*SPECIFIC CLEAVAGE OF Qβ-RNA AND IDENTIFICATION OF THE FRAGMENT CARRYING THE 3′-OH TERMINUS**

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The demonstration[1] that Qβ-replicase[2] can effect the synthesis of biologically competent copies, and that the RNA is the instructive agent,[3] provided a system which for the first time permitted an unambiguous *in vitro* analysis of a replicating mechanism.

Examination of the early course of the reaction revealed[4] the existence of a six-minute latent period which precedes the appearance of the first new infectious strands and which is accompanied by an "eclipse" of the initiating templates as infectious entities. A detailed analysis[5–7] of the early events in the latent period was made possible by the use of electrophoretic separation in acrylamide gels.[8, 9] In these experiments the input RNA was labeled with H^3 and the newly synthesized product with P^{32}, permitting a simultaneous examination of both. The data obtained indicate that all of the first product synthesized is initially associated with H^3-templates in complexes possessing the properties of Hofschneider[10] structures (HS). Subsequently Franklin[11] structures (FS) appear containing template and product. Soon after the formation of FS complexes, mature Qβ-RNA molecules are found.

Further insight into the nature of the Qβ-replicase reaction has been gained from the demonstration[12] that guanosine 5′-triphosphate (GTP) is the first subunit added. This may be related to the poly G polymerase observed[13, 14] in Qβ-replicase preparations, an activity which has been shown[14] to be associated with one of the two protein components required for complete synthetic function by Qβ-replicase.

The rapidity with which information is being generated by study of the Qβ-replicase reaction makes it desirable to begin a more detailed chemical analysis of the Qβ-RNA molecule, with the ultimate aim of obtaining its complete sequence. Partial degradation of RNA with endonucleases has been used in studies leading to the elucidation of the primary structure of sRNA.[15, 16] This technique has also been applied to the fragmentation of ribosomal RNA's;[17–19] and of 5*S* RNA using[20] alkylated ribonuclease.

In principle these procedures can be applied to any homogeneous population of RNA molecules. We report here a technique for the production of two specific fragments from the RNA of the coliphage Qβ.[21] The fragments have been characterized and their orientation with respect to the original RNA strand determined.

Materials and Methods.—(a) *Preparation of Qβ-RNA and P^{32} Qβ-RNA:* Qβ-phage were grown, harvested, purified, and the RNA extracted as described previously.[22, 23]

(b) *Preparation and isolation of fragments:* Purified Qβ-RNA was suspended in 0.2 *M* Tris-HCl, pH 8.5, at a concentration of 1 mg per ml. Pancreatic RNase was added to a final concentration of 0.2–2.0 mμg per ml, depending on the degree of fragmentation desired. The mixture was incubated for 30 min at 0°C and the reaction stopped by the addition of 50 μl of 10% sodium dodecyl sulfate (SDS). The mixture was then layered directly onto 30-ml gradients of 5–20% sucrose in 0.01 *M* Tris-HCl, pH 7.4, 0.005 *M* $MgCl_2$ (TM buffer) and centrifuged for 14–16 hr in the Spinco SW25 rotor at 25,000 rpm and a rotor temperature of 4°C. One-ml fractions were collected by

puncturing the bottom of the tube, and the optical density of each fraction read at 260 mμ. When the fragments were to be used for further experimentation, the tubes containing the desired fractions were pooled, the RNA precipitated twice with 2.5 vol of absolute ethanol, and resuspended in TM buffer.

For the preparation of P^{32}-fragments, where smaller amounts of RNA were treated, the concentration of RNase was reduced approximately tenfold. The rate of the fragmentation reaction is dependent upon the enzyme-to-substrate ratio as well as the concentration of the RNase, and some empirical explorations are necessary to determine the optimum fragmentation conditions.

(c) *Base composition of the fragments:* Base compositions of fragments uniformly labeled with P^{32}-orthophosphate were determined by procedures previously described[24] except that the size of the Dowex-1-X8-formate column was reduced to 5 × 20 mm. The reduction allowed for more rapid determinations because of a faster response to changes in eluting buffers.

(d) *Molecular weight and size determinations:* The molecular weights of the fragments were estimated from the distribution of radioactivity between the fragments after partial fragmentation of RNA uniformly labeled with P^{32}-orthophosphate. Since there was no loss in acid-precipitable counts during the reaction, the percentage of radioactivity found in each fragment is directly proportional to its molecular weight. To correct for breakdown of the large fragment, the radioactivity was measured in both the large and the small fragment in six different reactions where the fragmentation varied from 53 to 83%. The resulting data were then extrapolated to zero fragmentation of the large fragment.

