Characterization of Ribonuclease NU Cleavage Sites in a Bacteriophage 80-induced Ribonucleic Acid*

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SUMMARY

Ribonuclease NU, an endoribonuclease isolated from human KB tissue culture cells, can cleave a bacteriophage 80-induced RNA at four distinct sites. Nucleotide sequence analysis of the eight cleavage products has shown that the enzyme produces oligonucleotides terminating in 3'-phosphate groups, and that the four cleavage sites are in the only nonhydrogen-bonded region of the substrate. Various aspects of the cleavage reaction with this RNA and with other substrates are discussed.

RNase NU, an endoribonuclease isolated from human KB tissue culture cells, was detected by virtue of its ability to cleave an *Escherichia coli* tRNA\(^{34}\) precursor molecule (1). During the course of the purification and characterization of this enzyme, it was found that substrates normally stable in vitro, such as tRNA, 5 S RNA, and rRNA, were resistant to attack by RNase NU, but substrates unstable in vitro, such as the tRNA\(^{34}\) precursor and various bacterial mRNAs, were susceptible to attack. It is of obvious interest, if the above generalization concerning the action of RNase NU is correct, to understand what governs the specificity of this enzyme. To this end, it is useful to examine the cleavage sites in radiochemically pure substrates of known nucleotide sequence. Such an analysis has been carried out with the tRNA\(^{34}\) precursor molecule; two cleavage sites were found but no obvious similarities between these sites were identified. We have now extended this kind of analysis to include another substrate susceptible to RNase NU cleavage and of known nucleotide sequence. This substrate, a bacteriophage 80-induced RNA (M\(_2\)) 62 nucleotides long (2), is unstable in vitro. A comparison of the cleavage sites in M\(_2\) with those in the tRNA precursor suggests that cleavage of RNA by RNase NU will occur only in regions devoid of secondary or tertiary structure, or both.

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1 The abbreviations used are: RNase NU, endoribonuclease specific for unstable RNA (1); precursor, the RNA precursor (128 nucleotides) to *E. coli* tRNA\(^{34}\) mutant A25; M\(_2\), bacteriophage 80-induced RNA 62 nucleotides long called M\(_2\) by Pieczenik et al. (2); 5 S RNA, ribosome-associated RNA with sedimentation coefficient of 5 S.

EXPERIMENTAL PROCEDURE

All biological materials, chemicals, chromatographic media, enzymes, and biochemical methods were as described in the accompanying paper (1). Since M\(_2\) is coded for by bacteriophage 80 848 mutant A25, and it can be labeled with \(^{32}\)P-O\(^{32}\) in *Escherichia coli* infected with this phage, it was extracted from the same acrylamide gels used to prepare the tRNA\(^{34}\) precursor (1).

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**FIG. 1.** Autoradiograph of an acrylamide gel separation of the products of the reaction of RNase NU with \(M_2\). The reaction was carried out and the products analyzed in a 10% acrylamide gel as described in the accompanying paper (1). \(^{32}\)P-labeled bulk *Escherichia coli* 4 S tRNA, which was eluted as a single band from another gel and which had been subjected to electrophoresis as such again in 10% polyacrylamide gels, was added to the completed reaction before applying the sample to the gel. The various RNA species were eluted from the gel and identified by nucleotide sequence analysis. Numbers in parentheses refer to distance, in centimeters, of each band from the origin.
RESULTS

The products of the reaction of RNase NU with M₃₀ as visualized by autoradiography after acrylamide gel electrophoresis of the reaction mixture which had been incubated under standard conditions (I), are shown in Fig. 1. The bands numbered 1 to 8 have been identified as cleavage products of M₃₀ by standard nucleotide sequencing techniques. The production of eight bands suggests four discrete cleavage sites and this notion has been verified through the sequencing studies (see below). The electrophoretic mobilities of the various bands are also in agreement with the hypothesis that there are four cleavage sites which yield four pairs of bands, i.e., Site A (see Fig. 2, Structure A) gives Bands 1 and 8; Site B gives Bands 2 and 7; Site C gives Bands 3 and 6 and Site D gives Bands 4 and 5. Data from several preparative cleavage experiments indicate that the rates of cleavage at the different sites are in the following approximate ratios, A:B:C:D as 2:3:3:1. With our purest enzyme preparations, we never observed small fragments of M₃₀ which would result from cleavage at one of these sites (e.g., A, see Fig. 2) followed by cleavage in the same molecule at another (e.g., D). Therefore, it appears that RNase NU cleavage at any one site is not a prerequisite for cleavage at another site.

