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Use of T4 RNA ligase to construct model substrates for a ribosomal RNA maturation endonuclease

(ribosome synthesis/*Bacillus subtilis*/nucleotide sequence symmetry)

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ABSTRACT **RNase M5 of *Bacillus subtilis* specifically cleaves a 179-nucleotide precursor 5S rRNA to yield mature 5S rRNA (116 nucleotides) and two fragments derived from the termini. Possible recognition elements for RNase M5 within the precursor structure include nucleotide sequences arranged with 2-fold rotational and translational symmetry about the substrate bonds. We have used bacteriophage T4 RNA ligase to construct, from synthetic oligonucleotides and mature or precursor 5S rRNA fragments, test substrates lacking these symmetry elements. The susceptibilities of the artificial substrates to RNase M5 demonstrate that the symmetrically arranged sequences are not used in the RNase M5 interaction with the precursor. Additionally, the synthetic protocols permitted the invention of an acid-soluble assay for RNase M5 and, potentially, other specific endoribonucleases.**

Maturation of the precursors of ribosomal RNA (rRNA) in prokaryotes and eukaryotes includes a series of scissions that reduce the chain lengths of the precursor RNA molecules to their mature forms. The nucleases that effect these maturation cleavages are highly selective in their action and therefore, in principle, offer excellent models for exploring the mechanisms involved in specific interactions between proteins and polyribonucleotides. We therefore have undertaken to define in detail the action of an endonuclease responsible for the maturation of a precursor of 5S rRNA of *Bacillus subtilis*.

One immediate precursor of 5S rRNA of *B. subtilis*, termed $p5_A$, is 179 nucleotides long (1); the nucleotide sequence is shown in Fig. 1. We have isolated and extensively purified the specific endonuclease responsible for the production of mature 5S (m5) rRNA from this precursor (3). The enzyme, RNase M5, cleaves $p5_A$ rRNA at two sites (Fig. 1, arrows), releasing 42-nucleotide (termed F1) and 21-nucleotide (F2) segments from the 3′ and 5′ termini, respectively.

We have previously discussed several features of the $p5_A$ rRNA that, because of their low probability of random occurrence, might serve as sites of recognition for RNase M5 (3). These features include: (*i*) the duplex region within which the substrate bonds lie; (*ii*) two hexamer sequences (U-G-A-G-A-G, positions 1–6 and 116–121 in Fig. 1) disposed with 2-fold rotational symmetry about the substrate bonds; and (*iii*) two regions of translational symmetry (sequence repeats) that bound the substrate bonds. Since each of these structural features involves one or the other of the precursor-specific segments, we initially focused on the involvement of these segments in the reaction (4). It was possible to construct substrate precursors, lacking either F1 or F2, by isolating "halves" of $p5_A$ or m5 rRNA after partial digestion of the respective molecules with RNase T_2, and then annealing the 5′ or 3′ precursor half with, respectively, the 3′ or 5′ mature half. Reconstructed substrate molecules, lacking one or the other precursor-specific segment, proved to be susceptible to the enzyme, but with different efficiencies. The absence of F1 did not influence the release of F2, but in the absence of F2 the rate of release of F1 was diminished by about 80%. Therefore F2, but not F1, plays a significant role in the RNase M5–$p5_A$ recognition process.

F2, the 5′ precursor-specific segment of $p5_A$ rRNA, contributes to all of the features postulated to be involved in $p5_A$ recognition by RNase M5. In the present communication we explore their role in the RNase M5 interaction. We have used bacteriophage T4 RNA ligase (5, 6) to add synthetic oligonucleotides of defined sequence to the 5′ half of mature 5S rRNA, thereby in essence creating artificial F2 segments. Then these constructs were annealed to 3′ halves of $p5_A$ or m5 rRNA, thereby generating partially artificial RNase M5 test substrates. The results demonstrate that the elements of rotational and translational symmetry are not required by RNase M5 in its selection of substrates. Additionally, the synthetic protocols enabled us to develop a novel assay for RNase M5, and potentially any other maturation endonuclease, based on the acid solubility of terminally labeled oligonucleotides released from the partially artificial substrates upon cleavage.

