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Introduction of Specific Cleavages into RNAs of RNA Bacteriophages for Determination of Base Sequences

(R17 RNA/MS2 RNA/Qβ RNA/RNase T1)

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ABSTRACT **Large fragments of R17, MS2, and Qβ RNAs were obtained reproducibly by limited digestion by ribonuclease T1. After digestion of R17 and MS2 RNAs, two pairs of large fragments were obtained that probably resulted from specific cleavages of the whole molecules. The cleavages of MS2 RNA were produced at points 36 and 47% from the 5′ end.**

The RNA bacteriophages of *Escherichia coli* contain a genome that consists of about 3500 bases and are among the smallest known bacteriophages. Perhaps the most successful method used in the determination of the base sequences of bacteriophage RNAs has been the technique introduced by Sanger *et al.* (1) which links base sequence with the amino-acid sequence of a specific protein coded for by the phage RNA. Although this method has been used successfully by Fiers *et al.* (2) to determine the complete base sequence of the coat-protein cistron of bacteriophage MS2, it is more difficult to extend its application to other cistrons whose gene products are not readily obtainable in quantities sufficient to permit determination of their amino-acid sequences. In addition, this method cannot be used to study RNA that is not translated.

In this paper a method is proposed that may prove useful for the determination of the complete base sequence of high-molecular-weight RNA. Specific cleavage of an RNA molecule at a single site should generate a set of two large fragments. By subsequent labeling of the ends of the molecule, one can determine which of the fragments has the original 3′ or 5′ end. In this way the fragments can be ordered. After purifying the fragments and repeating the procedures several times, one should reduce the fragments to a size small enough to be analyzed by conventional RNA base-sequencing techniques. RNase T1 is known to be a guanylic acid-specific endonuclease that has a tendency to prefer single-stranded regions to double-stranded regions (3). It is also known that RNAs of RNA bacteriophages have a high degree of secondary structure (4–6). Thus, it may be possible to control the sequential series of cleavages of the molecules. The first step of this procedure, to obtain and order large fragments produced by a specific cleavage of the whole molecule, is shown in this paper with R17 and MS2 RNAs.

MATERIALS AND METHODS

Strains. Bacteriophages R17, MS2, and Qβ were gifts from Drs. Mario R. Capecchi, Walter Fiers, and Paul Kaesberg, respectively. *E. coli* S26 and *E. coli* Q13 were kindly made available by Drs. M. R. Capecchi and Robert Callahan, respectively. *E. coli* S26 was used as a host of bacteriophages R17 and MS2, and *E. coli* Q13 was used as a host of bacteriophage Qβ.

Preparation of Bacteriophages. Bacteriophages were grown in a medium containing 10 g of Bacto Tryptone (Difco), 1 g of yeast extract, 1 g of glucose, 8 g of NaCl, and 2 mmol of $CaCl_2$ per liter of water. ^{32}P-Labeled bacteriophage R17 was grown in a medium containing 10 g of Peptone (Difco), 5 g of NaCl, 1 g of glucose, and 2 mmol of $CaCl_2$ per liter of water. ^{32}P-Labeled bacteriophages MS2 and Qβ were grown in a medium containing 5 g of Casamino acids, 5 g of NaCl, 1 g of NH_4Cl, 4 ml of glycerol, 1 mmol of $MgSO_4$, 10 μmol of $FeCl_3$, 2 mmol of $CaCl_2$, and 10 mmol of Tris·HCl (pH 7.5) per liter of water. Cells were infected with bacteriophages at a multiplicity of infection of about 10 at a cell density of about 6×10^8 cells per ml. $H_3{}^{32}PO_4$ (New England Nuclear Corp.) was added to the medium to a final concentration of 20 μCi/ml about 5 min before the infection. After 3 hr of incubation, the bacteriophages were purified through two cycles of low- and high-speed centrifugations and finally spun down through step gradients of CsCl. The bacteriophage bands were taken out with capillary pipettes, and the CsCl was removed by dialysis.

Preparation of Ribosomes. E. coli Q13 was grown in the Casamino acids–glycerol medium described above. $H_3{}^{32}PO_4$ was added at the beginning of the cultivation, and the cells were harvested at late logarithmic phase. The cells were lysed with 0.5 mg/ml of lysozyme and 1% Brij 58 and incubated for 30 min at 37° with 2 μg/ml of DNase 1 (Sigma). The ribosomes were purified through two cycles of low- and high-speed centrifugations (7).

