Proc. Nat. Acad. Sci. USA
Vol. 69, No. 10, pp. 3033–3037, October 1972

Nucleotide Sequence at the Binding Site for Coat Protein on RNA of Bacteriophage R17

(RNA bacteriophages/translational repression/RNA synthetase)

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Communicated by J. D. Watson, August 7, 1972

ABSTRACT **The binding of a few molecules [1–6] of RNA bacteriophage coat protein to 1 molecule of RNA represses *in vitro* translation of the RNA synthetase cistron. Digestion of the complex, R17 coat protein–R17 RNA, by T1 RNase yields an RNA fragment bound to the coat protein. The nucleotide sequence of this fragment (59 residues) reveals that it contains the punctuation signal between the coat protein and RNA synthetase cistrons, suggesting that this is the site on the RNA where the coat protein acts as a translational repressor.**

Coat protein of RNA bacteriophages functions as a repressor of translation of the cistron coding for RNA synthetase (the phage-coded subunit of the RNA replicating enzyme) (1–8).

Isolated RNA and coat protein can interact to form two kinds of complexes: Complex I in which a few molar equivalents of coat protein [one (9)–six (10)] bind to one equivalent of RNA leading to formation of a complex that sediments at the same rate as free RNA, and Complex II in which about 180 coat-protein molecules bind to one molecule of RNA to form a complex that is phage-like, but not infectious (10). Complex I has been implicated in translational control of the RNA synthetase cistron since addition of coat protein to phage RNA reduces its messenger activity in an *in vitro* protein-synthesizing system and depresses the synthesis of the RNA synthetase (4–6, 9); it specifically inhibits the initiation step of that synthesis (8, 11–13). The mechanism by which the coat protein prevents translation of the RNA synthetase cistron is not known. We present evidence that the coat protein binds to the initiation site of the RNA synthetase cistron and directly prevents the ribosomes from translating that cistron. We show that when Complex I is formed *in vitro* and then incubated with T1 RNase, an RNA fragment is protected from nucleolytic degradation; this fragment (59 residues) contains the nucleotide sequence of the region of the R17 RNA preceding the cistron coding for the RNA synthetase and the first two codons of that cistron. The protection by the coat protein of this region from nuclease degradation is specific: when R17 RNA is mixed with the coat protein of Qβ (an RNA phage serologically unrelated to R17) and the mixture is degraded by T1 RNase, no comparable fragment is found.

MATERIAL AND METHODS

[^{3}H]Uridine-labeled R17 RNA (2.2×10^4 cpm/μg) and ^{32}P-labeled R17 RNA (3×10^6 cpm/μg) were prepared as in refs. 14 and 15, respectively. Coat proteins of phages R17 and Qβ were extracted by the acetic acid procedure (10); the ability of these protein preparations to repress *in vitro* synthesis of RNA synthetase was tested (4). Pancreatic RNase was obtained from Worthington and T1 and U2 RNases from Sankyo. Acid RNase and acid phosphatase B from spleen were a gift from Dr. G. Bernardi; they were prepared as described (16, 17).

