Involvement of Precursor-Specific Segments in the in Vitro Maturation of *Bacillus subtilis* Precursor 5S Ribosomal RNA†

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ABSTRACT: In vitro maturation of precursor 5S ribosomal RNA ($p5_A$) from *Bacillus subtilis* effected by RNase M5 yields mature 5S RNA (m5, 116 nucleotides), and 3′ precursor-specific segment (42 nucleotides), and a 5′ precursor-specific segment (21 nucleotides) (Sogin, M. L., Pace, B., and Pace, N. R. (1977), *J. Biol. Chem. 252,* 1350). Limited digestion of $p5_A$ with RNase T_2 introduces a single scission at position 60 of the molecule; m5 is cleaved at the corresponding nucleotide residue. The complementary "halves" of the molecules could be isolated from denaturing polyacrylamide gels. The isolated fragments of $p5_A$ are not substrates for RNase M5, suggesting that some recognition elements can be utilized by RNase M5 only when presented in double-helical form. In exploring the involvement of the precursor-specific segments in the RNase M5–$p5_A$ interaction, substrate molecules lacking the 3′ or 5′ precursor-specific segment were constructed by reannealing complementary "halves" from $p5_A$ and m5 RNA. The artificial substrate lacking the 5′-terminal precursor segment was cleaved very much more slowly than that lacking t′ segment; the 5′ precursor-specific segment therefore contains one or more components recognized by RNase M5 during its interaction with the $p5_A$ substrate.

The 23S, 16S, and 5S ribosomal RNAs of prokaryotes are derived from common RNA transcripts (Pace, 1973), which in *Escherichia coli* are specifically cleaved by RNase III (Dunn and Studier, 1973; Nikolaev et al., 1973; Ginsburg and Steitz, 1975) to yield the immediate precursors (termed p16, p23, and p5 rRNA[1]) of the mature rRNA forms. These precursors accumulate in the absence of protein synthesis, but during normal growth are rapidly converted by scissions and nucleoside modifications to their mature, functional counterparts (termed m16, m23, and m5 rRNA). During the maturation of 16S and 23S rRNA, precursor-specific sequences are removed from both 5′ and 3′ termini (Sogin et al., 1971, 1973; Brownlee and Cartwright, 1971; Hayes et al., 1971; Lowry and Dahlberg, 1971) apparently by specific endonucleases (Meyhack et al., 1974; Hayes and Vasseur, 1976).

Maturation of precursor 5S rRNA in *Bacillus subtilis* is quite analogous to that undergone by the high molecular weight rRNA molecules (Pace, 1973). At least two precursors of 5S rRNA accumulate in *B. subtilis* in the absence of protein synthesis (Pace et al., 1973). One of these, termed $p5_A$, is 179 nucleotides in length. In addition to the nucleotide sequence of m5 rRNA (116 nucleotides), $p5_A$ contains precursor-specific sequences at its 5′ and 3′ termini (Sogin and Pace, 1974). During maturation, both precursor segments are removed by a specific maturation endonuclease, RNase M5. This enzyme consists of two components, termed α and β, both of which are required for each of the scissions in $p5_A$ (Sogin et al., 1977). The nucleotide sequences of the precursor and mature 5S molecules have been determined (Sogin et al., 1976; Marotta et al., 1976), so the exact points of RNase M5 cleavage are known. Although distant in nucleotide sequence, the two cleavage sites are juxtaposed if the $p5_A$ primary structure is folded into its probable secondary structure. As indicated by the arrows in Figure 1, the enzyme makes one cut following nucleotide 21, to release the 5′-terminal precursor-specific fragment F2, and a second scission following position 137, to yield the 3′ precursor-specific fragment, F1. We do not yet know whether the cuts occur sequentially or simultaneously.

RNase M5 must recognize and act upon $p5_A$ rRNA in a highly specific manner. Three structural components of the precursor which possibly serve in the protein–polynucleotide interaction have been discussed (Sogin et al., 1977). These include: (a) the double-helical region in which both cleavage sites are located; (b) elements of twofold rotational symmetry about the substrate phosphodiester bonds (the U-G-A-G-A-G sequence at positions 1–6 and 116–121); and (c) elements of twofold translational ("positional") symmetry (four-nucleotide sequence repeats) about the substrate bonds.

In this paper we report the isolation of partial nuclease digestion products of $p5_A$ and m5 rRNA, the reassembly of these fragments into artificial precursors which lack one or the other precursor-specific terminus, and capacities of such constructs to serve as substrates for RNase M5. The results support the involvement of the duplex region containing the substrate bonds and one of the precursor-specific termini in the interaction with RNase M5.

Materials and Methods

Isolation and Partial Digestion of $p5_A$ and m5 rRNA. ^{32}P-Labeled $p5_A$ or m5 rRNA were isolated from, respectively, chloramphenicol-treated or exponentially growing cultures of *B. subtilis* 168 as detailed previously (Sogin et al., 1977). RNase T_2 partial digestion products were generated under conditions similar to those employed by Vigne et al. (1973) with *E. coli* m5 rRNA. ^{32}P-Labeled $p5_A$ or m5 rRNA dissolved in 0.003 M Na_2EDTA (pH 7.3) were adjusted to contain 0.01 M Tris-HCl (pH 7.3)–0.01 M $MgCl_2$, held at 60 °C

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[1] Abbreviations used are: $p5_A$, precursor 5S ribosomal RNA, species A; $p5_A$-I, 3′-terminal fragment of $p5_A$; $p5_A$-II, 5′-terminal fragment of $p5_A$; $p5_A$-I/$p5_A$-II, reannealed 3′- and 5′-terminal fragments derived from $p5_A$ rRNA; m5, mature 5S ribosomal RNA; m5-I, 3′-terminal fragment of m5; m5-II, 5′-terminal fragment of m5; m5-I/$p5_A$-II and $p5_A$-I/m5-II, reannealed "constructs" from complementary fragments of $p5_A$ and m5 rRNA; DEAE, diethylaminoethyl.

