and 125, the most accessible in the molecule. This pattern of limited RNase T_1 digestion is consistent with the m5 rRNA secondary structure proposed by Fox & Woese (1975), in that the accessible G residues are located in ostensibly single-strand regions of the $p5_A$ rRNA (see arrows in Figure 1). However, other G residues

TABLE I: Yield of Products of Complete RNase T_1 Digestion of Selected RNA Components.

oligonucleotide[a]	sequence	RNA components[b] 1	2	2A	2B	4	4A	4B	7	7A	7B
1	U-U-C-C-C-A-U-A-C-C-G-	1.0 (1)	1.0 (1)	1.1 (1)		1.1 (1)	0.9 (1)				
2	C-U-C-U-U-C-A-G-	1.1 (1)	1.2 (1)	1.0 (1)		1.2 (1)	1.0 (1)				
3	U-U-U-C-C-C-C-C-U-G-	1.1 (1)									
5	U-U-A-A-G-	1.0 (1)	1.2 (1)	1.1 (1)		1.2 (1)	1.1 (1)				
6	U-C-A-C-A-C-C-C-G-	0.9 (1)	1.0 (1)	0.9 (1)		1.0 (1)	0.9 (1)				
7	A-U-A-G-	1.1 (1)	1.1 (1)	1.0 (1)		1.2 (1)	1.1 (1)		1.3 (1)		1.1 (1)
8	A-U-G-	1.3 (1)	1.3 (1)	1.1 (1)		0.3 (0)					
9	U-A-G-	2.4 (2)	1.1 (1)		1.3 (1)						
10	A-A-C-A-C-G-	0.9 (1)	0.8 (1)	0.8 (1)		0.9 (1)	0.8 (1)				
11	U-C-G-	0.9 (1)									
12	U-G-	2.1 (2)	1.1 (1)	1.1 (1)		1.1 (1)	1.1 (1)		1.4 (1)		1.1 (1)
13	C-C-A-A-G-	0.9 (1)	0.9 (1)		1.0 (1)	0.9 (1)		1.1 (1)	1.1 (1)	1.1 (1)	
14	A-A-G-	2.2 (2)	2.1 (2)	2.1 (2)		2.4 (2)	2.1 (2)				
15	A-C-G-	0.8 (1)	0.8 (1)		0.9 (1)	0.8 (1)		1.1 (1)	1.0 (1)	1.0 (1)	
16	A-G-	6.3 (6)	4.3 (4)	3.2 (3)	1.0 (1)	4.6 (4)	3.1 (3)	0.8 (1)	3.3 (3)	0.9 (1)	2.1 (2)
17	C-C-G-	1.9 (2)	1.8 (2)	0.9 (1)	1.0 (1)	1.8 (2)	0.8 (1)	1.2 (1)	1.1 (1)	1.0 (1)	
18	C-G-	3.0 (3)	2.9 (3)	3.0 (3)		3.2 (3)	3.0 (3)		1.4 (1)		1.0 (1)
19	G-	13.6 (12)	7.4 (7)	4.0 (4)	3.2 (3)	7.1 (6)	4.1 (4)	2.4 (2)	5.1 (4)	2.4 (2)	1.9 (2)
20	C-U-C-A-A-U-G-	0.9 (1)	1.1 (1)		1.1 (1)	0.9 (1)		1.0 (1)	1.0 (1)	1.0 (1)	
21	C-U-U-A-A-A-C-C-C-A-G-	1.0 (1)	1.1 (1)		1.1 (1)	1.0 (1)		1.2 (1)	1.1 (1)	1.2 (1)	
22	A-A-C-A-C-U-C-U-C-A-A-U-U-U-G-	0.9 (1)	0.9 (1)	0.8 (1)		0.7 (1)	0.9 (1)		0.7 (1)		0.8 (1)
23	U-U-U-U-U-U-G-	0.6 (1)	0.6 (1)		0.8 (1)	0.6 (1)		0.8 (1)	0.6 (1)	0.8 (1)	
24	U-U-U-U-U-U-G_{OH}	0.6 (1)	0.6 (1)		0.8 (1)	0.6 (1)		0.8 (1)	0.6 (1)	0.8 (1)	
25	-U-G-	1.0 (1)	1.3 (1)	1.1 (1)		1.1 (1)	1.0 (1)		1.2 (1)		1.0 (1)
26	U-U-U-G-	1.3 (1)	1.5 (1)	1.3 (1)		1.5 (1)	1.3 (1)		1.5 (1)		1.2 (1)
28	C-A-A-G-	1.0 (1)	1.0 (1)		1.0 (1)	1.0 (1)		1.2 (1)	1.1 (1)	1.1 (1)	
29	C-U-G-	0.9 (1)	0.7 (1)		1.3 (1)	0.8 (1)		0.8 (1)	0.8 (1)	0.9 (1)	