Formation of Coat Protein–RNA Complex and Isolation of the Fragment Protected from Nuclease Degradation. The incubation mixture contained in a final volume of 0.6 ml: ^{32}P-labeled R17 RNA (1.15 nmol; 3.2×10^9 cpm), R17 coat protein (4.5 nmol), 100 mM Tris·HCl (pH 7.5), 10 mM Mg acetate, and 80 mM KCl (TMK buffer). After 10 min at 0°, 0.18 ml of a T1 RNase solution (0.7 mg/ml in 10 mM Tris·HCl pH 7.5–2 mM EDTA) were added. The mixture was incubated at 22° for 30 min, then chilled on ice and layered onto two sucrose density gradients (10–20% sucrose in TMK buffer) and centrifuged at 35,000 rpm for 18 hr at 4° in a SW41 Spinco rotor. The gradients were collected at 4° into plastic tubes (27 fractions). Aliquots (5 μl) were diluted with 1 ml of cold TMK buffer, immediately filtered under slow suction through Millipore filters (HAWP, 0.45 μm, 25 mm), washed 3 times with 1-ml portions of cold TMK buffer, dried, and counted in toluene scintillator. Fractions containing the radioactive peak were pooled and diluted 10 times with cold TMK buffer. Each 2-ml aliquot was filtered on a stack of three Millipore filters and washed with 20 ml of cold TMK buffer. The material on the filters was eluted by a modification of the procedure described (18). The filters were soaked for 5 min in 5 ml of 0.5% sodium dodecyl sulfate and 5 ml of phenol saturated with 0.5% sodium dodecyl sulfate containing 2 mM EDTA. After shaking on a Vortex mixer for 5 min, the mixture was centrifuged, the aqueous layer was saved, and the phenol phase was extracted three times. To the combined aqueous extracts were added: NaCl (final concentration 0.4 M), tRNA as a carrier (5 μg/ml), and 2 volumes of ethanol. After standing overnight at −20°, the precipitate was collected by centrifugation, dissolved in 1 ml of 0.2 M Tris-acetate buffer (pH 8.3), washed three times with ether, and precipitated with 2 volumes of ethanol. The precipitate was dissolved in 50 μl of 20 mM Tris-acetate buffer (pH 8.3) containing 2 mM EDTA and 10% sucrose; two dye markers (xylene F.F. cyanol and bromophenol) were added, and the solution was loaded onto a 12.5% polyacrylamide gel (20 × 40 × 0.3 cm) prepared as described (19). After electrophoresis (300 V, 34 hr at 4°) the gel was removed and autoradiographed. The bands were eluted (20).

Nucleotide Sequence Determination. The sequence of the total digestion products of the R17 RNA fragment by T1

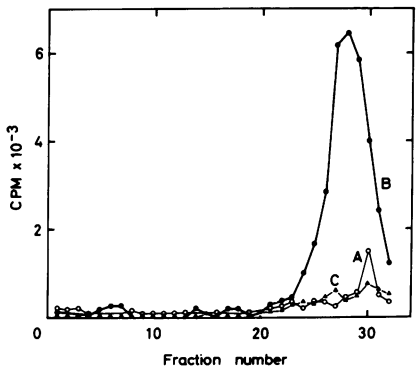

FIG. 1. T1 RNase degradation of R17 RNA complexed or not with R17 coat protein. (*A*) R17 RNA + T1 RNase. R17-[^{3}H]RNA (25 μl, 45 pmol, 2.9 $\times$ 10^6 cpm) was mixed with 10 μl of buffer (50 mM Tris·HCl, pH 7.5–50 mM Mg acetate–0.4 M KCl) and 15 μl of H_2O, and the mixture was left at 4° for 10 min. T1 RNase (6.3 μg) was added. The mixture was incubated at 22° for 30 min, then layered onto a sucrose gradient (5 ml, 5–20% sucrose in 10 mM Tris·HCl–10 mM Mg acetate–80 mM KCl) and centrifuged at 55,000 rpm for 3 hr in the SW 65 Spinco rotor. Fractions were collected into plastic tubes, 0.5 ml of cold TMK buffer was added, and the solution was filtered on Millipore filters under slow suction and washed three times with 1 ml of TMK buffer. The filters were dried and counted in toluene scintillation fluid, with an efficiency of 20%. Sedimentation is from *right* to *left*. (*B*) R17 RNA + R17 coat protein + T1 RNase. Same conditions as for *A*, except that R17 coat protein (7 μl, 225 pmol) was added to the RNA before T1 digestion. (*C*) R17 RNA + T1 RNase + R17 coat protein. Same conditions as for *A* except that R17 coat protein (7 μl, 225 pmol) was added after digestion of RNA by T1 RNase.

and pancreatic RNases was analyzed (21). The 5′-end of the oligonucleotides was determined with the following modification: acid phosphatase II from spleen was used instead of *E. coli* alkaline phosphatase; since the spleen enzyme is heat labile, it can be inactivated by heating the dephosphorylated product at 80° for 20 min. The dephosphorylated oligonucleotide is dried on a polythene sheet and then analyzed (21).