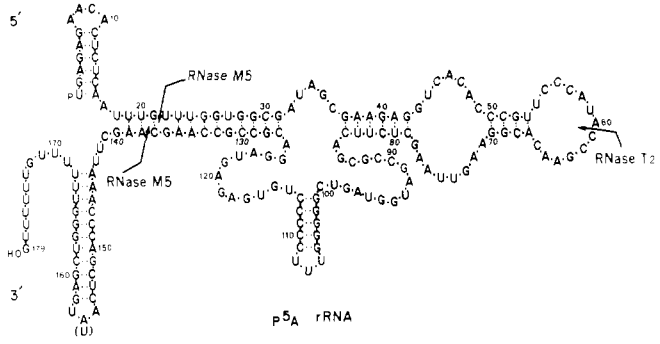

FIGURE 1: Secondary structure of $p5_A$ ribosomal RNA. The probable secondary structure of $p5_A$ (Sogin et al., 1976) is formed by folding complementary bases in the mature segment of the nucleotide sequence into the secondary structure suggested by Fox and Woese (1975). RNase M5, a specific maturation endonuclease, removes the precursor-specific segments F1 and F2 from the 3′ and 5′ termini, respectively; the cleavage sites are indicated by arrows labeled with RNase M5. Partial digestion of $p5_A$ with RNase T_2 results in a single scission at position 60, as indicated. Mature 5S rRNA is cleaved by RNase T_2 at the same position.

for 10 min and cooled slowly (over a 2-h interval) to room temperature. Samples were adjusted to contain 0.05 M Tris-HCl (pH 7.3), 0.02 M $MgCl_2$, 0.2 M NaCl, and 90 μg/mL total *E. coli* tRNA (purified from high molecular weight RNA and 5S rRNA by Sephadex G-100 chromatography). RNase T_2 (Sankyo) then was added to an enzyme:RNA mass ratio of 1:16, and the reaction mixtures were held at 0 °C for 15 min. Digestions were halted by the addition of sodium dodecyl sulfate to 0.1%, Na_2EDTA (pH 7.3) to 0.5 mM, and urea to 4 M, and products were resolved by electrophoresis through cylindrical, denaturing polyacrylamide gels constructed of 10% acrylamide and 0.1% bisacrylamide, containing E buffer (Loening, 1967) and 8 M urea. Gel slices (1 mm) were monitored for ^{32}P content by Cerenkov radiation, and appropriate regions were pooled and eluted by shaking with E buffer containing 0.1% sodium dodecyl sulfate. RNA was recovered by precipitation from ethanol and dissolved and stored in 0.003 M Na_2EDTA (pH 7.3).

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) (in 0.3 M ammonium formate (pH 3.5)–7 M urea–1 mM EDTA) were run in 0.4 M ammonium formate (pH 3.5)–1 mM EDTA at 80 V/cm for 30 min. In the second dimension the oligonucleotides were run on DEAE paper for 3.5 h at 20 V/cm in 7% formic acid. The shorter run in both dimensions allowed two fingerprints to be developed on one sheet of DEAE paper (40 × 80 cm). The separation of the oligonucleotides achieved was as good as obtained with more lengthy electrophoresis. Oligonucleotides were detected by radioautography, and then excised and monitored for radioactivity. Secondary characterization of selected oligonucleotides was carried out by completely digesting the oligonucleotides on the paper with RNase A (50 μg/mL) for 3 h at 37 °C, and resolving the products by electrophoresis on DEAE paper in 0.5% pyridine–5% acetic acid (pH 3.5).

Reannealing of Partial Digestion Products. $p5_A$ and m5 "halves", isolated following partial digestion with RNase T_2, were reannealed in equimolar amounts in the presence of 0.003 M Na_2EDTA (pH 7.3) and 0.43 M NaCl. The incubation mixtures (14 μL) were sealed in 50-μL capillary tubes and incubated for 5 min at 70 °C. The samples were then allowed to cool slowly to room temperature over a period of 2–3 h.

Purification of RNase M5. Enzyme purification was carried out as described by Sogin et al. (1977) with the following modifications. Buffer SB was replaced throughout the procedure by buffer NB, which contained 0.01 M Tris-HCl (pH 7.4), 0.001 M dithiothreitol (Sigma), and 15% (w/v) glycerol. Component β of RNase M5 was eluted from DE 52 cellulose by buffer NB containing 0.3 M NH_4Cl. This fraction was desalted by passage through a Sephadex G50 column equilibrated with buffer NB containing 0.01 M NH_4Cl and loaded onto a column of Ecteola-cellulose (Sigma) equilibrated in NB containing 0.01 M NH_4Cl. β was eluted by NB containing 0.06 M NH_4Cl and stored frozen at −80 °C. Component α of RNase M5 was purified by chromatography on phosphocellulose as described previously (Sogin et al., 1977).