[a] Oligonucleotides are numbered according to Sogin et al. (1976). [b] Molar yields of the various oligonucleotides were calculated from their relative ^{32}P contents (see Figure 4 for representative fingerprints). The expected molar yields, given in brackets, are based on the nucleotide sequence (Table II).

in putative single-strand sequences (e.g., G_{63}, G_{73}) are not available to RNase T_1. These inaccessible residues may be shielded by other components of the $p5_A$ rRNA chain, or they may be involved in "tertiary" hydrogen bonding and therefore not be susceptible to cleavage by RNase T_1.

Substrate Capacities of RNase T_1 Partial Digestion Products and Reannealed Fragments. All of the partially digested RNA species isolated under nondenaturing conditions and selected for study contain the precursor-specific sequences F1 and F2, and the complementary 5′ and 3′ termini of the mature component of the precursor. They differ, however, in that more or less extensive regions of the mature moiety of the precursor are absent. If these deleted sequences indeed are involved in the interaction with RNase M5, their absence should influence the rate of maturation, as measured by the release of the precursor-specific fragments F1 and F2 and the appearance of a "mature", partial molecule. The polyacrylamide slab gel autoradiogram presented in Figure 5 shows the results of such tests. The position of $p5_A$ rRNA is seen in slot 1 of the autoradiogram. Following digestion with RNase M5, the precursor is converted to m5 rRNA, with the release of F1 and F2 (slot 2). Species 1 RNA (slot 3), is cleaved as extensively as $p5_A$ itself (slot 4). Species 2 RNA (slot 5) is a competent substrate for RNase M5, as well (slot 6); the maturation products are F1, F2 and a mature, partial molecule consisting of 89 nucleotides. In contrast, species 4 RNA (slots 7 and 8) and species 7 RNA (slots 9 and 10) are not susceptible to the action of the enzyme. Radioactive regions of the dried polyacrylamide gel were excised and monitored for ^{32}P content; Table III summarizes the relative extents of cleavage of the various substrates during incubation with RNase M5.

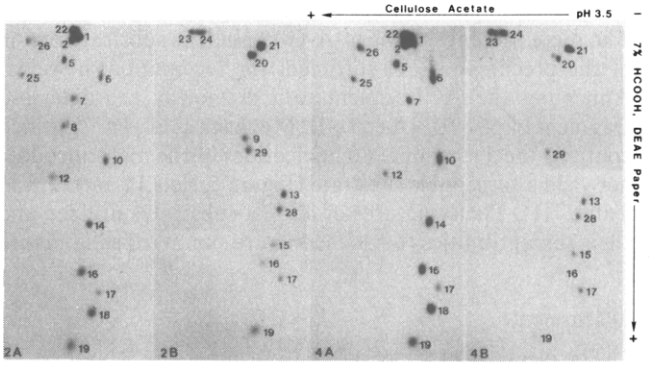

FIGURE 4: Oligonucleotide fingerprint analysis of isolated RNA fragments. The RNA fragments used in these experiments, as isolated from denaturing gels, were digested exhaustively with RNase T_1 and the resulting oligonucleotides were resolved by two-dimensional electrophoresis as detailed in Materials and Methods. The oligonucleotide fingerprints of representative fragments (2A, 2B, 4A, 4B) are shown.