The nucleotide sequence analysis of the RNase NU cleavage products was made simple because the complete nucleotide sequence of M₃₀ has been determined by Piefenriek et al. (2). The complete sequence, drawn in a hypothetical structure, is shown in Fig. 2. There are 12 different products resulting from the action of T₁ ribonuclease on M₃₀. These are listed in Table I in the order (aside from the mononucleotide Gp) they appear in the sequence as it is read in the 5' to 3' direction. Table I summarizes those products of T₁ digestion found in each of the eight RNase NU cleavage products and lists also any new products found.

The four RNase NU cleavage sites of M₃₀ were unequivocally identified as follows.

Site A—Band 8 contains T₁-produced oligonucleotides 1 to 3 as well as a new product which was further identified by ribonuclease A digestion as being UpApApCpApCp. Band 1 contains all the 1; products expected for M₃₀ except for those found in Band 8 and the Product 4; it has instead an extra mole of ApGp. Thus Site A is identified as being between nucleotides 17 and 18 from the 5' end of the molecule.

Site B—Band 7 contains the first four (from the 5' end) T₁ products in the M₃₀ sequence as well as an extra spot in the position of Up migration. Band 2 contains all the T₁ products missing in Band 7 as well as an extra mole of Gp. The location of Site B is defined by the products of ribonuclease A digestion of Bands 2 and 7 (Table II). That is, if the cleavage site is located between nucleotides 20 and 21 from the 5' end of the molecule, a ribonuclease A digestion of Band 2 should contain 1 mol only of GpUp and not, for example, ApGpUp, and this is seen to be the case.

Site C—Band 6 contains the same four T₁ products as found in Band 7 plus 1 mol of UpGp and 1 mol of Up. Band 3 contains the sequences missing in Band 6 as well as an extra mole of Gp. The unequivocal position of this site between nucleotides 22 and 23 from the 5' end depends upon the identification of GpApUp in the RNase A digest of Band 6. Both of these conditions are fulfilled.

Site D—Band 5 contains the first five oligonucleotides from the
The RNase A digest of Band 5 indicated that all the nucleotides of the rRNA precursor molecule. This molecule is a precursor to the Ti products missing in Band 5, except for Product 6. Two moles of a ribonuclease for one kind of structure or another can also be illustrated by the reaction of RNase III with the phosphate-terminated products of cleavage were found as was the case for the Ti products. Only 5'-phosphate-terminated oligo- or mononucleotides. Only 3'- which can be drawn in potential hydrogen-bonded structures (7) up to and including nucleotide 25 from the 5' end (the product 16 S and 23 S rRNA and is cleaved only once by RNase III (3, between nucleotides 25 and 26. Studies have shown that RNase III will digest double-stranded regions of RNA (5, 6) and sequences do exist in the mature rRNA (9). Shorter segments may be devoid of such structure but could still be protected from nuclease action by tertiary folding that the hydrogen-bonded structure of MS, as shown, probably exists in vitro. The four RNase A digestion sites are located in the only single-stranded region in this molecule. Similarly, the cleavage sites of RNase NU in the E. coli tRNA Trr precursor are also located in the single-stranded region of a hypothetical hydrogen-bonded structure for that molecule (Fig. 2, Structure B). The similarity in RNase NU specificity with the two substrates susceptible to RNase NU attack since it is known, for example, that there are more ribosomal binding sites exposed in the fragmented, compared to intact, RNA of the related bacteriophage R17 (8). Extensive nucleotide sequence studies of a related bacteriophage RNA reveal that at least one long segment of this molecule is almost totally involved in secondary structure (9). Shorter segments may be devoid of such structure but could still be protected from nuclease action by tertiary folding of the molecule. In addition to the result cited above, we have found that one particular fragment of Qβ RNA (which is 26 nucleotides long and cannot be represented in a hydrogen-bonded structure) is not cleaved at all by RNase NU, suggesting again that an appropriate substrate for this enzyme must be more.