Extraction of RNA. RNA was extracted from phages and ribosomes with phenol equilibrated with 0.1 M NaCl, 0.1 M Tris·HCl (pH 7.5), and 0.01 M EDTA. The extraction was repeated three times. The solution was dialyzed against 0.1 M sodium acetate–0.01 M magnesium acetate.

Digestion by RNase T1. Equal volumes (usually 5 μl) of the RNA solution and RNase T1 (Sankyo, Tokyo) aqueous solution, resulting in salt concentration of 50 mM sodium acetate–5 mM magnesium acetate were incubated at 0° for 90 min.

Polyacrylamide Gel Electrophoresis was carried out with a vertical gel electrophoresis apparatus EC 470 (E-C Apparatus Co., Philadelphia, Pa.) following the procedures of Peacock and Dingman (8). The bands were made visible by autoradiography.

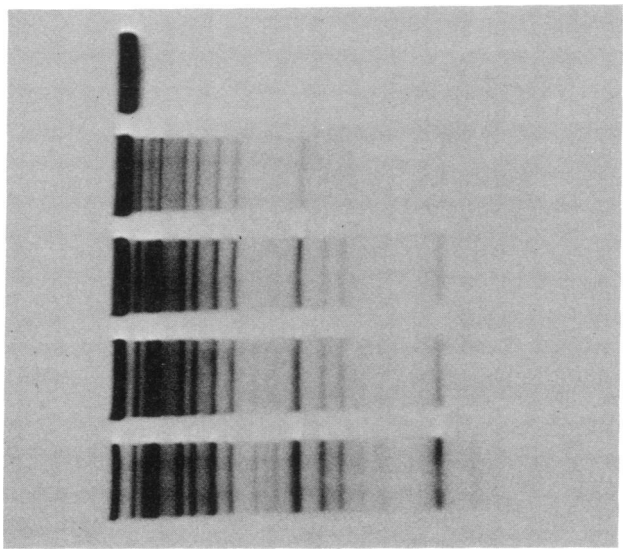

FIG. 1. Polyacrylamide gel electrophoresis of MS2 RNA treated with various amounts of RNase T1. From *top* to *bottom*, the concentration of RNase T1 used was 0, 0.25, 0.5, 0.75, and 1.0 unit/ml. The concentration of RNA was 122 μg/ml for each case. A 3.5% acrylamide gel was used; electrophoresis was performed at 200 V for 2 hr at 19°. All five samples were run simultaneously.

Labeling of 5′ Ends of RNA Fragments. Polynucleotide kinase was purified by the procedures of Richardson (9) and Takanami (10). *E. coli* Q13 was infected by phage T4amN82 to eliminate contaminating RNases and cell lysis. [γ-^{32}P]ATP (13 Ci/mmol) was purchased from New England Nuclear Corp.

RESULTS

Appearance of Bands of Large Fragments. RNA of MS2 bacteriophage was incubated with RNase T1 at 0° for 90 min and the samples were analyzed by polyacrylamide gel electrophoresis (Fig. 1). A limited number of clearly separable bands were observed; by increasing the amount of RNase T1, the bands of smaller fragments became darker. Furthermore the positions of these bands were reproducible. Clearly separable bands also appeared with R17 and Qβ RNAs. The three samples shown in Fig. 2 were run together on the same polyacrylamide gel plate for comparison. One can find no similarity between the patterns of the bands of the fragments of Qβ RNA and R17 or MS2 RNA, while there are many fragments that have the same molecular weights in R17 and MS2 RNAs. These results are consistent with the fact that bacteriophages R17 and MS2 belong to the same group while bacteriophage Qβ is among a different class of bacteriophages and contains an RNA molecule that is larger than either of R17 and MS2 RNAs. In Fig. 2, the bands of large fragments that are visible are numbered. When the autoradiographic films were exposed for longer time periods, more bands appeared. By comparing 10 bands of R17 RNA and 12 bands of MS2 RNA shown in Fig. 2, corresponding bands are found in the two RNAs except for bands 1 and 9 of MS2 RNA. Band 9 of MS2 RNA is a minor band, and the corresponding band of R17 RNA can be seen after a longer film exposure. No band of R17 RNA corresponding to band 1 of MS2 RNA has been seen. Furthermore, it may be noticed that bands 4 and 5 of R17 RNA are present in much smaller amounts than the corresponding