Species 2 RNA is cleaved by RNase M5, even though nucleotides 95–121 are deleted by limited RNase T_1 digestion. This deleted portion of the precursor therefore cannot be important to the RNase M5–$p5_A$ recognition process. However, species 4 RNA, which is only slightly more abbreviated than species 2 RNA (see Table II), is not susceptible to maturation cleavage. Species 4 RNA differs from species 2 RNA in lacking additional residues 92–94 (A-U-G) and 122–125 (U-A-G-G); either or both sequences could be responsible for the loss in substrate capacity. To resolve the importance of these two sequences in the recognition process, we annealed

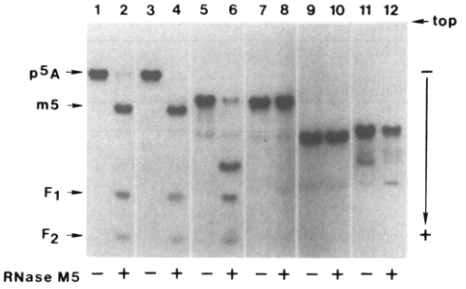

FIGURE 5: Analysis of RNA species susceptibility to RNase M5. Standard maturation assays were carried out as described in Materials and Methods. RNase M5 was included where indicated at the bottom of the figure; (+) RNase M5 included; (−) RNase M5 not present. The maturation products were separated on polyacrylamide slab gels and located by autoradiography. Slots 1 and 2, $p5_A$; slots 3 and 4, species 1 RNA; slots 5 and 6, species 2 RNA; slots 7 and 8, species 4 RNA; slots 9 and 10, species 7 RNA; slots 11 and 12, the product of annealing the 60-nucleotide fragment $p5_A$-II (see text) to fragment 2B. The positions of $p5_A$, m5, F1, and F2 are indicated. Note that species 2, 4, and 7 RNA are of smaller size than $p5_A$; the mature molecule generated by RNase M5 cleavage of species 2 RNA (slot 6) is correspondingly smaller than m5 rRNA. However, the F1 and F2 fragments released during the cleavage are of normal size. Slots 11 and 12 are from a separate gel with slightly different migration rates of the respective RNA constituents.

appropriate fragments isolated from denatured species 2 and species 4 RNAs (see Figure 3) and tested the constructs as substrates for RNase M5, using the gel electrophoresis assay as above. The results are summarized in Table III. Again, reconstituted species 2 RNA (construct 2A/2B) is an excellent substrate for RNase M5, as is the construct lacking the A-U-G element (construct 4A/2B). However, the alternate construct (construct 2A/4B), which lacks the sequence U-A-G-G, is not susceptible to the enzyme. Clearly the region encompassed by the sequence U-A-G-G at positions 122–125 in the $p5_A$ structure is important to substrate recognition by RNase M5. The mere presence of the U-A-G-G plus the substrate region of the precursor is not sufficient for recognition, however. Annealing the 2B fragment to a previously characterized fragment of $p5_A$ rRNA ($p5_A$-II; Meyhack et al., 1977), which contains the 5′-terminal 60 nucleotides of the molecule, does not yield a susceptible substrate (Figure 5, slots 11 and 12; and Table III). The structures of all test substrates utilized and their susceptibilities to RNase M5 are summarized in Figure 6.

Discussion

The cleavage of the 5S rRNA precursors by RNase M5 is a highly specific process. We have tested as RNase M5 substrates a variety of synthetic ribopolymers, including poly(A-U) duplex and duplex RNA from *Penicillium chrysogenum,* which are excellent substrates for RNase III of *Escherichia coli* (Robertson & Dunn, 1975). Additionally, we have examined the capacity of RNase M5 to cleave the immediate precursor of mature 16S rRNA from *B. subtilis* and several tRNA precursors derived from *E. coli* (Schedl et al., 1976) which undergo maturation cleavage by extracts from *B. subtilis.* However, under conditions exhaustive for the cleavage of $p5_A$ rRNA by RNase M5, none of these other potential substrates was acted upon.