**DISCUSSION**

The four cleavage sites of M12 by RNase NU are depicted in Fig. 2, Structure A. Tiezenek et al. (2) have provided evidence that the hydrogen-bonded structure of M12, as shown, probably exists in vitro. The four RNase NU cleavage sites are located in the only single-stranded region in this molecule. Similarly, the cleavage sites of RNase NU in the E. coli tRNA Trr precursor are also located in the single-stranded region of a hypothetical hydrogen-bonded structure for that molecule (Fig. 2, Structure B). The similarity in RNase NU specificity with the two substrates susceptible to RNase NU attack since it is known, for example, that there are more ribosomal binding sites exposed in the fragmented, compared to intact, RNA of the related bacteriophage R17 (8). Extensive nucleotide sequence studies of a related bacteriophage RNA reveal that at least one long segment of this molecule is almost totally involved in secondary structure (9). Shorter segments may be devoid of such structure but could still be protected from nuclease action by tertiary folding of the molecule. In addition to the result cited above, we have found that one particular fragment of Qβ RNA (which is 26 nucleotides long and cannot be represented in a hydrogen-bonded structure) is not cleaved at all by RNase NU, suggesting again that an appropriate substrate for this enzyme must be more.

**Table I**

| Products of T1 ribonuclease digestion of RNase NU cleavage products of M12 | Site A | Site B | Site C | Site D |
|---|---|---|---|---|
| T1 Products | Band 5 | Band 1 | Band 7 | Band 2 | Band 6 | Band 3 | Band 5 | Band 4 |
| 1. PPPGP | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| 2. UpUpApCpUpUpAp | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| 3. CpcPcPp | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| 4. UpApApCpApCpApGp | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| 5. UpGp | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| 6. ApUpApGp | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| 7. CpcPcPpUpUpUpGp | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| 8. ApApGp | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| 9. UpUpApCpApCpGp | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| 10. CpcPcPpUpUpApCp | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| 11. CpcPcPpUpUpUpOH | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| 12. UpGp | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |

Products are listed in the order they occur in M12 RNA going in the 5' to 3' direction, except for mononucleotides. These have all been characterized extensively by Tiezenek et al. (2) in their determination of the nucleotide sequence of M12. If a product occurs more than once, it is not listed twice. A number 1 indicates that the normal molar yield of the product (as listed in reference 2) was found and 0 indicates absence of the product.

In all analyses no evidence was found for the generation of new 5'-phosphate-terminated oligo- or mononucleotides. Only 3'-phosphate-terminated products of cleavage were found as was also the case for the *E. coli* tRNA Trr precursor molecule after reaction with RNase NU.

The similarity in RNase NU specificity with the two substrates susceptible to RNase NU attack since it is known, for example, that there are more ribosomal binding sites exposed in the fragmented, compared to intact, RNA of the related bacteriophage R17 (8). Extensive nucleotide sequence studies of a related bacteriophage RNA reveal that at least one long segment of this molecule is almost totally involved in secondary structure (9). Shorter segments may be devoid of such structure but could still be protected from nuclease action by tertiary folding of the molecule. In addition to the result cited above, we have found that one particular fragment of Qβ RNA (which is 26 nucleotides long and cannot be represented in a hydrogen-bonded structure) is not cleaved at all by RNase NU, suggesting again that an appropriate substrate for this enzyme must be more.
complex than single-stranded RNA lacking any secondary or tertiary structure, or both.

Finally, it is worthwhile mentioning the possible usefulness of RNase NU in nucleotide sequence analysis of RNA. This enzyme has a specificity different from that of the commonly used nucleases. It may be expected to provide fragments containing "hairpins" with single-stranded tails.

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### TABLE II

| Products of ribonuclease A digestion of RNase NU cleavage products of M1 | Site A | Site B | Site C | Site D |
|-------------------------------------------------------------------------|-------|-------|-------|-------|
| RNase A Products                                                        |       |       |       |       |
| GpUp                                                                  | 1     | 1     | 2     | 2     |
| ApGpUp                                                                | 0     | 1     | 0     | 1     |
| GpAUp                                                                 | 1     | 1     | 0     | 1     |

The molar yield of each product is listed. These are the critical di- or tri-nucleotides in determining the exact RNase NU cleavage sites. All other RNase A products were found in the molar yields expected for each band assuming the four cleavage sites shown in Figure 2, structure A. No RNase A digestion was carried out on Bands 7 or 8.
Characterization of ribonuclease NU cleavage sites in a bacteriophage phi80-induced ribonucleic acid.
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