Maturation Assay. Assays were carried out in siliconized 10 × 75 mm glass tubes in an assay buffer consisting of 0.01 M Tris-HCl (pH 7.3), 0.005 M $MgCl_2$, 30% (w/v) glycerol, and 0.001 M dithiothreitol. Reactions generally contained about 5 μg of RNase M5 component β preparation, 0.5 μg of RNase M5 component α preparation, and substrate to yield a final volume of 60 μL. After appropriate incubation at 37 °C, assay tubes or aliquots thereof were chilled on ice and 5 μL of 0.1 M EDTA, 5% sodium dodecyl sulfate, and bromophenol blue (as electrophoresis dye marker) were added. The reaction products were separated by electrophoresis through polyacrylamide slab gels which consisted of 15% acrylamide and 0.15% bisacrylamide, in E buffer overlaid with a stacking gel composed of 6% acrylamide and 0.06% bisacrylamide, in 1:4 E buffer. Electrophoresis was carried out for 12 h at 8 V/cm at 4 °C to minimize heat accumulation and consequent denaturation of reannealed products. The slab gels were dried onto Whatman No. 3MM paper under vacuum at 95 °C. RNA bands were located by radioautography on standard x-ray film and, when necessary, excised from the slab for scintillation counting.

Results

Isolation and Analysis of $p5_A$ and m5 Fragments. During the cleavage of $p5_A$ rRNA by RNase M5 we have not observed the accumulation of reaction intermediates lacking one or the other of the precursor termini. In order to explore the role of the precursor segments in the RNase M5 reaction, it therefore was necessary to construct such "intermediates" artificially. This proved to be possible because of the extensive secondary structural interaction between the 5′ and 3′ regions of m5 and $p5_A$. We could cleave the purified precursor and mature molecules at a single site (Figure 1), denature and isolate the component "halves", and then reanneal precursor with complementary mature fragments to yield test substrates lacking one or the other precursor-specific terminus. Vigne et al. (1973) have determined that, under appropriate conditions, the susceptibility of several prokaryotic 5S rRNA molecules to digestion by RNase T_2 is confined to a common region located about 40 nucleotides from the 5′ ends of the m5 molecules. The $p5_A$ and m5 rRNA molecules isolated from *B. subtilis* displayed analogous sensitivity to digestion by RNase T_2. As detailed under Materials and Methods, ^{32}P-labeled $p5_A$ or m5 were heated to 60 °C in the presence of Mg^{2+} and then slowly cooled, to allow the molecules to assume their optimum conformation. RNase T_2 digestion then was carried out at 0 °C and 0.2 M NaCl to stabilize resident secondary structure. These conditions of digestion resulted in the introduction of a single RNase T_2 cleavage in both $p5_A$ and m5 rRNA. Because of the extensive secondary structure of these RNA molecules, the cleavage was manifest only following denaturation; the resulting RNA fragments therefore were recov-

amide gels containing 8 M urea. Figure 2 shows preparative ered following electrophoresis through cylindrical polyacryl- electropherograms of the products of RNase T_2 digestion of p5A (Figure 2A) and m5 (Figure 2B) rRNA; the RNA components are identified in the figures. It is evident that digestion of $p5_A$ (179 nucleotides) releases two fragments, $p5_A$-I and $p5_A$-II, of lengths (estimated by electrophoretic mobility) of approximately 120 and 60 nucleotides, respectively. The fragments are generated in equimolar yield. Digestion of m5 rRNA (116 nucleotides) generated approximately equimolar m5-I (ca. 80 nucleotides) and m5-II (ca. 40 nucleotides).

Following elution from the preparative gel slices, the original locations of the RNase T_2 generated fragments within the intact molecules were determined by analysis of their nucleotide sequences. As detailed under Materials and Methods, aliquots from the fragment preparations were completely digested with RNase T_1 and the resulting oligonucleotides were resolved by two-dimensional electrophoresis. Radioautograms of the resulting "fingerprints" of $p5_A$ and m5 rRNA and the various RNase T_2 generated fragments are displayed in Figure 3 (A–F). We previously have reported the analyses of m5 (Marotta et al., 1976) and $p5_A$ (Sogin et al., 1976) rRNA from *B. subtilis*, using equivalent electrophoretic procedures to separate RNase T_1 oligonucleotides. Most of the oligonucleotides present in the fingerprints of Figure 3 therefore could be identified by their positions within the patterns of known T_1 oligonucleotides. The quantitative yields of the various oligonucleotides were calculated from their relative ^{32}P contents; molar yields from these analyses are accumulated in Table I. By reference to the known termini of intact $p5_A$ and m5 rRNA, the origins of the fragments derived from the intact molecules were obvious. It is noteworthy that fragment $p5_A$-I (Figure 3B) contains T_1 oligonucleotide 24 (U-U-U-U-U-G_{OH}), the 3′ terminus of $p5_A$ (Figure 3A), and all other oligonucleotide sequences from nucleotide 64 of the precursor RNA to the 3′ end (see Figure 1 for the sequence). Fragment $p5_A$-II yields pU-G- (spot 25), the 5′ terminus of $p5_A$, and all T_1 oligonucleotides to position 52 of the precursor. Oligonucleotide 1 (U-U-C-C-C-A-U-A-C-C-G-), which is present in $p5_A$ (position 53–63), is detectable in neither $p5_A$-I nor $p5_A$-II fragments (Figures 3B and 3C). However, the molar yield of C-C-G- from fragment $p5_A$-I was 3 as compared to 2 for the entire $p5_A$ sequence (Table I). Moreover, T_1 RNase digestion released from fragment $p5_A$-II a novel oligonucleotide, indicated as "X" in Figure 3C. Secondary digestion of oligonucleotide X with RNase A released A-U-, A-, C-, and U-, but no A-C-. Presuming that oligonucleotide X is derived from the missing oligonucleotide 1, the sequence of X must be U-U-C-C-C-A-U-A-. The residuum of the missing oligonucleotide 1, C-C-G-, must be represented by the extra mole of C-C-G- recovered per mole of fragment $p5_A$-I. The scission of $p5_A$ by RNase T_2 therefore must have been at A_{60}, to release the 5′-terminal 60-nucleotide fragment $p5_A$-II and the 3′-terminal 119-nucleotide fragment $p5_A$-I.