The process of substrate cleavage by RNase M5 involves at least two recognition events. The enzyme first must bind the precursor RNA substrate; it then must identify and act upon the two susceptible phosphodiester bonds. Thus far we have been able to examine only the overall reaction, i.e., the capacity of a test substrate to undergo cleavage by RNase M5. In an initial investigation we focused on the contribution of the precursor-specific elements of $p5_A$ rRNA to the recognition/cleavage events. It was possible to construct test substrates lacking either the 5′ or the 3′ precursor segment, and we could show that the 5′ precursor segment was essential for RNase M5 action. However, the most important element of the 5′ precursor segment proved to be only residue G_{21}, which evidently interacts by hydrogen bonding to residue G_{137}, to complete the duplex region within which RNase M5 cleaves (see Figure 1). Thus, although RNase M5 is sensitive to the conformation of nucleotides comprising the substrate bonds, the high specificity of the reaction must derive from information contained within the mature moiety of the precursor.

TABLE II: Nucleotide Sequences of RNase T_1 Partial Digestion Products of $p5_A$ rRNA.[a]

RNA species	sequence	denatured fragments	sequence
1	1–179		
2	1–94/122–179	2A	1–94
		2B	122–179
3	1–94/122–179	3A	1–94
		3C	122–179
	1–91/126–179	3B	1–91
		3D	126–179
4	1–91/126–179	4A	1–91
		4B	126–179
5	1–91/126–179		
6	1–35/126–179		
7	1–35/126–179	7A	126–179
		7B	1–35

[a] Native RNA species (no. 1–7) were isolated as RNase T_1 partial digestion products of $p5_A$ on nondenaturing polyacrylamide gels (see Figure 2). Noncovalently associated fragments of the native RNA species were separated on denaturing polyacrylamide gels (see Figure 3) and labeled by letters. The nucleotide sequences were deduced by fingerprint analysis (see Figure 4 for representative fingerprints). The position of the RNase T_1 cut at G_{94} vs. G_{95} was deduced solely on the basis of the molar yields of GMP in complete RNase T_1 digests of fragment 2A; 4 mol of GMP per mol of fragment 2A suggests cleavage at G_{94}. The cleavage at G_{125} vs. G_{126}, which yields fragment 4B, was deduced similarly.

TABLE III: RNase M5 Cleavage of Test Substrates.[a]

RNA species	construct	conversion (%)	sequence
1		95	1–179
2		75	1–94/122–179
4		5	1–91/126–179
7		0	1–35/126–179
	2A/2B	89	1–94/122–179
	4A/2B	83	1–91/122–179
	2A/4B	2	1–94/126–179
	4A/4B	2	1–91/126–179
	$p5_A$-II/2B	0	1–60/122–179

[a] Native RNA species and constructs were prepared as test substrates for RNase M5 as detailed in Materials and Methods. The nucleotide sequences are as listed in Table II. Standard maturation assays were carried out as described in Materials and Methods. The maturation products were separated on polyacrylamide slab gels, located by autoradiography and excised from the dried gels for quantitative analysis. The substrate capacity of several test substrates for RNase M5 is given in percent molar conversion.

