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The Sequence at the 5′ Terminus of a Self-Replicating Variant of Viral Qβ Ribonucleic Acid*

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ABSTRACT: Nearest-neighbor analysis and resolution by DEAE column chromatography was used to identify the 5′-terminal sequence of a self-replicating variant derived from viral Qβ ribonucleic acid. The data indicate that the *plus* strand begins at the 5′ terminus with the octanucleotide $ppp(Gp)_4(Ap)_2(Cp)_2\ldots$ RNase T_1 digests of the appropriately labeled 5′ fragments confirms the sequence specified. The 5′ end of the complementary *minus* strand of the variant was shown to contain a much longer purine sequence starting with guanosine triphosphate. The 3′ end of the *minus* strand probably terminates with . . . GpGpUpUpCpCpCpC.

The availability of purified (Pace and Spiegelman, 1966a) Qβ replicase (Haruna and Spiegelman, 1965) which can mediate a virtually unlimited synthesis of biologically functional viral RNA (Spiegelman *et al.*, 1965) has made possible a variety of informative investigations into the mechanism of RNA replication. The use of a temperature-sensitive mutant led to the demonstration (Pace and Spiegelman, 1966b) that the RNA is the instructive agent in the replicative process. This finding rigorously justified the conclusion that the *in vitro* synthesis of a self-replicating molecule had been achieved.

Among the interesting possibilities thus generated was the feasibility of performing Darwinian experiments in which the molecules are exposed during replication to a variety of selection pressures. Mills *et al.* (1967) reported experiments designed to select molecules which could replicate faster than the original viral RNA. During the course of the serial transfers involved, variants of decreasing length made their appearance sequentially. The mutant isolated after the 74th transfer replicated some 15 times faster and contained only 550 of the 3600 residues present in the original Qβ-RNA. It has recently been shown (Levisohn and Spiegelman, 1968) that synthesis of copies by Qβ replicase can be initiated with a single strand of variant RNA. The clones produced provide the sort of uniformity required for chemical analysis of the RNA molecules and for sequence studies in particular.

A replicating molecule which has eliminated nonessential residues possesses evident advantages for a detailed examination of the replicative process. Of immediate interest is its obviously greater accessibility to sequence determination. Available methods for separating oligonucleotides (Tomlinson and Tener, 1963; Sanger *et al.*, 1965), combined with the advantages inherent in being able to synthesize chains with any one or all bases labeled, make it possible to obtain the sequence at the two ends of the molecules. Since the ends are probably involved in the initiation and termination of replication, information on the sequences at the termini should help to illuminate both the process of replication and the recognition mechanism which enables the replicase to distinguish one RNA molecule from another.

The present paper demonstrates that the 5′ end of the plus strand of a cloned Qβ-RNA variant begins with pppGpGpGpGpApApCpCp.[1]

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: GTP (pppG), guanosine triphosphate; pppGp, guanosine tetraphosphate; Ap, Gp, Cp, and Up are the 2′,3′-monophosphates of adenosine, guanosine, cytosine, and uridine, respectively. The term plus strand RNA refers to the variant RNA strands which have a sequence derived from Qβ viral RNA and *complementary* to the variant minus strand RNA which is synthesized during the replication process (Mills *et al.*, 1968). All double-stranded RNA forms, composed of plus and minus strands hydrogen bonded together, are termed HS RNA (Bishop *et al.*, 1967a).

Experimental Section

Enzyme, Substrates, Assays, and Materials. Purification of Qβ replicase through the stages of cesium chloride and sucrose sedimentation (Pace and Spiegelman, 1966a), assay for enzyme activity under standard conditions, and liquid scintillation counting of acid-precipitable product on membrane filters have been detailed by Haruna and Spiegelman (1965). The synthesis of [β,γ-^{32}P]GTP (1×10^{10} cpm/μmole) (Bishop *et al.*, 1967b), or [α-^{32}P]ATP, -UTP, or -GTP (1.5×10^{9} cpm/μmole) (Haruna *et al.*, 1963) has been described. [α-^{32}P]CTP (1×10^{9} cpm/μmole) and [^{3}H]ATP (1 mCi/μmole) were obtained from Schwarz BioResearch. Pancreatic ribonuclease and bacterial alkaline phosphatase were obtained from Worthington Biochemicals. Ribonuclease T_1 was obtained from Sankyo through Calbiochem.