RESULTS

Degradation of the Complex R17 RNA–Coat Protein by T1 RNase. In order to analyze degradation of the RNA–coat protein complex by T1 RNase, use was made of the observation that coat protein is retained on Millipore filters. When R17 [^{3}H]RNA is digested with T1 RNase in TMK buffer and the resulting mixture is analyzed by sucrose density-gradient centrifugation followed by Millipore filtration of the fractions, very little of the radioactivity is found, as expected (Fig. 1*A*). In contrast, when the complex, R17 coat protein–R17 RNA (molar input ratio 5), is subjected to the same treatment, some radioactive material (about 2% of the input) is retained on Millipore filters and sediments as a peak near the top of the gradient (Fig. 1*B*). If coat protein is added to the RNA after T1 RNase digestion, one does not observe any significant radioactive peak (Fig. 1*C*): this experiment rules out the possibility of an artifact resulting from the affinity of the protein to any of the oligonucleotides produced by degradation of the RNA by T1 RNase.

Isolation and Sequence Analysis of the R17 RNA Fragment Protected by the Coat Protein. Highly labeled R17 [^{32}P]RNA complexed to R17 coat protein was degraded by T1 RNase, and the protected fragment was isolated. Fig. 2*A* shows the result of gel electrophoretic analysis of the material retained on Millipore filters and Fig. 2*B* that of the material before Millipore filtration. The major band (Fig. 2*A*) migrates with a mobility corresponding to that of an oligonucleotide about 50–60 nucleotides in length; minor bands were present but varied in number, intensities, and electrophoretic mobilities in different experiments. The major band was eluted and digested with T1 or pancreatic RNases. The products were analyzed by the fingerprinting method (Fig. 3). The sequence of all oligonucleotides was determined directly after elution from the paper except for one spot from the T1 RNase digest (T8 + T9 on Fig. 3), which was resolved into its two components by monodimensional homochromatography on DEAE thin layer with solvent C (22). The sequence determination of the four larger T1 oligonucleotides is presented in Table 1, and sequences of the other T1 products are shown in Fig. 4. Sequences of the pancreatic RNase digestion products are indicated in Fig. 3.

Except for one base change, the sequence of the T1 and pancreatic RNases products of the RNA fragment is identical

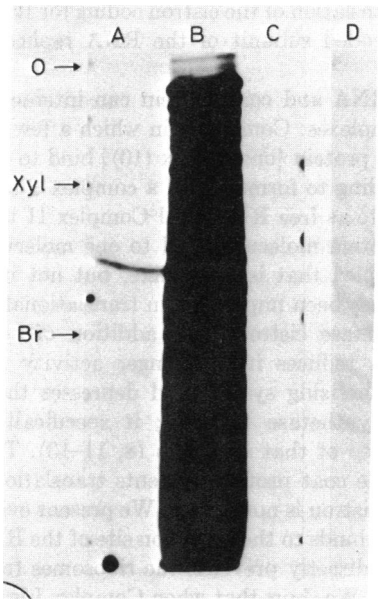

FIG. 2. Gel electrophoresis of T1 RNase digests of ^{32}P-labeled R17 RNA and of its complex with R17 or Qβ coat protein. See *Methods* for conditions for electrophoresis. *O*, point of application of the sample; *Xyl* and *Br*, position of the xylene cyanol marker dye and of bromophenol blue, respectively.

(*A*) The complex R17[^{32}P]RNA–R17 coat protein was formed, degraded by T1 RNase, and the protected fragment was isolated. 3 $\times$ 10^6 cpm were loaded on the gel, which was autoradiographed for 60 min. (*B*) The complex R17[^{32}P]RNA–R17 coat protein was formed and digested with T1 RNase as in *A*, but the Millipore filtration step was omitted. 1.2 $\times$ 10^9 cpm were applied to the gel, which was autoradiographed for 60 min. (*C*) Same as *A* except that Qβ coat protein was used instead of R17 coat protein and the experiment was scaled down 4-fold. 0.3 $\times$ 10^6 cpm were loaded on the gel. Autoradiography was for 60 min. Another autoradiogram of the same gel was done for 16 hr, revealing only a diffuse background without any discrete bands. (*D*) Same as *A* but R17 coat protein was omitted. 0.8 $\times$ 10^6 cpm were applied to the gel, which was autoradiographed for 16 hr.