MATERIALS AND METHODS

Enzymes and Assays. The RNase M5 α and β subunits were purified from *B. subtilis* 168 as detailed (3). Standard reaction mixtures (60 μl) contained 10 mM Tris·HCl (pH 7.3), 5 mM $MgCl_2$, 30% (wt/vol) 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 periods, reaction products were analyzed by gel electrophoresis or thin-layer chromatography.

T4 RNA ligase, isolated as detailed by Walker *et al.* (6), was a generous gift from Richard Gumport (University of Illinois, Urbana, IL). The intermolecular reaction catalyzed by RNA ligase was carried out in a mixture (30 μl) containing 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 8.3), 0.1 mM ATP, 20 mM $MgCl_2$, 3.3 mM dithiothreitol, 10 μg of bovine serum albumin per ml, 250 units of RNA ligase per ml, 1 mM oligonucleotide, and 5 μM of either ^{32}P-labeled or nonradioactive m5-II fragment. In these syntheses, it was found

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Abbreviations: $p5_A$ rRNA, precursor of 5S rRNA of *Bacillus subtilis*; m5 rRNA, mature 5S rRNA. F1 and F2 are, respectively, the 42-nucleotide 3′ and 21-nucleotide 5′ precursor-specific segments of $p5_A$ rRNA; they are released by RNase M5 during generation of m5 rRNA. Under appropriate conditions, RNase T_2 cleaves $p5_A$ and m5 rRNA to yield "half" molecules (see Fig. 1). The resulting 3′ and 5′ halves of $p5_A$ and m5 are referred to as, respectively, $p5_A$-I and $p5_A$-II or m5-I and m5-II. The m5-II fragments with synthetic oligomers appended to the 5′ terminus are denoted as (oligomer)m5-II [e.g., (U_3G)m5-II]. $NaDodSO_4$, sodium dodecyl sulfate.

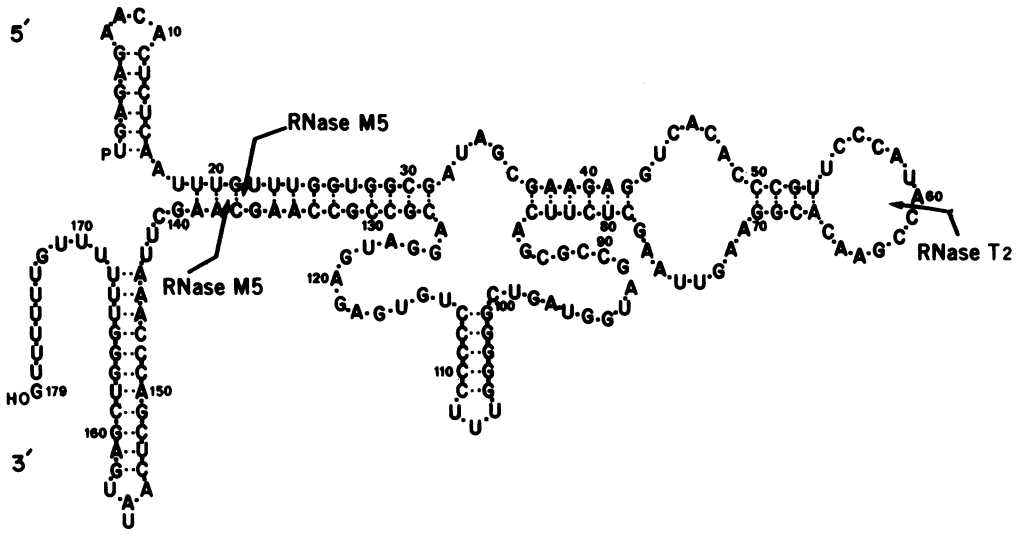

FIG. 1. Structure of $p5_A$ ribosomal RNA precursor. The nucleotide sequence of *B. subtilis* $p5_A$ rRNA (1) is folded into its probable secondary structure, as suggested by Fox and Woese (2). The sites of cleavage by RNase M5 and partial digestion by RNase T_2 are indicated. Precursor segment F2 includes residues 1–21, m5 rRNA includes residues 22–137, and F1 consists of residues 138–179.