Synthesis of ^{32}P-Labeled RNA, Electrophoretic Conditions. The synthesis and purification of [α-^{32}P]-CTP-, -UTP-, -ATP-, and -GTP-labeled variant plus and minus strands using one or all four ribonucleoside triphosphates at the same specific activity or, alternatively, [β,γ-^{32}P]GTP and [^{3}H]ATP was identical in detail with that described by Mills *et al.* (1968). The broad outline of the purification process used is as follows. Plus strand RNA was obtained after phenol extraction and gel electrophoresis of a 10- to 20-fold reaction mixture primed with the cloned variant RNA (V-2) isolated by Levisohn and Spiegelman (1968). The RNA was self-annealed in 0.4 M NaCl–0.003 M EDTA (pH 7.4) at 70° for 30 min to remove traces of minus strands and further purified from resulting double-stranded RNA by electrophoresis through swollen bisacrylamide cross-linked 3.6% polyacrylamide gels. Minus strand RNA was obtained by melting the original double-stranded (HS) RNA in 0.003 M EDTA at 100° for 1 min and annealing the product to Qβ viral RNA. The hybrid was separated by sucrose gradient centrifugation, melted, and the single-stranded variant minus strand was obtained after electrophoresis. Self-annealing was finally used to ensure freedom from plus strand contamination. The gel electrophoresis buffers and conditions are identical with those described by Bishop *et al.* (1967c) and Pace *et al.* (1967).

Determination of Base Ratios, Ribonuclease Digestion, Separation of Oligonucleotides by DEAE Column Chromatography, and Paper Electrophoresis. Alkali degradation of RNA to mononucleotides and separation of nucleotides on Whatman No. 52 paper by high-voltage electrophoresis were identical with that described by Sanger *et al.* (1965). Ribonuclease T_1 digestion and electrophoresis of oligonucleotides on DEAE paper in 7% formic acid also followed the procedures recommended by Sanger *et al.* (1965). Pancreatic ribonuclease digestion in 0.05 M EDTA–0.10 M NaCl–0.01 M Tris-HCl buffer (pH 7.4) of RNA was performed by mixing an RNA sample with 11 mg of *Escherichia coli* RNA and 0.5 mg of pancreatic ribonuclease and incubating the 1-ml mixture at 37° for 30 min. The digest was diluted tenfold with 7 M urea and loaded directly on a DEAE column.

DEAE column resolution of oligonucleotides according to their charge at pH 7.8 in 7 M urea was similar to that described by Tomlinson and Tener (1963). DEAE (Bio-Rad 0.95 mequiv/g) was freed from fines by suspension in distilled water (200 g/10 l.) and decanting the supernatant; then it was adjusted to pH 2 with HCl (4 l.), diluted to 10 l. with water, and the supernatant was again decanted. The DEAE was washed twice with 10 l. of water and made 2 M with respect to ammonium carbonate (4-l. total volume), diluted subsequently to 10 l. with water, and the supernatant was again decanted. After a further two washes with 10-l. volumes of water, the slurry was made up to 2 M NaCl (4-l. total volume) diluted to 0.2 M NaCl, the supernatant was decanted, and the slurry was finally made up to 7 M urea–0.003 M EDTA–0.01 M Tris–0.2 M NaCl (pH 7.8). Columns (27 × 0.8 cm) were supported on sintered-glass disks and packed under 5 psi. Prior to use, a column was washed with 100 ml of 7 M urea–0.003 M EDTA–0.01 M Tris (pH 7.8) at a rate of 0.5 ml/min, regulated by a Harvard Apparatus Co. multi-speed transmission peristaltic pump.

The isolation of an isopleth from a DEAE column was as follows. The pooled fractions were diluted six-fold with water and passed through a 9 × 0.5 cm column of DEAE packed under 5 psi and previously washed with 50 ml of 0.05 M triethylamine bicarbonate buffer (pH 7.4). The oligonucleotides, which were absorbed by the DEAE, were freed from residual urea and NaCl by subsequent washing the columns with 30 ml of 0.05 M triethylamine bicarbonate buffer and then eluted with 2 M triethylamine bicarbonate (pH 7.4). Finally, the triethylamine bicarbonate was removed by distillation with water under reduced pressure at 40°. The oligonucleotides were converted into a sodium salt by Dowex (H^+) 50 treatment and neutralizing the supernatant with 0.1 N NaOH.