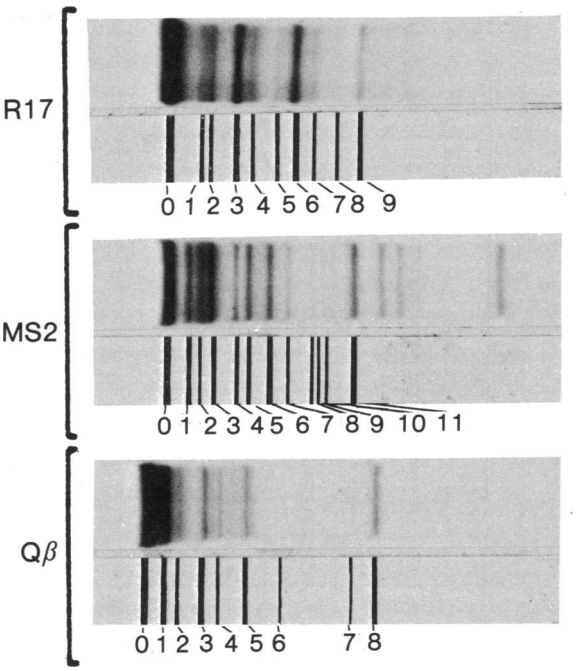

FIG. 2. Comparison of the patterns of three RNAs of RNA bacteriophages treated with 0.5 unit/ml of RNase T1 and simultaneously analyzed by gel electrophoresis as described in the legend of Fig. 1. The concentration of RNA was 180 μg/ml (R17), 199 μg/ml (MS2), and 164 μg/ml (Qβ). Bands labeled 0 are intact molecules.

bands 5 and 6 of MS2 RNA. This fact may be explained by some differences in the secondary structures of the two RNAs, which will be discussed later.

Selection and Ordering of Pairs of Fragments of R17 and MS2 RNAs. The molecular weights of the fragments of R17 RNA were determined from the distances the fragments migrated in a 2.0% acrylamide–0.5% agarose gel (Fig. 3). In a gel of this acrylamide concentration, a linear relation exists between distances migrated and the logarithm of the molecular weights of molecules smaller than intact R17 RNA (8). Intact R17 RNA, and 23S, 16S, and 5S ribosomal RNAs were used as standards. In Table 1, the values of the molecular weights determined in Fig. 3 are listed. From this table, one can find four pairs of fragments whose molecular weights suggest that they might be derived by single cleavages of the whole molecules. These are fragments 3 and 9, and 5 and 6, from R17 RNA and fragments 4 and 11, and 6 and 7, from MS2 RNA.

Next, the relation of the two pairs of fragments from MS2 RNA was studied. RNA of RNA phages has pppGp at the 5′ end and does not act as a substrate for polynucleotide kinase (10). On the other hand, a 5′ end newly formed by RNase T1 does not have a phosphate (3) and, thus, can act as a substrate for polynucleotide kinase. Therefore, one can expect that one of the fragments of a pair will act as a substrate for polynucleotide kinase while the other should not. To test this, unlabeled MS2 RNA was incubated together with RNase T1, polynucleotide kinase, and [γ-^{32}P]ATP, and then run in a polyacrylamide gel to compare it with uniformly ^{32}P-labeled MS2 RNA incubated with RNase T1 (Fig. 4). One can see that fragments 4 and 6 act as substrates for polynucleotide kinase. On the other hand, fragments 7 and 11 do not serve as substrates for polynucleotide kinase. These results suggest that