necessary to use the m5-II fragment rather than intact m5 rRNA because RNA ligase, under the reaction conditions used, will not utilize duplex donor molecules, and the 5′ and 3′ termini of intact m5 rRNA are juxtaposed in double-helical array (Fig. 1). The preparation of oligonucleotides $(Ap)_3C$, $(Ap)_6C$, $(Up)_3G$, and $(Cp)_6G$ is described elsewhere (7, 8). The mixtures were incubated at 37° for 1 hr; then the reaction was halted by the addition of sodium dodecyl sulfate ($NaDodSO_4$) to a final concentration of 0.1%, EDTA to 0.5 mM, and urea to 4 M. The reaction products were resolved on polyacrylamide slab gels composed of 20% acrylamide/0.4% bisacrylamide in E buffer (9), and a denaturing stacking gel which consisted of 6% acrylamide/0.06% bisacrylamide/8 M urea in H_2O. The gels were run in E buffer containing 0.1% $NaDodSO_4$. Gel slices (1 mm) were monitored for radioactivity by Cerenkov radiation, and RNA was recovered from appropriate gel slices as described (4). The resolution of the ligase reaction products from unreacted material was equivalent to that shown in Fig. 4.

Polynucleotide kinase was isolated from bacteriophage T4 *am* E4314 as detailed by Cameron and Uhlenbeck (10). Kinase reaction mixtures (60 μl) contained 50 mM 2(*N*-cyclohexylamino)ethanesulfonic acid (pH 7.5), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 50 μg of bovine serum albumin per ml, 32 units of polynucleotide kinase per ml, 70 μM [γ-^{32}P]ATP (about 1000 Ci/mmol), and 5 μM RNA. The mixture was incubated for 1 hr at 37°C. After addition of 1 ml of 10 mM Tris·HCl (pH 7.5)/1 mM EDTA, the RNA was extracted once with phenol, separated from residual ATP by passage through Sephadex G50 (50-ml bed volume equilibrated in 10 mM Tris·HCl, pH 7.3/1 mM EDTA/0.1% $NaDodSO_4$), and isolated by gel electrophoresis as described above.

RNA and Analyses. ^{32}P-Labeled $p5_A$ or m5 (^{32}P-labeled or nonradioactive) rRNA were isolated from, respectively, chloramphenicol-treated or exponentially growing cultures of *B. subtilis* 168 as detailed previously (1). "Half" molecules of $p5_A$ and m5 rRNA were generated by partial digestion of the intact molecules with RNase T_2 and isolated by gel electrophoresis, all as detailed previously (4). At low temperature and high ionic strength, RNase T_2 cuts $p5_A$ rRNA only at position A-60 (see Fig. 1) and m5 rRNA at the corresponding residue. We have reported the detailed structural analysis of the $p5_A$ and m5 rRNA half molecules (4). The products of the RNA ligase-catalyzed condensation of the various synthetic oligonucleotides with uniformly ^{32}P-labeled m5-II fragments were characterized by oligonucleotide fingerprint analysis, essentially as described by Volckaert *et al.* (11). Complete RNase T_1 digests of the purified RNA constructs were resolved first by electrophoresis at pH 3.5 (4), followed by transfer to plastic-backed, polyethyleneimine-impregnated cellulose thin-layer plates (Brinkman) and development at 65° in the second dimension with homochromatography solvent C (12).