Results

Identification of the 5'-Terminal Sequences from the Variant Plus and Minus Strand RNA. The DEAE column profiles of pancreatic digests of unlabeled *E. coli* RNA and [β,γ-^{32}P]GTP- and [^{3}H]ATP-labeled variant plus or minus strands are shown in Figure 1. The majority of ^{32}P label from the plus strand (Figure 1A) was recovered between the nona- and decanucleotides of the *E. coli* digest. Occasionally, a small peak is also obtained in the nonanucleotides. None of the ^{32}P label from the *minus* strand preparation (Figure 1B) is eluted between 0 and 0.35 M NaCl in the gradient. All of the added radioactivity is, however, obtained in the high (2 M) salt wash. To determine if this oligonucleotide could be degraded by more extensive digestion, the high salt fraction was diluted 50-fold, freed from urea and salt by passage through DEAE, and eluted as the triethylamine salt as described in the Experimental Section. After conversion into the sodium salt, the material with no added RNA was digested with 100 μg of pancreatic ribonuclease in 0.05 M EDTA–0.1 M NaCl–0.05 M Tris (pH 7.4) for 60 min at 37°. Its sub-

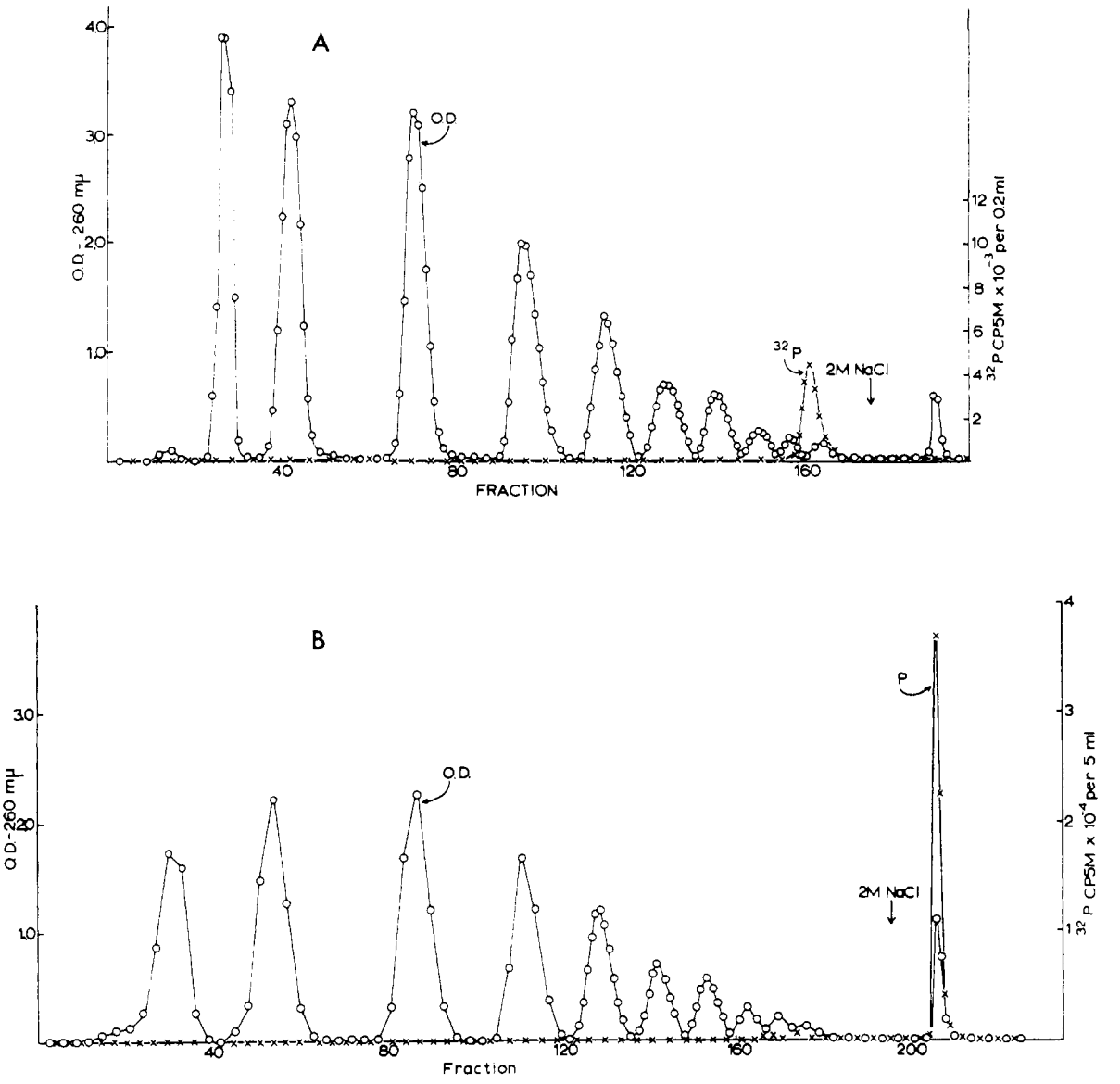

FIGURE 1: DEAE chromatography of pancreatic ribonuclease digests of [β,γ-^{32}P]GTP-labeled variant plus and minus RNA. Pancreatic ribonuclease digests of unlabeled *E. coli* RNA with (A) variant plus or (B) minus strand RNA labeled with [β,γ-^{32}P]GTP and [^{3}H]ATP (not plotted in figure) and isolated as described by Mills *et al.* (1968) were resolved on DEAE columns by a linear gradient to 0–0.35 M NaCl in 7 M urea buffer as described in the Experimental Section. At the end of the gradient, the columns were stripped by passage of 2 M NaCl in 7 M urea–0.003 M EDTA–0.01 M Tris-HCl buffer (pH 