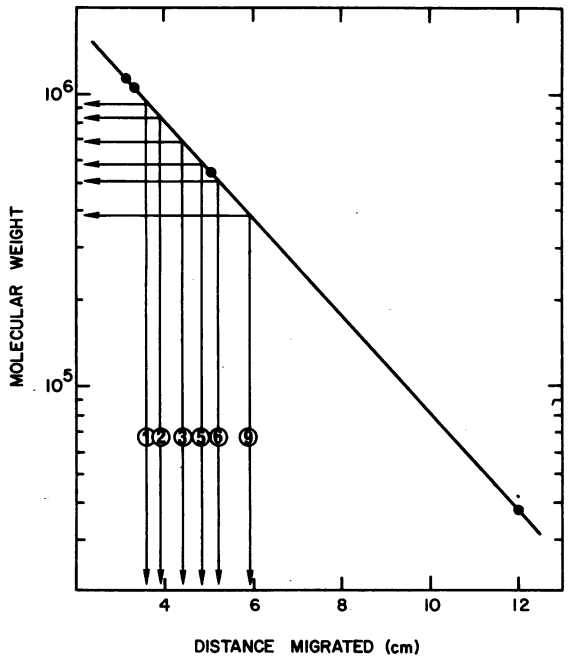

FIG. 3. Determination of molecular weights of six fragments of R17 RNA. A 2.0% acrylamide–0.5% agarose plate was used, and samples were run at 100 V for 4 hr at 19°. Four RNA molecules were used as standards (*filled circles*): intact R17 RNA, and 23S, 16S, and 5S ribosomal RNA molecules. The *circled numbers* indicate the band positions (Fig. 2) of the six fragments of R17 RNA.

MS2 RNA has very sensitive sites for RNase T1 at points 36% and 47% from its 5′ end (Fig. 5). Similar studies of R17 RNA revealed that of the paired fragments 3 and 9, only fragment 3 acts as a substrate for polynucleotide kinase. Attempts to label the fragments 5 or 6 from R17 RNA are complicated

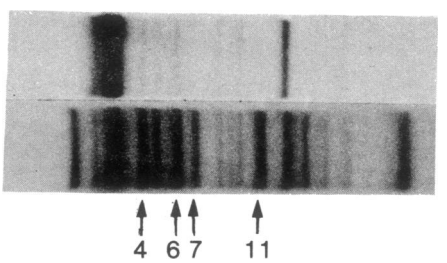

FIG. 4. Demonstration that fragments 7 and 11 of MS2 RNA have original 5′ ends of the molecules. The two samples were run together in a 3.5% acrylamide gel plate for 2 hr at 200 V. The *upper sample* is unlabeled MS2 RNA incubated with RNase T1, polynucleotide kinase, and [γ-^{32}P]ATP. The *lower sample* is uniformly ^{32}P-labeled MS2 RNA incubated with RNase T1. Both samples were incubated at 0° for 90 min.

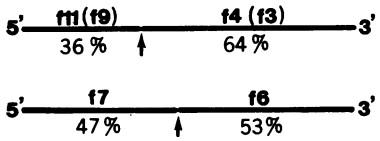

FIG. 5. Model for cleavage of MS2 and R17 RNAs into pairs of fragments. The *parentheses* indicate the fragments of R17 RNA.

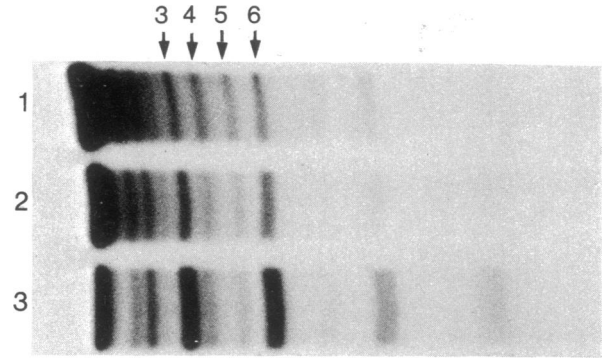

FIG. 6. The change in the amount of fragment 5 of R17 RNA according to the concentration of RNase T1 in the reaction mixture. The concentration of RNase T1 in samples 1, 2, and 3 was 0.025, 0.05, and 0.1 unit/ml, respectively. The concentration of RNA was 175 μg/ml for each sample. A 3.5% acrylamide gel plate was used and electrophoresis was performed at 300 V for 8 hr at 19°.

by the fact that fragment 5 is a minor component. If fragments 5 and 6 of R17 RNA were produced in a pair from an intact molecule and remained as they had been formed, the amount of RNA in fragment 5 should be larger than that of fragment 6 because the molecular weight of fragment 5 is larger than that of fragment 6. The data in Figs. 2 and 4, however, do not show this, probably because fragment 5 is degraded soon after it is formed. It is clearly seen that when R17 RNA is digested to a lesser degree, fragment 5 is present in an amount similar to that of fragment 6 (Fig. 6). As cleavage proceeds, however, the amount of fragment 6 increases and the amount of fragment 5 decreases.