with the sequences already established by Nichols (25) and Argetsinger–Steitz (26) for Fragments A and B, respectively (Fig. 4). This leaves no doubt that the sequence of the major band found in Fig. 2*A* comprises part of the cistron coding for the coat protein, the intercistronic region, and the codons for the first two amino acids of RNA synthetase. The oligonucleotides located at both ends of the RNA fragment were present in the same molar yields as those in the internal region, indicating that the fragment has no frayed ends and that it is cut cleanly by T1 RNase. We have also looked for hidden breaks by submitting the fragment to electrophoresis on cellulose acetate strips at pH 3.5 in the presence of 7 M urea followed by homochromatography at 60°, using solvent A (22). The radioactivity still moved as a single component whose fingerprint, after T1 RNase digestion, was identical to that of the original band eluted from the polyacrylamide gel.

Specificity of the Protection of the RNA Fragment by Coat Protein. R17 is serologically related to the phages f2 and MS2 but not Qβ. f2 coat protein can repress *in vitro* synthesis of MS2 RNA synthetase and vice versa, but Qβ coat protein cannot (5, 6, 27). To test the specificity of our protection experiment we incubated Qβ coat protein with R17 RNA and submitted the mixture to the same treatment described for the homologous system. Although the percentage of radioactivity retained on the Millipore filters was close to that found in the homologous mixture, the material analyzed by gel electrophoresis showed no discrete bands but only a diffuse background (Fig. 2*C*). As a further control the same experiment shown in Fig. 2*A* was done, but coat protein was omitted. As expected, only a very small percentage of the radioactivity was retained by the Millipore filters (see also Fig. 1*A*), and the gel electrophoretic analysis of that material revealed only faint bands (Fig. 2*D*).

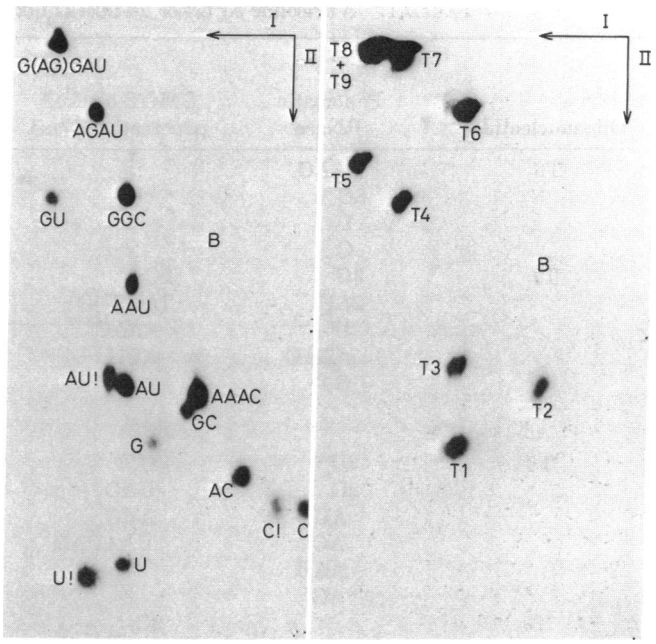

FIG. 3. Fingerprints of the pancreatic or T1 RNase digests of the RNA fragment. The material eluted from the band (Fig. 2*A*) was digested with pancreatic or T1 RNase at 37° for 30 min (enzyme-to-substrate ratio 1:20). Electrophoresis was done on cellulose acetate pH 3.5 (*arrow I*) and on DEAE paper in 7% formic acid (*arrow II*). *B* indicates the position of the blue dye marker. *Left:* pancreatic RNase digest. *Right:* T1 RNase digest. The spot labeled T1 is *G*.