Analysis of the RNase T_2 fragments derived from m5 rRNA followed similar logic, with equivalent results. Fragment m5-I contained 1 additional mol of C-C-G- above the 2 mol expected from m5 rRNA (Table I) and RNase T_1 released from fragment m5-II a novel oligonucleotide, Y (Figure 3E), which proved to be identical with oligonucleotide X from fragment $p5_A$-II. The $p5_A$ and m5 molecules therefore are cleaved at identical positions by the mild RNase T_2 digestion, to yield, in the case of the m5 rRNA, a 5′-terminal 39-nucleotide fragment (m5-II) and a 3′-terminal 77-nucleotide segment (m5-I). The susceptibility of the cleaved bond to RNase T_2 digestion is consistent with the secondary structure drawn in Figure 1; that phosphodiester bond is located in a single-strand region of the molecule.

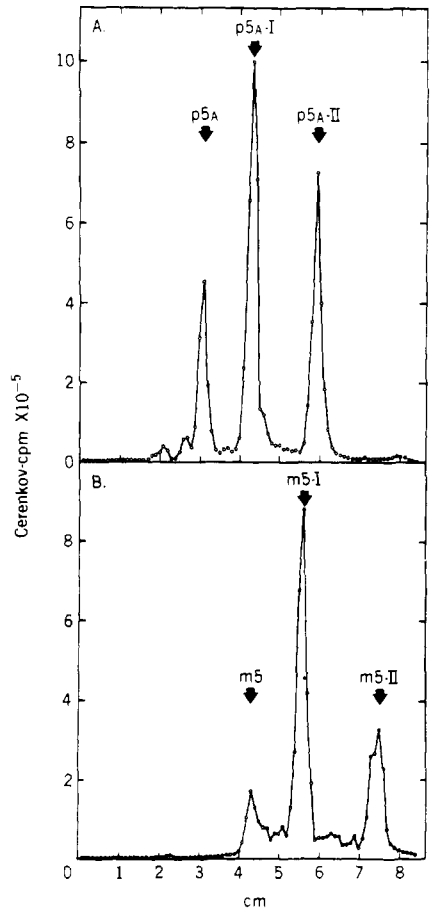

FIGURE 2: Separation of RNase T_2 partial digestion products. ^{32}P-Labeled $p5_A$ (A) and m5 (B) rRNA were incubated with RNase T_2 as detailed under Materials and Methods. The reaction products were resolved by electrophoresis through cylindrical gels (9 × 100 mm) under denaturing conditions (10% acrylamide with 8 M urea). Gels were sliced and the positions of the products determined by Cerenkov radiation.

Substrate Capacity of Reannealed $p5_A$ and m5 Complementary Fragments. If in fact the fragments I and II derived from m5 and $p5_A$ rRNA are aligned in the native molecules in complementary fashion, as shown in Figure 1, they should be capable of reannealing under appropriate conditions. This expectation was realized; it therefore was possible to test the effect upon the RNase M5 interaction of deleting one or the other of the precursor-specific termini from the RNA substrate. Additionally, we tested the capacity of the isolated $p5_A$ fragments I and II to serve as substrates for RNase M5. If the information required for recognition of a given cleavage site resides only in the nucleotide sequence in the vicinity of the cleaved phosphodiester bond, then the isolated fragments should be acted upon by the enzyme. The polyacrylamide slab gel radioautogram presented in Figure 4 summarizes data pertinent to this test. The position of intact $p5_A$ rRNA is seen in slot 1 of the radioautogram. Following digestion with RNase M5, as detailed under Materials and Methods, $p5_A$ is converted to m5 rRNA with the release of the 3′ precursor-specific segment, F1, and the 5′ segment, F2 (slot 2). The isolated, 3′-terminal 119-nucleotide fragment $p5_A$-I (slot 3) is not cleaved by the enzyme (slot 4). Similarly, the 5′ fragment $p5_A$-II (slot 5) is not accessible to RNase M5 (slot 6). However, upon annealing of fragments $p5_A$-I and $p5_A$-II as detailed under Materials and Methods, a structure having the electrophoretic mobility of intact $p5_A$ is generated (slot 7) and the capacities