Effect of $ZnSO_4$ on the Pattern of R17 RNA Fragments. Although fragment 5 of R17 RNA is unstable and the fragment is a minor component, the corresponding fragment 6 of MS2 RNA is not. This difference may arise from a difference in the secondary structures of the two RNAs. $ZnSO_4$ inhibits the action of RNase T1 (3), and it is thought to act as an inhibitor by changing the secondary structures of RNA molecules. Fig. 7 shows the result of incubating R17 RNA with RNase T1 and 0.3 mM $ZnSO_4$. It can be seen that fragment 5 becomes stable under these conditions and the pattern of the bands of R17 RNA becomes similar to that of MS2 RNA.

TABLE 1. *Molecular weights of six fragments of R17 RNA determined in Fig. 3*

Fragment of R17 RNA	Corresponding fragment of MS2 RNA	Molecular weight*
1	2	9.4
2	3	8.4
3	4	6.9
5	6	5.8
6	7	5.1
9	11	3.9

Numbers of corresponding fragments of MS2 RNA (see Fig. 2) are listed. The sum of molecular weights of fragments 3 and 9, and 5 and 6, of R17 RNA are 1.08×10^6 and 1.09×10^6, respectively. Molecular weight of the intact RNA is 1.10×10^6 (4).

* Values have been multiplied by 10^{-5}.

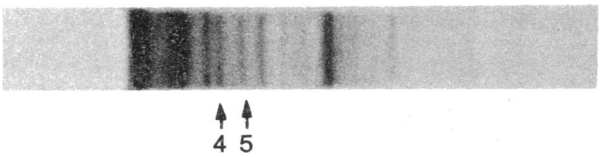

FIG. 7. The change of the pattern of bands of fragments of R17 RNA by addition of $ZnSO_4$ to the reaction mixture. A reaction mixture that contained R17 RNA (123 μg/ml), RNase T1 (6.7 units/ml), $ZnSO_4$ (0.3 mM), ammonium acetate (33 mM), and magnesium acetate (3 mM) was incubated for 90 min at 0°. Electrophoresis of this sample was performed as described in the legend of Fig. 1.

DISCUSSION

It has been shown with MS2 and R17 that three pairs of fragments can be obtained and ordered. Obtaining and ordering pairs of fragments that can be considered to have resulted from specific cleavages of the whole molecules may serve as the first step in the determination of the complete base sequences of these RNAs. Two points, however, must be investigated before these procedures can be extended: (*1*) whether the cleavages are introduced at exactly specific points at the level of base sequences and (*2*) whether or not any small fragments exist between the two pairs of large fragments. These points are related, for if there are small fragments between pairs of the larger fragments, the newly formed ends will probably be heterogeneous. The first question can be answered by analyzing the newly formed ends of R17 and MS2 fragments. The second point may be answered by using a system with unlabeled RNA, RNase T1, polynucleotide kinase, and [γ-^{32}P]-ATP. According to Richardson (9), 3'-AMP and the dinucleotides bearing a 5'-hydroxyl end group serve as better substrates for polynucleotide kinase than 5'-hydroxyl-terminated RNA. Therefore, if some small fragments exist between the pair of major fragments, they ought to be detected without difficulty even if they are as small as one base.

The methodology proposed here may be applied to any RNA molecules that have some secondary structures. Ribosomal RNA, for example, is known to have considerable secondary structure (11), and preliminary results show that *E. coli* 23S and 16S ribosomal RNA can be cleaved into large fragments by RNase T1. These procedures may also be useful in determining the sequence of single-stranded DNA using T4-induced endonuclease IV (12, 13).

MS2 RNA was cleaved by RNase T1 at points 36% and 47% from its 5' end. R17 RNA was cleaved at the 36% point and probably also at the 47% point, though the result was not so clear. These positions of the cleavages might be the same as those of cleavages introduced by RNase IV into R17 RNA. Gesteland and Spahr showed that large fragments of R17 RNA can be produced by RNase IV and that the cleavages are located at two points around the point 40% from the 5' end, one in coat-protein cistron and the other out of the cistron (14). The specificity of RNase IV is not known, but both RNases T1 and IV cleave single-stranded regions better than double-stranded regions and sometimes the two RNases cleave the same position in a molecule (15).

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