RESULTS

Construction of partially synthetic precursors

The RNA ligase elicited in bacteriophage T4-infected *Escherichia coli* is capable of catalyzing the ATP-dependent formation of 3′ → 5′ phosphodiester bonds between 5′-phosphorylated "donor" oligoribonucleotides and oligoribonucleotide "acceptors" with 3′-hydroxyl groups (5, 6). In the present investigation we used as RNA ligase donor the 39-nucleotide, 5′ fragment of *B. subtilis* m5 rRNA (m5-II), which contains a 5′-phosphoryl group derived from native m5 rRNA and a 3′-phosphoryl group as a result of RNase T_2 action. The presence of the 3′ phosphate prevents cyclization or self-addition of the donor (12). As RNA ligase acceptors we used the synthetic oligonucleotides $(Up)_3G$, $(Cp)_6G$, $(Ap)_3C$, and $(Ap)_6C$. These were chosen on the basis of availability and nucleotide sequence to provide relatively unambiguous tests for the involvement of particular features within the $p5_A$ rRNA molecule in the RNase M5 recognition process. After ligation of the nonradioactive, synthetic oligonucleotides to the 5′ terminus of [^{32}P]m5-II, the products were purified by gel electrophoresis and their structures verified by two-dimensional oligonucleotide fingerprint analysis after digestion with RNase T_1. As an example, the fingerprint of the (A_6C)m5-II product is shown in Fig. 2. Noteworthy is the absence of $p(Up)_3Gp$, the 5′-terminal oligonucleotide released by RNase T_1 from the m5-II fragment (4). Instead, a novel component appears in the position expected for $(Ap)_6Cp(Up)_3Gp$. All other RNase T_1 oligonucleotides in the fingerprint have previously been analyzed as components of the m5-II fragment (4).

After their isolation and characterization, each of the ligase

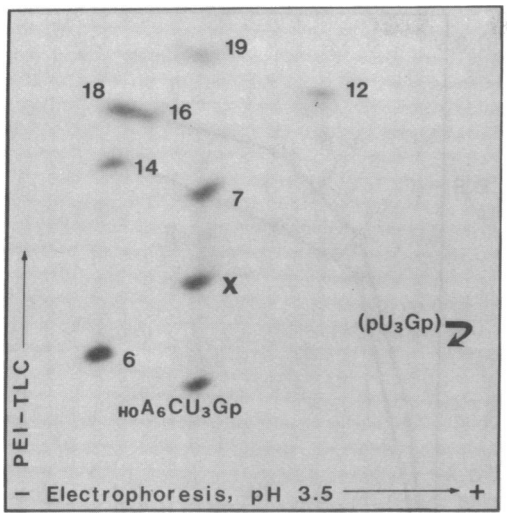

FIG. 2. Fingerprint analysis of an RNase T_1 digest of (A_6C)m5-II. The products of complete RNase T_1 digestion of the (A_6C)m5-II construct were resolved by electrophoresis and homochromatography (11); an autoradiogram of the resulting fingerprint is shown. The position of $p(Up)_3Gp$, the 5′ terminus of m5-II, is indicated with an arrow. Other oligonucleotides derived from the m5-II fragment are labeled as previously (4). PEI-TLC, polyethyleneimine-cellulose thin-layer chromatography.

products as annealed with the complementary 3′ halves of the $p5_A$ precursor ($p5_A$-I) or m5 rRNA (m5-I) to yield constructs differing from the native substrate of RNase M5 with regard to the 5′-terminal, "precursor-specific" segment.

Tests of symmetry involvement in F1 release

We initially directed attention toward the influence of the 5′ end of the precursor rRNA upon the release of F1 by RNase M5. Partially synthetic "precursor" halves, as well as the original m5-II, were individually annealed with $p5_A$-I derived from uniformly labeled $p5_A$ to form test substrates containing F1, the native 3′-terminal precursor segment, but having short, synthetic 5′ "precursor" segments. The kinetics of RNase M5 release of F1 from these substrates are summarized in Fig. 3; the curves are designated according to the structure of the 5′ half molecules. It is evident that the character of the 5′ precursor segment markedly influences the rate of F1 release. Thus, as we have previously shown (4), the restored $p5_A$ molecule (curve $p5_A$-II) exhibits a maximum rate of F1 release, whereas the absence of any 5′ precursor segment (m5-II) results in poor substrate capacity. However, the entirety of F2 is not required for maximum rate of cleavage by RNase M5: mere addition of U_3G to the m5–II fragment restores full susceptibility of F1 to cleavage [curve (U_3G)m5-II]. Therefore, at least one of the elements of 2-fold rotational symmetry, the U-G-A-G-A-G sequence, which resides at the 5′ end of the $p5_A$ molecule, is not required by RNase M5 for the release of F1 at optimal rate. Furthermore, translational symmetry is not required for good substrate activity; substitution of U_3G by C_6G as the 5′ precursor segment also yields a quite effective substrate with respect to cleavage of F1 [curve (C_6G)m5-II]. Not all 5′ precursor structures potentiate the release of F1 by RNase M5, however. The synthetic 5′ precursor structures A_3C and A_6C, which cannot restore duplex character to the substrate site by hydrogen bonding to nucleotide residue C-137 (adjacent to F1), do not facilitate recognition and cleavage of the F1 segment by RNase M5 [curves (A_3C)m5-II and (A_6C)m5-II].