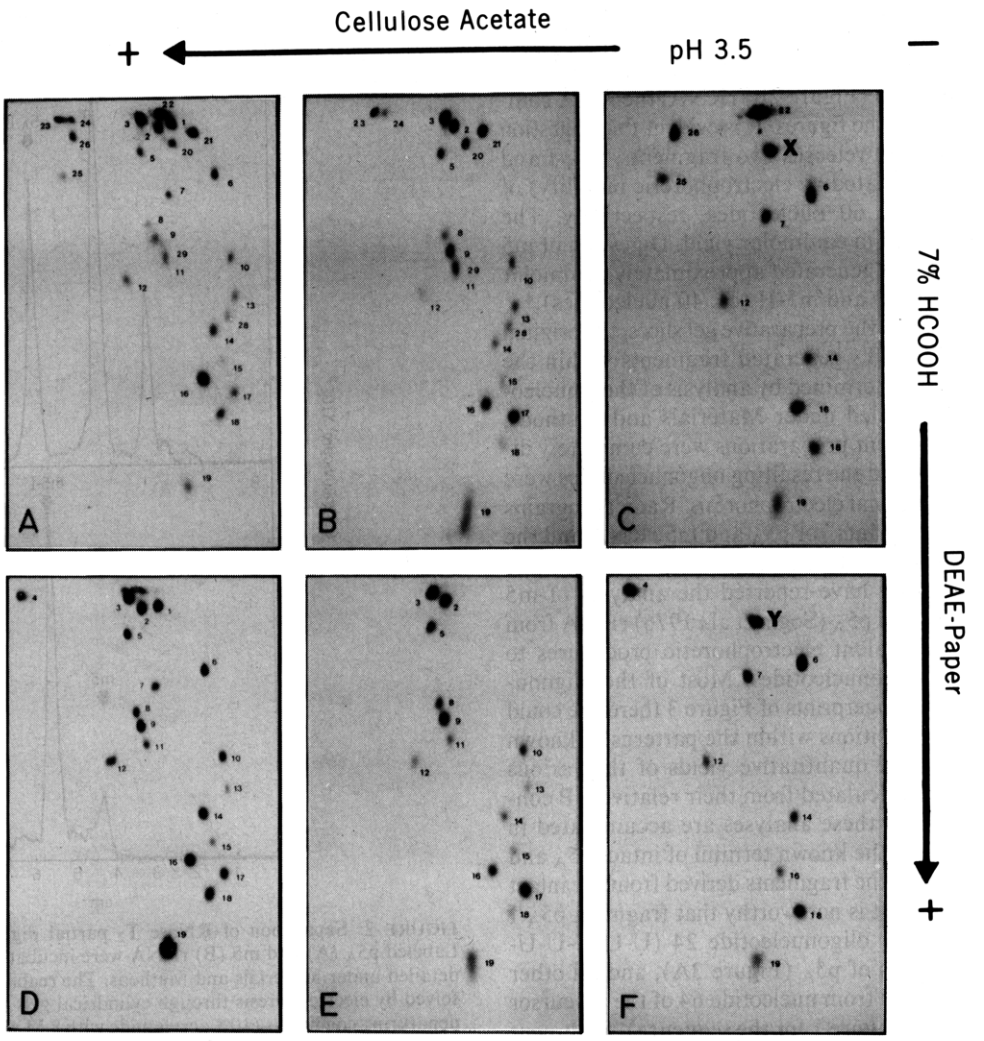

FIGURE 3: RNase T_1 fingerprints. Complete RNase T_1 digests of several RNA molecules were analyzed essentially by the method of Sanger et al. (1965) (see Materials and Methods): (A) $p5_A$; (B) $p5_A$-I; (C) $p5_A$-II; (D) m5; (E) m5-I; (F) m5-II.

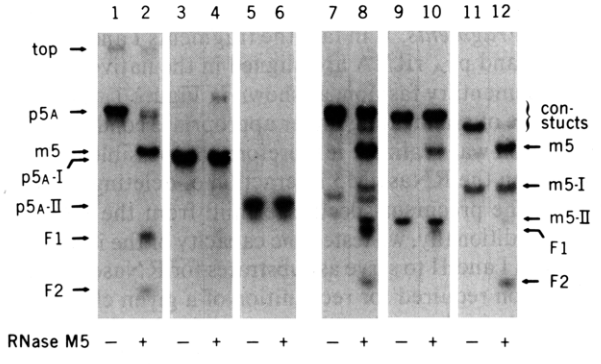

FIGURE 4: Analysis of maturation products. Standard maturation assays were carried out as described under Materials and Methods. RNase M5 was included where indicated at the bottom of the figure: (+) RNase M5 included; (−) RNase M5 not present. The maturation products were separated on polyacrylamide slab gels and located by radioautography; the positions of the various bands are indicated in the figure: slots 1 and 2, $p5_A$; slots 3 and 4, $p5_A$-I; slots 5 and 6, $p5_A$-II. Several substrates were generated by annealing complementary RNase T_2 "halves" derived from $p5_A$ or m5 rRNA (constructs), including slots 7 and 8 ($p5_A$-I/$p5_A$-II), slots 9 and 10 ($p5_A$-I/m5-II), and slots 11 and 12 (m5-I/$p5_A$-II). Residual RNase T_2 "halves" which did not anneal are labeled as such. Lane 8 exhibits bands corresponding to residual $p5_A$ "halves" (as in lane 7) plus two additional bands corresponding in size to the *mature* 5S halves (m5-I and m5-II). Presumably these result from the denaturation of a small fraction of the m5 rRNA generated in the RNase M5 reaction.

of the fragments to serve as substrate, with the release of both precursor-specific segments, are restored (slot 8). RNase M5 therefore requires for both cleavage events information residing in both fragments $p5_A$-I and $p5_A$-II. This suggests that the enzyme is capable of utilizing at least part of the required polynucleotide information only in double-helical form.