7.8). Samples were collected at 5-min intervals, the optical density was measured, and radioactivity was determined on suitable aliquots using Kinard's scintillation fluid (Kinard, 1957) and a Packard Tri-Carb scintillation counter. For aqueous samples of more than 1-ml volume, when a two-phase mixture was present in the counting vial, 50% gain and 50 to ∞ discriminator settings were used and the unquenched radioactivity was determined by subsequently adding a known amount of [α-^{32}P]UTP and determining the counting efficiency. The counts obtained are expressed at 95% ^{32}P counting efficiency.

sequent behavior on DEAE chromatography indicated no change in its elution properties.

These results suggest that the *minus* strand begins with pppGp at the 5′ end followed by a long sequence rich in purines. The *plus* strand also begins with pppGp but contains a relatively shorter stretch of purines.

Chromatographic Separation of Oligonucleotides from α-^{32}P-Labeled Plus and Minus Strand RNA. Preliminary data on the approximate distribution of oligonucleotides labeled *in vitro* with each or all four [α-^{32}P]-ribonucleoside triphosphates were obtained for both plus and minus strands of the variant and several interesting features stand out. As may be seen from Figures 2 and 3, no nona- or decanucleotides are liberated by pancreatic ribonuclease digestion of the *minus* strand and no decanucleotides are obtained from the *plus* strand RNA (Figure 4). A large proportion of the [α-^{32}P]GTP or [α-^{32}P]ATP *minus* strand label was recovered in the high salt fraction (Figure 2) in agreement with the previous suggestion that the *5′-terminal* sequence of the *minus* is rich in guanosine and adenosine. For the *plus* strand oligonucleotides (Figure 4), it is