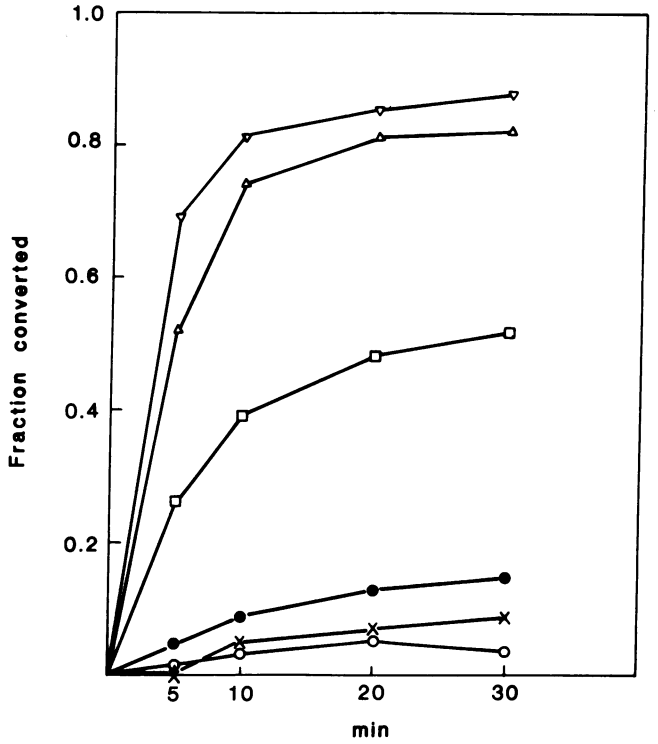

FIG. 3. Release of F1 from partially artificial precursors. The m5-II fragments with various oligonucleotides appended to the 5′ termini were annealed with ^{32}P-labeled $p5_A$-I fragment. Equivalent molar amounts of these substrates were individually incubated with RNase M5 in the standard maturation assay for different times and the products were resolved by gel electrophoresis (4). After autoradiography, radioactive bands were excised from the dried gel and monitored for ^{32}P content; release of F1 from $p5_A$-I was scored by the appearance of the 77-nucleotide m5-I fragment. The curves in the figure are designated according to the 5′ half molecules which were annealed to $p5_A$-I to generate the test substrates. ▽, $p5_A$-II; △, (U_3G)m5-II; □, (C_6G)m5-II; ●, m5-II; ×, (A_3C)m5-II; ○, (A_6C)m5-II.

Release of artificial 5′ "precursor" segments by RNase M5

Clearly the entirety of F2 is not required for optimal rate of RNase M5 release of F1. We next examined the features within F2 that are required for its own release. This was approached by examining the susceptibility of the synthetic 5′ precursor segments discussed above to cleavage by RNase M5. The ^{32}P-labeled, m5-II fragments containing nonradioactive, synthetic oligomers at their 5′ termini were annealed with nonradioactive m5-I and presented to RNase M5 at high enzyme: substrate levels. Reaction products then were resolved on denaturing polyacrylamide gels (containing urea) to separate the 5′ and 3′ halves of the substrate molecules; the autoradiogram of the relevant gel lanes is shown in Fig. 4. It is evident that the m5-II fragment is separable from m5-II containing the added synthetic "precursor"-specific oligonucleotides (− RNase M5). The individual RNase M5 subunits, α and β, are incapable of effecting maturation (data not shown). However, upon presentation of the substrates to the RNase M5 holoenzyme (+ RNase M5) each is wholly or in part reduced to the length of the 39-nucleotide, m5-II fragment. The efficiencies of cleavage of the various substrates qualitatively follow the same pattern as the facilitation of F1 release by the corresponding synthetic