In assessing the contribution of each of the $p5_A$ precursor-specific segments to the information required by RNase M5, we constructed substrates lacking either 5′ or 3′ precursor-specific sequences. Substrate molecules lacking the 21-nucleotide, 5′ precursor-specific segment were formed by annealing fragments $p5_A$-I and m5-II (Figure 4, slot 9); the substrate lacking the 42-nucleotide, 3′ precursor-specific segment was formed from m5-I and $p5_A$-II (Figure 4, slot 11). Both of these artificial "intermediates" proved susceptible to RNase M5, but the substrate molecule lacking the 5′-terminal precursor-specific segment is cleaved quite slowly compared to that lacking the 3′ segment (compare Figure 4, slots 10 vs. 12). We therefore evaluated the rates of cleavage of the substrates. As detailed under Materials and Methods and in the legend to Figure 5, maturation assays with approximately equimolar amounts of the various reannealed substrates were intitiated by adding identical amounts of RNase M5 and then, at time intervals, further reaction in aliquots of the reaction mixtures was halted by addition of sodium dodecyl sulfate and EDTA. Reaction products in all samples were resolved by slab

TABLE I: Yield of Products of Complete RNase T_1 Digestion of Several RNA Species.

Oligonucleotide[a]	Sequence	Yield: $p5_A$ Obsd *b*	$p5_A$ Obsd *c*	$p5_A$ Calcd[d]	$p5_A$-I Obsd[e]	$p5_A$-I Calcd[d]	$p5_A$-II Obsd[e]	$p5_A$-II Calcd[d]	m5 Obsd[c]	m5 Calcd[d]	m5-I Obsd[e]	m5-I Calcd[d]	m5-II Obsd[e]	m5-II Calcd[d]
1	U-U-C-C-C-A-U-A-C-C-G-	1.0	0.9	1					0.8	1				
2	C-U-C-U-U-C-A-G-	1.0	1.1	1	1.1	1			1.1	1	1.1	1		
3	U-U-U-C-C-C-C-C-U-G-	0.7	1.2	1	1.2	1			1.2	1	1.0	1		
4	-U-U-U-G-								0.7	1			1.0	1
5	U-U-A-A-G-	1.0	1.0	1	1.0	1			1.0	1	1.0	1		
6	U-C-A-C-A-C-C-C-G-	1.0	0.7	1			0.8	1	0.7	1			0.8	1
7	A-U-A-G-	0.9	1.0	1			1.0	1	1.0	1			1.0	1
8	A-U-G-	1.2	1.2	1	1.3	1			1.3	1	1.2	1		
9	U-A-G-	2.0	2.4	2	2.4	2			2.2	2	2.3	2		
10	A-A-C-A-C-G-	0.8	0.7	1	0.7	1			0.8	1	0.8	1		
11	U-C-G-	0.8	1.0	1	0.9	1			1.1	1	0.9	1		
12	U-G-	2.2	2.2	2	1.1	1	1.1	1	2.3	2	1.1	1	1.1	1
13	C-C-A-A-G-	0.9	0.8	1	0.9	1			0.5	1	0.5	1		
14	A-A-G-	1.8	2.1	2	1.1	1	1.0	1	2.1	2	1.1	1	0.9	1
15	A-C-G-	0.8	0.8	1	0.8	1			0.9	1	0.8	1		
16	A-G-	5.5	6.6	6	3.3	3	3.1	3	3.7	3	2.4	2	1.1	1
17	C-C-G-	1.7	1.6	2	2.7	3			1.7	2	2.8	3		
18	C-G-	2.9	3.0	3	1.0	1	2.1	2	3.1	3	1.0	1	2.0	2
19	G-		13.0	12	10.1	9	3.4	3	12.4	10	7.5	7	3.4	3
20	C-U-C-A-A-U-G-	0.6	0.7	0.5	0.8	1								
21	C-U-U-A-A-A-C-C-C-A-G-	0.7	0.9	1	0.9	1								
22	A-A-C-A-C-U-C-U-C-A-A-U-U-U-G-	0.9	0.9	1			0.8	1						
23	U-U-U-U-U-U-G-	0.7	0.6	1	0.8	1								
24	U-U-U-U-U-U-G_{OH}	0.7	0.4	1	0.3	1								
25	-U-G-	0.8	1.0	1			0.9	1						
26	U-U-U-G-	0.9	1.1	1			1.2	1						
27	C-U-C-A-U-U-G-	0.3		0.5										
28	C-A-A-G-	0.7	0.8	1	0.9	1								
29	C-U-G-	0.7	0.9	1	0.9	1								
X,Y	U-U-C-C-C-A-U-A-						0.9	1					0.9	1

[a] The numbers refer to oligonucleotides shown in Figure 3. [b] Observed molar yields as published by Sogin et al. (1976). [c] Observed molar yields from one preparation. [d] Expected molar yields on the basis of the nucleotide sequence. [e] Observed molar yields averaged from three different preparations.

polyacrylamide gel electrophoresis and visualized by radioautography, as presented in Figure 5. With reference to the film, radioactive regions of the dried gel were excised and monitored for ^{32}P content; Figure 6 summarizes the relative rates of cleavage of the various substrates. It is apparent that the artificial substrate m5-I/$p5_A$-II, which lacks the 3′ precursor-specific segment, is as effective in interaction with RNase M5 as are native $p5_A$ or reconstructed precursor RNA ($p5_A$-I/$p5_A$-II). The 3′ precursor-specific segment associated with $p5_A$ therefore has no informational role in the release of the 5′ precursor-specific segment. In contrast the $p5_A$-I/m5-II construct, which lacks the 5′ precursor-specific segment, is markedly diminished (ca. 80–85%) in the rate at which it undergoes cleavage by RNase M5. This rate difference is not a consequence of only a fraction of substrate molecules being susceptible to the enzyme. At very high enzyme concentrations and long incubation periods, nearly all of the $p5_A$-I/m5-II construct is cleaved with the release of F1.