Sedimentation coefficients of the fragments were determined by mixing isolated P^{32}-labeled fragments with H^3-labeled bulk RNA from *E. coli* and centrifuging through sucrose gradients as described in section (*b*) above. Fractions were precipitated with cold 5% trichloroacetic acid (TCA), filtered onto membrane filters (Schleicher and Schuell, B-6), washed with 5% TCA, dried, and the radioactivity determined in a liquid scintillation spectrometer.

(e) *Reaction with H^3-iso-nicotinic hydrazide:* Qβ-RNA was coupled with H^3-*iso*-nicotinic hydrazide by a modification of the procedure of Hunt.[25] Qβ-RNA was suspended in 0.1 *M* sodium acetate buffer, pH 5.2, at a concentration of 1 mg per ml. A 100-fold molar excess of sodium metaperiodate was added and incubated for 30 min at 0°C. The oxidized RNA was precipitated with 2.5 vol of cold absolute ethanol, centrifuged, dried, and resuspended at the original concentration in fresh buffer. A 500-fold excess of H^3-*iso*-nicotinic hydrazide (200 μc/μmole; Nuclear-Chicago Corp.) was added and the reactants incubated for 60 min at 25°C. The RNA was precipitated twice with 2.5 vol of ethanol to remove most of the excess *iso*-nicotinic hydrazide. The RNA was then purified by centrifugation through sucrose gradients as described above, isolated, precipitated with ethanol, and resuspended in 0.2 *M* Tris-HCl, pH 8.5, for fragmentation. The fragmentation of the RNA, isolation of the fragments, and radioactivity measurements were carried out as described for unlabeled RNA (§*b*).

Results.—(a) *Fragmentation of Qβ-RNA:* The course of the fragmentation reaction is shown in Figure 1. It is clear that, under the conditions described, the initial cleavage of Qβ-RNA by pancreatic RNase occurs almost exclusively in a very restricted region of the strand to produce two fragments. At higher concentrations of ribonuclease or after more prolonged digestions, further degradation is observed, especially of the initial large fragment. The products of this more extensive breakdown are approximately the same size as the original small fragment, but are heterogeneous as can be seen by the increasing width of the peak in the small fragment region of Figure 1*C*.

(b) *Base composition of the fragments:* The base composition of the fragments and whole Qβ-RNA are given in Table 1. The base compositions of both fragments are similar to that of the whole RNA.

(c) *Molecular weights and sizes of the fragments:* The molecular weight of whole Qβ-RNA has been determined by light scattering to be 1×10^6 daltons.[23] After correction for secondary breakdown of the large fragment, it was found that the large fragment constituted 68 per cent of the original strand and the small fragment

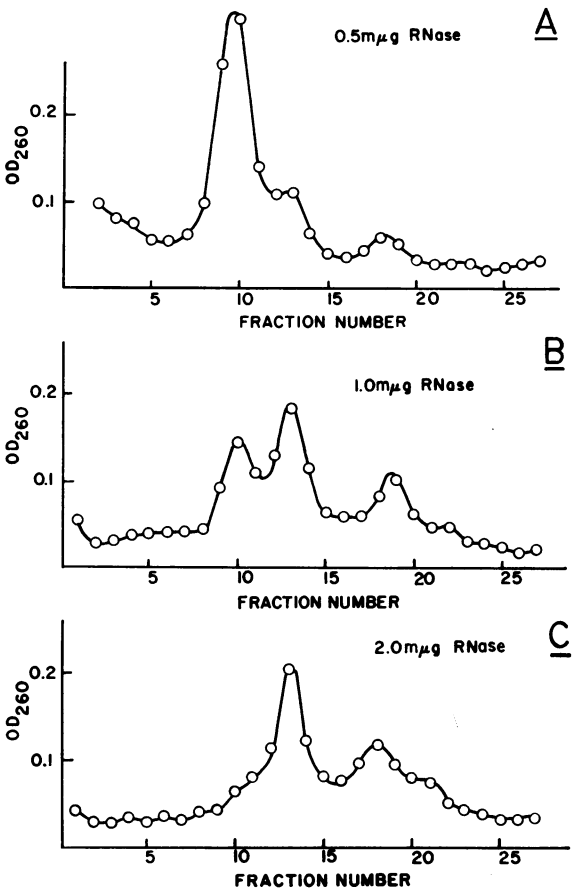

FIG. 1.—Fragmentation of Qβ-RNA by RNase: 200 μg Qβ-RNA in 1 ml of 0.2 *M* Tris-HCl buffer, pH 8.5, incubated 30 min with 0.5 mμg RNase (*A*), 1.0 mμg RNase (*B*), and 2.0 mμg RNase (*C*) at 0°C. The reaction was stopped with 20 μl 10% SDS, layered onto 5–20% linear sucrose gradients, and centrifuged 15 hr at 25,000 rpm in Spinco SW25 rotor.