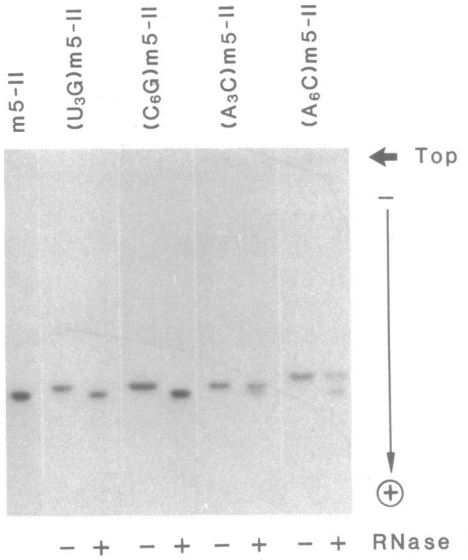

FIG. 4. Release of synthetic precursor segments from artificial precursors. Uniformly ^{32}P-labeled m5-II fragments with nonradioactive 5′-terminal oligonucleotides as indicated were annealed to nonradioactive m5-I fragment and then incubated in the standard maturation assay with (+) or without (−) RNase M5. Reaction products were resolved by gel electrophoresis; autoradiograms of the gel lanes are shown. [^{32}P]m5-II is a marker for the expected RNase M5 reaction product.

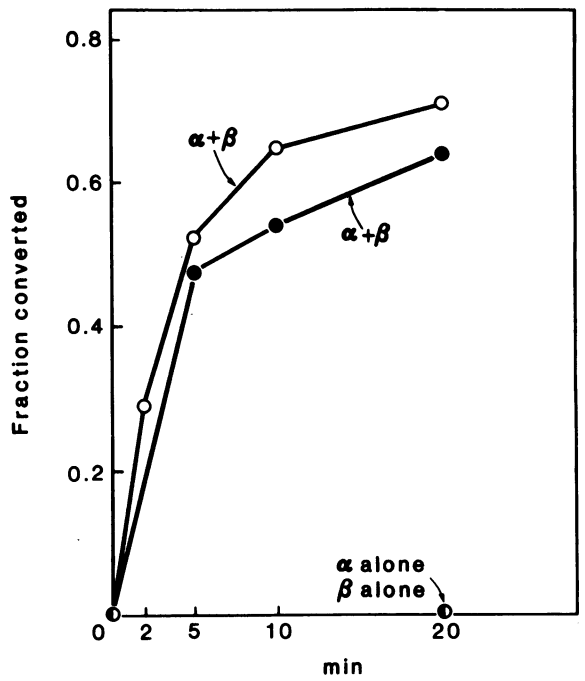

FIG. 5. Release of acid-soluble radioactivity from a terminally labeled, partially synthetic RNase M5 substrate. [5′-^{32}P]p(U_3G)m5-II (○) was annealed with equimolar m5-I fragment and incubated in the standard maturation assay with RNase M5 holoenzyme or the individual RNase M5 subunits as indicated. Aliquots withdrawn at the indicated intervals were precipitated with 10% trichloroacetic acid and collected on membrane filters; acid-soluble radioactivities are expressed as a fraction of total input radioactivity. In a parallel reaction, uniformly ^{32}P-labeled $p5_A$ rRNA (●) was incubated with RNase M5 and the products were resolved by gel electrophoresis (4). Radioactive bands were excised from the dried gel and monitored for ^{32}P content. The mole fractions of total input substrate converted to m5 rRNA are plotted against incubation time.