One conceivable explanation for the differing rates of cleavage of the artificial intermediates is that excess m5 fragments included in the reannealing mixtures might differentially inhibit the RNase M5 interaction with the substrate. We therefore evaluated, as described in the legend to Figure 7, the rate of cleavage of $p5_A$ in the presence of fragments m5-I or m5-II; the kinetics are presented in Figure 7. It is evident that the m5 fragments do not influence the RNase M5–$p5_A$

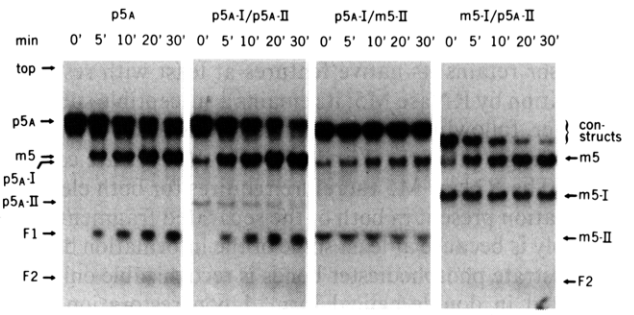

FIGURE 5: Maturation kinetics of several precursor RNA constructs. The standard maturation assay was modified as follows: assay buffer, precursor RNA as indicated, and component β of RNase M5 were preincubated in 50 μL for 5 min at 37 °C; then 0.07 μg of RNase M5 component α preparation was added. The mixture (60 μL) was further incubated at 37 °C for the time indicated; reaction products then were separated on a polyacrylamide slab gel and following drying of the gel, detected by radioautography. With reference to the film, appropriate regions of the gel were excised and monitored for radioactivity. Radioactivities present in the gel positions corresponding to the various products at zero time were subtracted from the value observed in samples drawn at subsequent times. Radioactivity occupying the m5 region of zero time slots represents the $p5_A$-I fragment or, in the case of the m5-I/$p5_A$-II construct, some material which annealed to yield a conformation hydrodynamically distinct from the "native" construct and therefore different in electrophoretic mobility. Maturation products are labeled m5, F1, and F2. Some residual RNase T_2 fragments which did not reanneal are labeled as such.

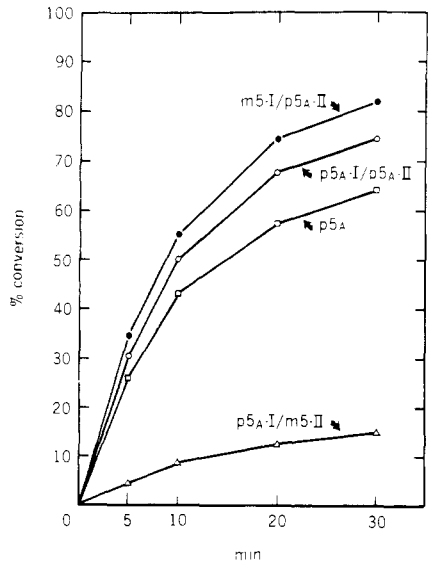

FIGURE 6: Maturation kinetics of several precursor RNAs. The percent conversion of several precursor constructs to m5 rRNA was calculated from data obtained from the same gel as the radioautograph shown in Figure 5. The radioactivity of the spots was determined by cutting and counting corresponding areas of the dried gel.

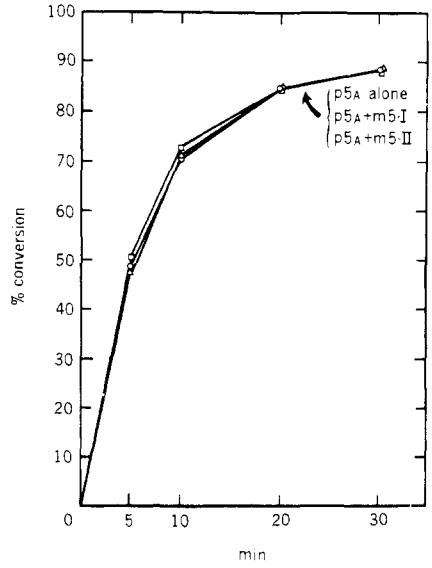

FIGURE 7: Kinetics of maturation of $p5_A$ in the presence of m5-I and m5-II. Maturation assays of $p5_A$, as detailed in the legend to Figure 5, were compared to those performed in the presence of 0.1 μg of unlabeled m5-I or 0.06 μg of unlabeled m5-II, respectively.

interaction. Moreover, the difference in the relative rates of cleavage of $p5_A$-I/m5-II and m5-I/$p5_A$-II observed in Figure 6 is exhibited upon exposure of mixtures of these two substrates to RNase M5 (data not shown), so the relative retardation in $p5_A$-I/m5-II cleavage is not due to the presence of some unknown inhibitor in preparations of the $p5_A$-I/m5-II construct. Therefore, the difference in the relative rates of maturation suggests that part, but not all, of the information utilized by RNase M5 for substrate recognition is associated with the 5′ portion of the precursor rRNA molecule.