32 per cent. These values correspond to molecular weights of 6.8×10^5 and 3.2×10^5.

Sedimentation coefficients for the large and the small fragments are 22*S* and 14*S*, respectively. The combined sedimentation patterns of isolated fragments and bulk *E. coli* RNA provided three markers with known sedimentation coefficients

TABLE 1

BASE COMPOSITIONS OF UNIFORMLY LABELED WHOLE Qβ-RNA AND LARGE AND SMALL FRAGMENTS

	C	A	U	G
Whole Qβ	24.7	22.3	29.4	23.7
Large fragments	23.4	23.5	29.0	24.1
Small fragments	24.9	22.1	29.6	23.4

Determinations were carried out as described in section (c) of *Materials and Methods*. The numbers recorded are in mole per cent.

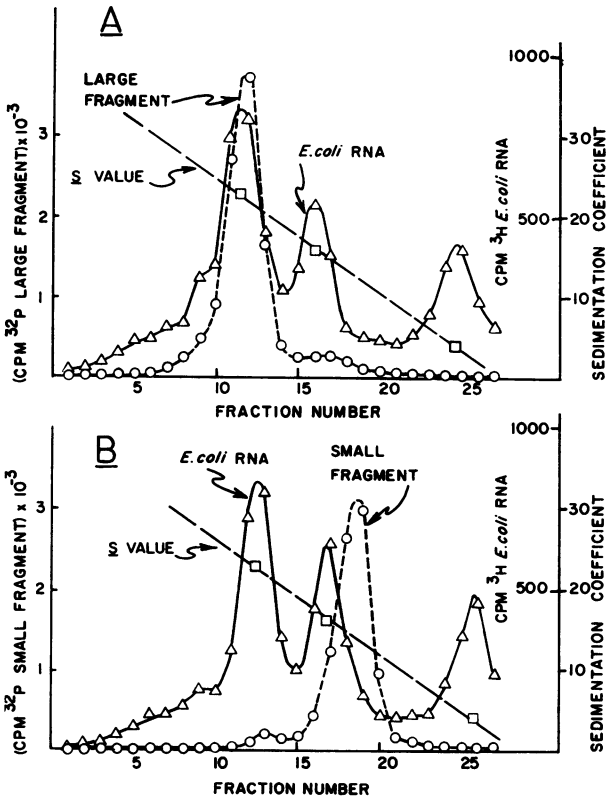

FIG. 2.—Sucrose gradient centrifugation of isolated [P^{32}] large fragment (*A*) and [P^{32}] small fragment (*B*) with [H^3] *E. coli* bulk RNA: 10,000 cpm each of fragment and *E. coli* RNA mixed, layered onto 5–20% linear sucrose gradients, centrifuged 15 hr at 25,000 rpm in Spinco SW25 rotor, and fractionated by puncturing the bottom of the tubes. Fractions were precipitated with 5% TCA, washed onto membrane filters, and counted in a liquid scintillation spectrometer. Sedimentation coefficients were established by the known values of the three *E. coli* components.

which are linearly related. This allowed an estimation of the sedimentation coefficients of the fragments. The accuracy of this method was checked by running an identical experiment using whole Qβ-RNA. The value obtained was 28*S*, in agreement with the value established by other procedures.[22, 23]

(d) *Orientation of the fragments:* After oxidation, coupling with H^3-*iso*-nicotinic hydrazide, and repurification of the RNA, the molar ratio of *iso*-nicotinic hydrazide to RNA was calculated to be 0.8. The reaction was essentially complete after 30 minutes. This is in contrast to the results reported[25] with ribosomal RNA which took 20 hours for complete reaction. This discrepancy could be due to differences in the availability of the oxidized terminal nucleotide for coupling. The use of greater excesses of sodium periodate in the oxidation reaction resulted in higher and nonspecific levels of *iso*-nicotinic hydrazide binding.