5′ sequence. That is, when reannealed with m5-I, (U_3G)m5-II and (C_6G)m5-II are completely cleaved by RNase M5, whereas (A_3C)m5-II and (A_6C)m5-II are marginally acceptable. Therefore, the elements of rotational and translational symmetry associated with F2 are not important to removal of either the F1 or F2 precursor segments by RNase M5.

Construction of a convenient substrate for RNase M5

The high degree of substrate specificity of RNase M5, and processing endonucleases in general, has rendered their purification and study difficult. We have thus far identified no substrates for RNase M5 except those containing the m5 rRNA sequence and, therefore, have necessarily used a cumbersome and expensive assay, which involves submitting the labeled precursor ($p5_A$) to the enzyme and scoring the various digestion products after their resolution by gel electrophoresis (3). The acceptability to RNase M5 of substrates consisting of m5 rRNA with appended, synthetic precursor sequences suggested a simplified assay, based on the release of an isotopically labeled, acid-soluble fragment, which also would be suitable for quantitative studies never before possible with specific maturation endonucleases.

The substrate for the simplified assay was constructed by first using RNA ligase to condense nonradioactive m5-II (donor) with nonradioactive $(Up)_3G$ (acceptor). The product, (U_3G)-m5-II, then was phosphorylated at the 5′ terminus, with polynucleotide kinase and [γ-^{32}P]ATP as phosphate donor. After isolation of the [5′-^{32}P](U_3G)m5-II by gel electrophoresis, it was annealed with nonradioactive m5-I fragment to yield the 5′-^{32}P-labeled substrate for RNase M5. As shown in Fig. 5, presentation of this substrate to RNase M5 holoenzyme, but not to the α or β subunits individually, results in the release of acid-soluble radioactivity at a rate and extent comparable to the maturation of the native substrate, $p5_A$ rRNA. Proof that the ^{32}P radioactivity was associated with the synthetic precursor segment, and that release of acid-soluble radioactivity from the substrate by RNase M5 was due to the specific cleavage of the synthetic precursor segment, was obtained by chromatography of various nuclease digestion products of the terminally labeled substrate (Fig. 6). Lanes 1–3 are references and contain, respectively, [5′-^{32}P]p($Up)_3G$ with no treatment, after treatment with RNase T_1 (no reaction expected), and after digestion with RNase A ([5′-^{32}P]pUp expected). ^{32}P radioactivity associated with the artificial substrate does not migrate in the solvent (lane 4). As anticipated from the proposed structure, digestion of the substrate with RNase T_1 releases labeled p($Up)_3Gp$ (lane 5); digestion with RNase A releases labeled pUp exclusively (lane 6). The RNase M5 subunits α or β (lanes 7 and 8) individually have no effect on the substrate, but the action of the holoenzyme generates [5′-^{32}P]p($Up)_3G$, the synthetic "precursor" segment (lane 9).

DISCUSSION

On the basis of the low probability of their random occurrence, we proposed (3) that nucleotide sequences disposed in the $p5_A$ rRNA molecule with 2-fold rotational symmetry and 2-fold translational symmetry about the bonds cleaved by RNase M5 might be involved in substrate recognition by this highly specific enzyme. However, the results presented here seem to rule out a requirement for these elements of symmetry in RNase M5 action. Replacement of F2 with synthetic oligomers, lacking one or both of the symmetry elements, may result in a substrate