Discussion

Upon mild digestion with RNase T_2, the *B. subtilis* $p5_A$ and m5 rRNA molecules are cleaved at a unique point, but the covalently separated components remain associated by virtue of secondary and/or tertiary interactions. The nicked $p5_A$ precursor retains its native features at least with respect to recognition by RNase M5; it remains a susceptible substrate. However, following denaturation and separation of the component fragments ($p5_A$-I and $p5_A$-II), all substrate capacity is lost. The RNase M5 therefore requires for both cleavages information present in both of the separated fragments. This probably is because at least some of the information defining the substrate phosphodiester bonds is recognizable only when presented in double-helical form. Upon restoration of the secondary structure of $p5_A$ rRNA by annealing the isolated RNase T_2 fragments, substrate capacity is fully restored.

The ability to reconstruct $p5_A$ or *m5* rRNA by reannealing the isolated RNase T_2 fragments permitted us to test the role of the precursor-specific segments in the recognition of $p5_A$ rRNA by RNase M5. The structure generated by annealing the 5′ fragment derived from $p5_A$ ($p5_A$-II) with the 3′ fragment from m5 rRNA (m5-I) lacks the 3′ precursor-specific segment, and yet remains as susceptible to RNase M5 as the parent precursor (Figure 6). The 42-nucleotide, 3′ precursor-specific segment therefore plays no role in the interaction of RNase M5 and its substrate which precedes release of the 5′ precursor-specific fragment, F2. The deleted segment of course may play some role in defining its own scission: the experiments performed do not bear on this question. Presumably the 3′ precursor-specific component of $p5_A$ enjoys some other role(s) in cell function; we previously have discussed the possible involvement of this portion of the molecule in the termination of DNA transcription (Sogin et al., 1976).

The 21-nucleotide, 5′ precursor-specific segment is required for the release of the 3′ precursor-specific segment, F1, at optimal rate. This precursor segment might influence the RNase M5-$p5_A$ interaction in two general ways. It might be one component of several features sought by RNase M5 or, conceivably, the segment might in some manner "activate" the RNase M5 independently of the binding of the remainder of the substrate. However, addition of purified F2, the 5′ precursor-specific segment, has no influence upon the rate at which the $p5_A$-I/m5-II construct is cleaved. Apparently then, the 5′ precursor-specific segment contains one or more of the informational elements recognized by RNase M5, and this information can be utilized only in the context of the intact precursor. The fact that the constructed precursor $p5_A$-I/m5-II (which lacks the 5′ precursor-specific segment) remains an effective, albeit comparably poor, substrate for RNase M5 demonstrates that other aspects of the precursor molecule are also involved in this protein-polynucleotide interaction. The information sought by RNase M5 therefore probably constitutes a matrix of recognizable features rather than a single element.

The structure of the $p5_A$ rRNA precursor, as shown in Figure 1, exhibits two elements within the 5′ precursor-specific segment which we have suggested (Sogin et al., 1976) might be involved in recognition by RNase M5. One of these is the 5′-terminal sequence -U-G-A-G-A-G-, which is one of two such sequences at positions 1-6 and 116-121 which are disposed about the substrate phosphodiester bonds with twofold rotational symmetry. We have discussed the roles which these symmetrically placed sequences might serve in the interaction with RNase M5 (Sogin et al., 1976). Another feature of the 5′ precursor-specific segment is the sequence -U-U-U-G-, at positions 18-21, which is complementary to a portion of the 3′ precursor-specific segment and the 3′-terminal C residue of the *m5* segment (positions 137-139). If the RNase M5 action which releases the 3′ precursor fragment prefers the double-helical substrate, then removal of the 5′ precursor-

specific component could markedly influence the rate of reaction. On the other hand, the phosphodiester bond whose scission releases the 5′ precursor-specific segment likely is involved in a double helical array even if the 3′ precursor-specific component is absent (see Figure 1). The observation that the substrate construct m5-I/$p5_A$-II (which lacks the 3′ precursor-specific segment) is as susceptible to RNase M5 action as intact $p5_A$ is consistent with this latter interpretation; the point of cleavage is just within the duplex stalk.

The $p5_A$ secondary structure displayed in Figure 1 juxtaposes the two RNase M5 substrate phosphodiester bonds. If RNase M5 possesses a single catalytic site, it seems unlikely that both bonds could be cleaved without reorientation of the substrate between the two scissions. This is because the amino acids involved in catalysis very likely must align with respect to the polarity of the substrate phosphodiester bonds and these, if inspected simultaneously, would confront the catalytic site with opposite polarities. If such reorientation of the substrate RNA on the enzyme surface occurs, it apparently does not involve the release of a reaction intermediate, lacking one or the other of the precursor-specific segments. Neither of the "artificial intermediates" constructed and tested for substrate capacity interacts with RNase M5 substantially more favorably than $p5_A$ itself. Thus a released intermediate, which would necessarily compete for the enzyme with unreacted $p5_A$ present at substantially higher concentration, would be expected to accumulate during the early course of the RNase M5 reaction. We previously have pointed out that such intermediate accumulation is not evident; a similar conclusion may be drawn from the radioautogram presented in Figure 5. If reorientation of the substrate with respect to the catalytic amino acids occurs during the reaction course, it must do so without release of the RNA.

Finally, we note that the different rates at which the artificial intermediates are cleaved by RNase M5 suggest, but do not prove, that the 3′ precursor-specific segment is released prior to the 5′ segment, if indeed the scissions are effected sequentially. Initial release of the 5′ precursor-specific segment would yield a substrate analogous to the artificial intermediate $p5_A$-I/m5-II, which undergoes hydrolysis quite slowly relative to the intact precursor or to the construct m5-I/$p5_A$-II.

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