DISCUSSION

The nucleotide sequence of the R17 RNA fragment protected from nuclease degradation contains at its 5′-end the codons for the last 6 amino acids of the coat protein. However, the R17 RNA–coat protein complex can still direct *in vitro* synthesis of the coat protein to the same extent as the RNA alone and must, therefore, allow translation of the codons for the C-terminal amino acids of that protein. This apparent contradiction could be explained if the fragment we have obtained bears a nucleotide sequence that is not directly complexed with the coat protein but is not cleaved away by T1 RNase. This possibility appears likely in view of the secondary structure of the nucleotide sequence at the 3′-end of the coat protein cistron. Nichols (25) has isolated, from a partial T1 RNase digest of R17 RNA, a fragment (Fragment A, on Fig. 4) the sequence of which contains the codons for the last six amino acids of the coat protein and a portion of the intercistronic region. Part of this sequence can be arranged in a hairpin loop where 9 out of 10 base pairs are

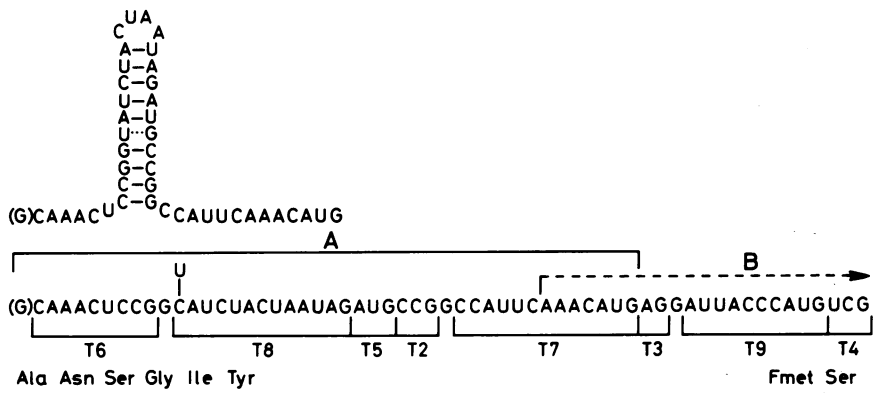

FIG. 4. Nucleotide sequence of the RNA fragment. All the oligonucleotides (T2–T9) obtained after complete digestion with T1 RNase of the band eluted from the gel (Fig. 2*A*) are aligned according to the sequence determined by Nichols (25) (*line A*) and by Argetsinger–Steitz (26) (*line B*). The amino-acid sequence on the *left* is that of the C-terminal end of the coat protein and on the *right* that of the N-terminus of the RNA synthetase. The hairpin loop structure proposed for Sequence A (25) is illustrated on top of the figure.

TABLE 1. *Nucleotide sequence analyses of oligonucleotides T6, T7, T8, and T9 shown in Fig. 4*

Oligonucleotide	Digestion products: Pancreatic RNase	CMCT-blocked pancreatic RNase	Spleen acid RNase	U2 RNase	Sequence
T6	AAAC		CAAAC	A,A	CAAACUCCG
	3C		UCCG	C,A	
	U			C,C,C,U,G	
	G				
T7	3C	2C	C,U,AAAC	UG	CCAUUCAAACAUG
	2AU	(AU,U)C	U,U,C,AA	C,C,A	
	U	AAAC	UG	A,C	
	AAAC	AUG	AAAC	AAAC,AU	
	G		AC	3A	
				2U	
				1C	
T8	2U	C	U,AU,C,U,AC	C,A	CAUCUACUAAUAG
	2C	UAC	UAG	G	
	AU	AUC	U,AA	UA	
	AC	UAAUAG	AC,AAU,U,U	C,U,A	
	AAU			UAG	
	AG			U,U,C,A	
T9		2C		A,C	AUUACCCAUG
		AUG		AU,U	
		AUUAC		CCA	
				UG	