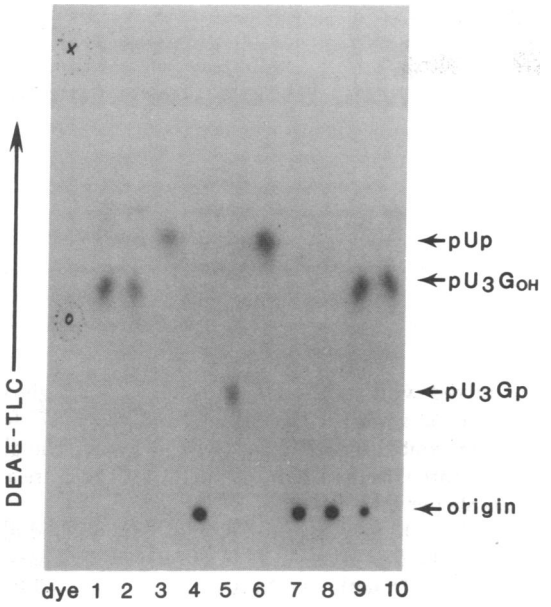

FIG. 6. Structural analysis of terminally labeled RNase M5 substrate. [5′-^{32}P]p(U_3G)m5-II annealed with m5-I was incubated for 30 min with the indicated enzymes. Products were resolved on a plastic-backed DEAE thin-layer plate (20 × 20 cm) in one dimension with homochromatography mixture C (12). [5′-^{32}P]p$(Up)_3$G is shown as marker (lanes 1 and 10) and after digestion with 15 μg of RNase T_1 per ml (lane 2) or 15 μg of RNase A per ml (lane 3). The artificial precursor, [5′-^{32}P]p(U_3G)m5-II, annealed with m5-I (lane 4) was digested with 15 μg of RNase T_1 per ml (lane 5) or 15 μg of RNase A per ml (lane 6) or incubated in the standard maturation assay with RNase M5 subunit α alone(lane 7), subunit β alone (lane 8), or RNase M5 holoenzyme (lane 9).

that is nearly as susceptible to RNase M5 as the native precursor, with regard to release of both the 5′- and 3′-terminal precursor-specific fragments. However, the enzyme is not indifferent to the structure of the synthetic segments used in constructing the partially artificial precursors. The substrate molecules containing U_3G and C_6G as 5′-terminal precursor segments are acceptable to RNase M5, but those containing A_3C and A_6C are acted upon only poorly. Nevertheless, even these relatively poor substrates are cleaved with fidelity by the enzyme. Therefore, although the enzyme is sensitive to the nucleotide sequence and/or conformation in the immediate vicinity of the bonds cleaved, this observation suggests that the most significant recognition elements used by RNase M5 in its search for the substrate lie within the mature 5S rRNA component of the precursor. This conclusion is bolstered by experiments to be presented elsewhere that examine the substrate capacity of chemically modified $p5_A$ and $p5_A$ with regions deleted by partial nuclease digestion.

To date we have tested only the limited number of oligomers discussed here as precursor segments appended to the m5 rRNA. However, the results are consistent with the notion that RNase M5 prefers to cleave adjacent to a hydrogen-bonded base pair and explain the facilitation of F1 release by the presence of F2. Thus, the G residues of the U_3G and C_6G segments associated with the more competent substrates may pair with the 3′-terminal C residue of the mature component of the precursor, whereas the C residue of the A_3C or A_6C segments cannot do so. However, definition of this limited specificity requires the quantitative evaluation of the susceptibility of precursor constructs with numerous combinations and permutations in the nucleotide sequence of the artificial precursor segment.

The discovery that mature 5S rRNA containing an appended, artificial "precursor" segment is a substrate for RNase M5 permitted us to devise a simple assay system for this maturation endonuclease, based on the release of the terminally labeled, acid-soluble oligonucleotide. The methodology in principle could be extended to the construction of substrates for other specific maturation endonucleases that require gel electrophoresis for the analysis of reaction products. In addition to providing a convenient assay for the relevant enzymes, the protocol permits the construction of substrates of known specific radioactivity for the inspection of the kinetic parameters of specific endonulcease interaction with nearly native substrates. The isolation of precursor RNA molecules from cells generally involves pulse-labeling, after treatment of cultures with inhibitors of RNA maturation, or alternatively the labeling of mutant populations under conditions not permitting growth. Only trace amounts of the RNA precursors can generally be isolated, and these are usually of uncertain purity and therefore of unknown specific radioactivity. Consequently, it has not been possible to explore the reaction kinetics and hence the reaction mechanism of any maturation endonuclease. Such studies now are feasible, using substrates constructed from the mature RNA species as described here.

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