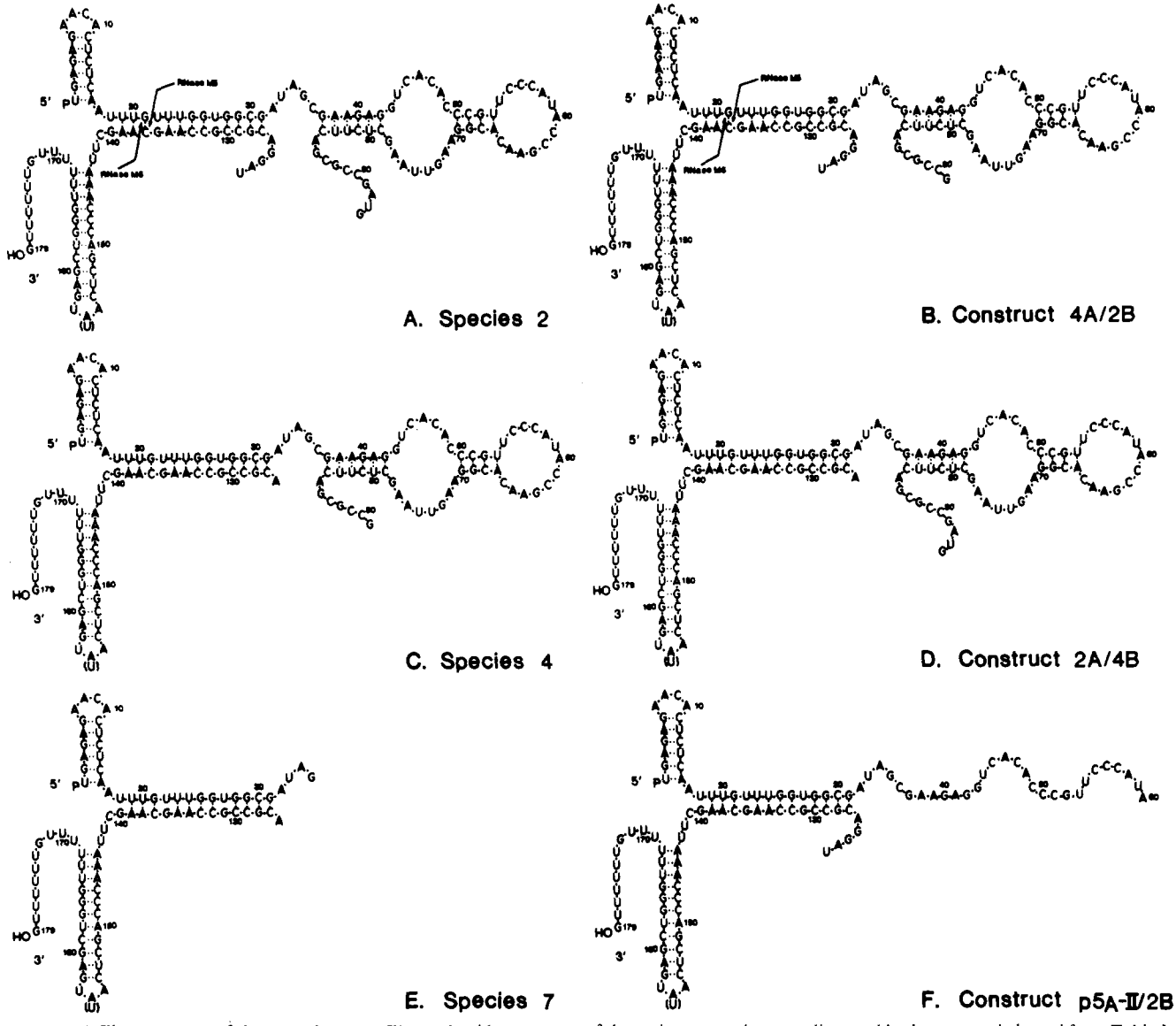

FIGURE 6: The structures of the test substrates. The nucleotide sequences of the various test substrates discussed in the text, as deducted from Table I, are drawn to correspond with the intact $p5_A$ rRNA structure shown in Figure 1.

In the present investigation we have constructed test substrates for RNase M5 which lack various regions of the mature moiety of $p5_A$. As documented in Results, RNase T_1 primarily cleaves $p5_A$ rRNA at G residues 35, 91, 94, 121, and 125. Cleavages at two of these sites or the reannealing of appropriate $p5_A$ fragments generated the test substrates whose structures are shown in Figure 6. Each of these contains both precursor-specific segments, and the primary and secondary structures in the close vicinity of the cleavage sites presumably are unchanged. Since the immediate site of RNase M5 action seemingly is not perturbed, it is likely that the determination of the capacities of the various substrates to undergo maturation is essentially a test for the initial recognition and binding of the test substrates by RNase M5.