Conditions for digesting the oligonucleotides with pancreatic and U2 RNases have been described (23). CMCT [*N*-cyclohexyl-*N*-(β-morpholinyl-(4)-ethyl) carbodiimide-methyl-*p*-toluene sulphonate] — modified oligonucleotides were digested with pancreatic RNase and the product was identified (19); in some cases the blocking groups were removed before electrophoretic separation (24). The oligonucleotides eluted from DEAE thin-layer plates (T8 & T9) with spleen acid RNase were digested with 3 units (16) of enzyme in 5 μl of 50 mM Na-acetate–10 mM EDTA for 2 hr at 37°; the same conditions were used for the oligonucleotides eluted from DEAE paper (T6 & T7), except that carrier R17 RNA (10 μg) was added and 2 units (16) of enzyme were used for 15 min at 37°. The digestion products were examined by electrophoresis on DEAE paper at pH 1.9.

hydrogen bonded (Fig. 4). That this structure is likely to exist is shown by the resistance to T1 RNase of the G residues in the double helical region of the loop. It is therefore possible that the left-hand portion of the fragment we have isolated, which contains that hairpin loop, is not directly protected by the coat protein but is not cleaved by the T1 RNase away from the truly protected sequence.

Our results strongly suggest that the sequence actually protected by the coat protein must comprise the punctuation between the two cistrons and the first codon of the RNA synthetase cistron. Even under mild conditions of degradation of the RNA by T1 RNase there is no production of a fragment bearing the intact intercistronic region, but only of a fragment containing part of that region (Fragment A on Fig. 4); this indicates that some G residues in the intercistronic region are easily accessible to degradation by T1 RNase under mild conditions. In contrast, even under our conditions of total T1 RNase degradation of the RNA–coat protein complex we find a fragment that contains that intercistronic region where all the G residues are totally resistant to T1 RNase since there are no hidden breaks in the fragment. That at least the first codon of the RNA synthetase cistron found at the 3′-end of our protected fragment (AUG, UCG) also interacts with the coat protein is suggested by the fact that in the fragment the G residue of the triplet AUG is totally resistant to T1 RNase and we do not observe any hidden breaks or frayed ends. It seems, therefore, that at least part of the intercistronic region (and possibly all) and the first codon of the RNA synthetase cistrons are the nucleotide sequence that is recognized by the coat protein acting as a repressor.

When the sequence determined by Nichols (25) is compared with that reported here, there is a change of one nucleotide, U to C in our sequence (Fig. 4). This change occurs at the third position of the triplet coding for the last but two amino acids at the C-terminal end of the coat protein and does not alter its coding properties. It is the second spontaneous mutation we observed since our phage stock was separated from that used by Nichols (25); the first mutation involved a G to A change in the sequence preceding the initiation of the coat protein cistron (29).

The coat protein of bacteriophage Qβ cannot act as a repressor of *in vitro* translation of the RNA synthetase cistron of phages f2 or MS2. The fact that we find no protected fragment when Qβ coat protein is reacted with R17 RNA, lends support to our evidence that the R17 coat protein interacts specifically with the intercistronic region and the beginning of the RNA synthetase cistron. This specificity of interaction between RNA and coat protein is reflected in the similarity of the nucleotide sequence of the intercistronic region of the RNA phages, whose coat protein and RNA can crossreact, and of one region of the amino-acid sequence of their coat proteins. Thus, for MS2 (30), R17 (25), and for the part of f2 that has been analyzed (28), the nucleotide sequence of the intercistronic region is identical but for one base change in R17. The amino-acid sequence of the coat protein of MS2 (30) differs in three positions from that of R17 (31) and in a

fourth place from that of f2 (32), whereas that of fr [the coat protein of which can repress *in vitro* f2 RNA (6)] has as many as 20 amino-acid changes (33); yet the amino-acid sequence of a region containing 35 residues (position 20–54) is identical in all four coat proteins. Since the coat proteins of these phages can crossrepress their RNAs this region probably plays an important role in the process of recognition between coat protein and RNA.

We thank Drs. R. F. Gesteland and D. Kolakofsky for reading the manuscript. This work was supported by a grant from the Fonds National Suisse de la Recherche Scientifique.

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