The sedimentation profile of the partially fragmented RNA specifically labeled in

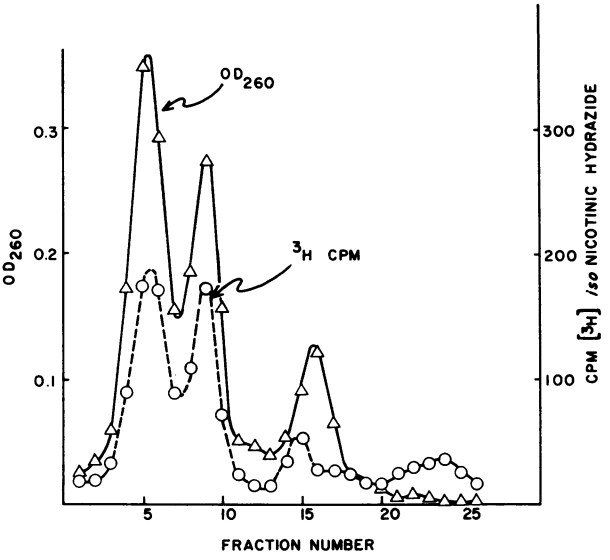

FIG. 3.—RNA coupled with [H^3] *iso*-nicotinic hydrazide, purified, and fragmented as described in *Materials and Methods* (§*b*).

the 3′-terminal nucleotide is shown in Figure 3. The specific activity of the unfragmented RNA is unchanged by the fragmentation reaction. The major proportion of the radioactivity in the lighter region of the gradient coincides with the large fragment. The specific activity of this fraction is approximately 30 per cent higher than that of the unfragmented fraction, in good agreement with the increase expected on the basis of coupling to a strand approximately two thirds as large as whole Qβ-RNA. The secondary breakdown of the large fragment into smaller pieces of approximately the same size as that of the original small fragment creates some "noise" in this region of the gradient. The radioactivity in the small fragment region is clearly polydisperse, and there is no peak coincident with that of the small fragment. Furthermore, the specific activity in this region is much lower than even that of the whole Qβ. On these grounds one can definitely identify the large fragment as the carrier of the 3′-end of the original strand.

Discussion.—The specific fragments produced from Qβ-RNA and the further breakdown of the large fragment are similar to the action of endonuclease on sRNA[15] and ribosomal RNA's.[17] Particular regions of the RNA strand show a much greater sensitivity to attack than the remainder of the molecule. This sensitivity may be due to sequences of nucleotides especially vulnerable to nuclease, or to regions being in exposed positions because of the secondary structure of the RNA.

Similar fragmentation of both MS-2 and Qβ-RNA is observed during protein synthesis using these RNA's as messages in an *in vitro* amino acid-incorporating system derived from *E. coli*. The rate of fragmentation varies with the strain of *E. coli* used for the S-30 extracts, but the general pattern is the same with extracts from wild type, A 19, a mutant lacking RNase activity, and Q 13, a mutant lacking

RNase and polynucleotide phosphorylase activities.[26] Thus the specificity of the reaction seems to be a function of the RNA, and not the nuclease. This would indicate that the secondary structure of the RNA may be the essential determinant of the reaction.

In addition to sequence studies, the availability of specific fragments of phage RNA makes possible a number of informative experiments. It has been shown that RNA replicases require their homologous RNA as templates.[2, 27, 28] It has further been shown[29] that the molecules must be intact for significant activity to occur. A careful examination of the limited reaction mediated by the two fragments singly and in combination should illuminate the nature of the recognition mechanism. Because of the polycistronic character of the phage genome,[30] studies of the template activity of fragments can provide information about the mechanism of translation of complex message RNA. Knowledge of the orientation of the fragments in the original strand provides a method for the determination of the sequence of cistrons in the complete strand. A preliminary report of experiments along these general lines has been reported recently.[31]

Summary.—RNA from Qβ-phage is broken into two specific fragments by treatment with very low concentrations of pancreatic RNase. The larger fragment has a molecular weight of approximately 6.8×10^5 and a sedimentation coefficient of 22*S*. The smaller fragment has a molecular weight of 3.2×10^5 and a sedimentation coefficient of 14*S*. The base composition of both fragments is similar to that of complete strands of Qβ-RNA. The orientation of the fragments in the complete strand was determined by specific coupling of H^3-*iso*-nicotinic hydrazide with the 3′-hydroxy-terminal nucleotide of the complete strand followed by fragmentation. The distribution of the radioactivity in the fragments showed that the large fragment was derived from the portion of the Qβ strand containing the 3′-hydroxy-terminal nucleotide.

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