Each of the partial $p5_A$ molecules shown in Figure 6 lacks at least nucleotides 95–121. This region includes the short, double-helical region termed the "prokaryotic loop" by Fox & Woese (1975) because of its ubiquity in the 5S rRNA of prokaryotes. Two of the partial $p5_A$ molecules (Figures 6A and 6B) are cleaved by RNase M5, so evidently the prokaryotic loop, a significant structural landmark, is not important for recognition by the enzyme. However, as pointed out in Results, the adjacent sequence, U-A-G-G, at positions 122–125, does have a role in the recognition process. The molecules drawn in Figures 6C and 6D, which are not substrates for RNase M5, differ from the substrates depicted in Figures 6A and 6B only in that they lack this region. The RNase M5 may utilize the nucleotide sequence U-A-G-G as one component of a matrix of requisite information or, more likely, the U-A-G-G participates in some secondary and/or tertiary structural interaction involved in folding the RNA into a conformation acceptable to the enzyme. However, structural features in addition to this U-A-G-G plus the substrate region of the $p5_A$ molecule certainly are required. The construct depicted in Figure 6F, which is not acted upon by RNase M5, contains the U-A-G-G element, but lacks nucleotides 61–121. It seems clear, then, that the recognition of $p5_A$ rRNA by RNase M5 is not solely dependent upon nucleotide sequence information in the locale of the substrate bonds, in a manner analogous to the selection of cleavage sites by most DNA restriction endonucleases (Roberts, 1976). Rather, the RNase M5 apparently recognizes the overall conformation of potential substrates, neglecting the prokaryotic loop. This conclusion is bolstered by experiments in which we have chemically modified residues evidently in-

volved in the tertiary folding of the mature segment of the $p5_A$ molecule, thereby rendering the precursor insusceptible to RNase M5 (L. Ribnik, B. Meyhack, & N. R. Pace, manuscript in preparation).

The substrate requirements of three RNA maturation endonucleases have now been examined in some detail. Besides RNase M5 from *B. subtilis,* these include the *E. coli* enzyme RNase P, which is responsible for one of the maturation steps in tRNA biosynthesis (Robertson et al., 1972) and RNase III, also from *E. coli,* which specifically cleaves the primary transcript of the ribosomal DNA as well as certain mRNA classes (Dunn & Studier, 1973; Nikolaev et al., 1973). At least in the case of RNase P, it seems clear that nucleotide sequences in the immediate vicinity of substrate sites do not play the major role in their recognition by the enzyme. The cleavage points do not display common nucleotide sequences, and mutations in tRNA precursors which abolish or drastically reduce their susceptibility to RNase P most commonly are associated not with the substrate sites, but rather with regions of the precursors which are known to be involved in the secondary and/or tertiary folding of the mature tRNA moiety of the precursor (Smith, 1974; McClain, 1977). As with RNase P cleavage points, the substrate sites for RNase III which thus far have been sequenced do not contain consistently identical or similar nucleotide sequences (Robertson, 1976). All are associated with short double-helical regions of RNA, but features additional to those in the immediate locale of these duplex regions seem to be required by the enzyme (Rosenberg & Kramer 1977). In addition to these processing endonucleases, the aminoacyl-tRNA synthetases have been shown by a variety of studies to have a high dependence upon substrate conformation, rather than simple, localized nucleotide sequences, in their selection of substrates (Rich & Schimmel, 1977). Thus, the rule seems to be emerging that polyribonucleotide structures which are recognized with high specificity by proteins must be envisaged as three-dimensional arrays, with the informational elements aligned in the requisite matrices by the overall conformations of the substrates.

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