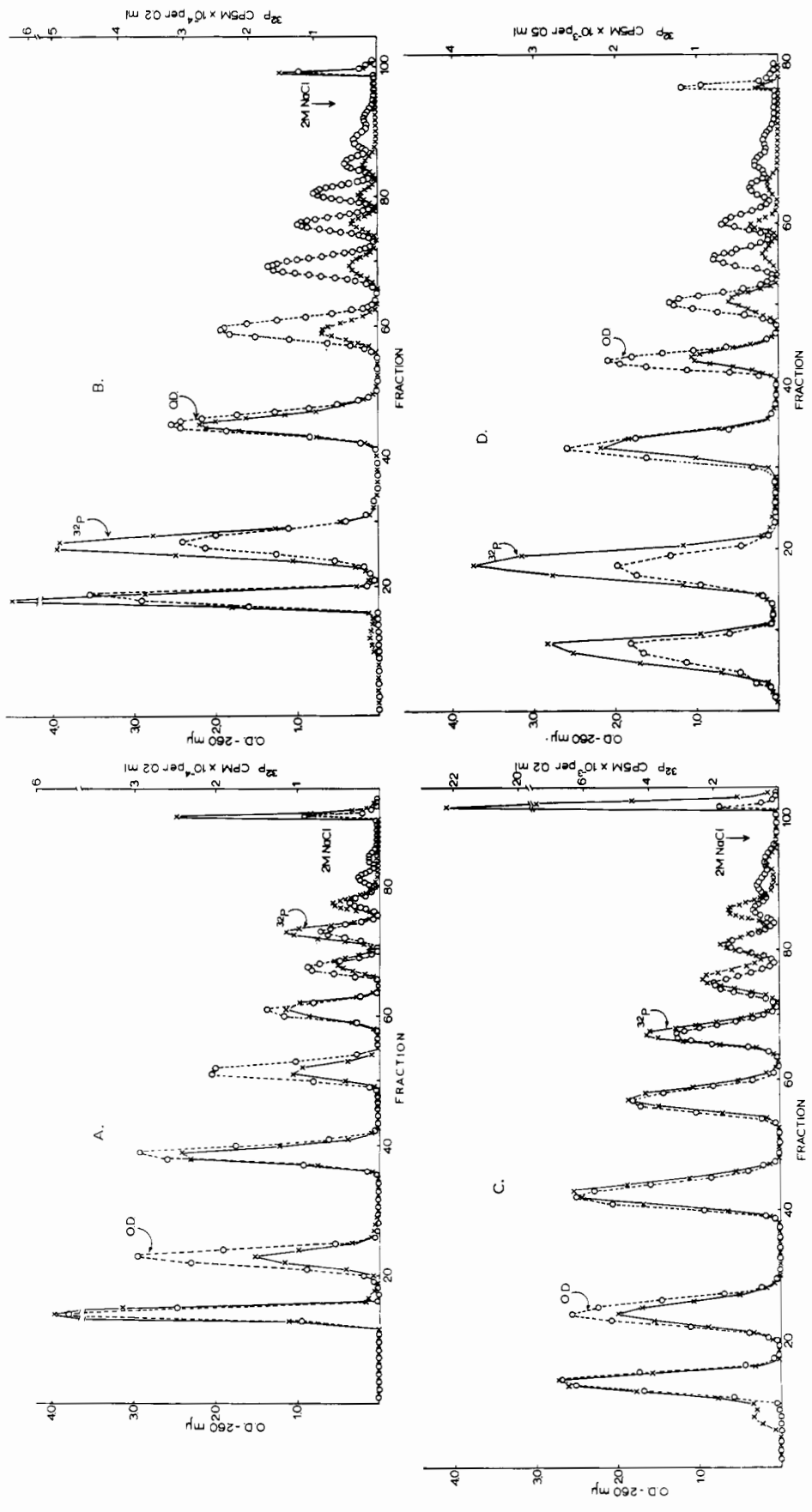

FIGURE 2: DEAE chromatography of pancreatic ribonuclease digests of [α-^{32}P]ATP-, [α-^{32}P]UTP-, [α-^{32}P]GTP-, or [α-^{32}P]CTP-labeled variant *minus* strand RNA. The preparation of minus strand RNA and conditions used for ribonuclease digestion, DEAE chromatography in 7 M urea, sampling, and counting are described in the Experimental Section and legend to Figure 1. Minus strand RNA was labeled with (A) [α-^{32}P]ATP, (B) [α-^{32}P]UTP, (C) [α-^{32}P]GTP, or (D) [α-^{32}P]CTP. To increase resolution, fractions were collected at 10-, 5-, and 2.5-min intervals. However, the fraction numbers shown are normalized to a 10-min interval.

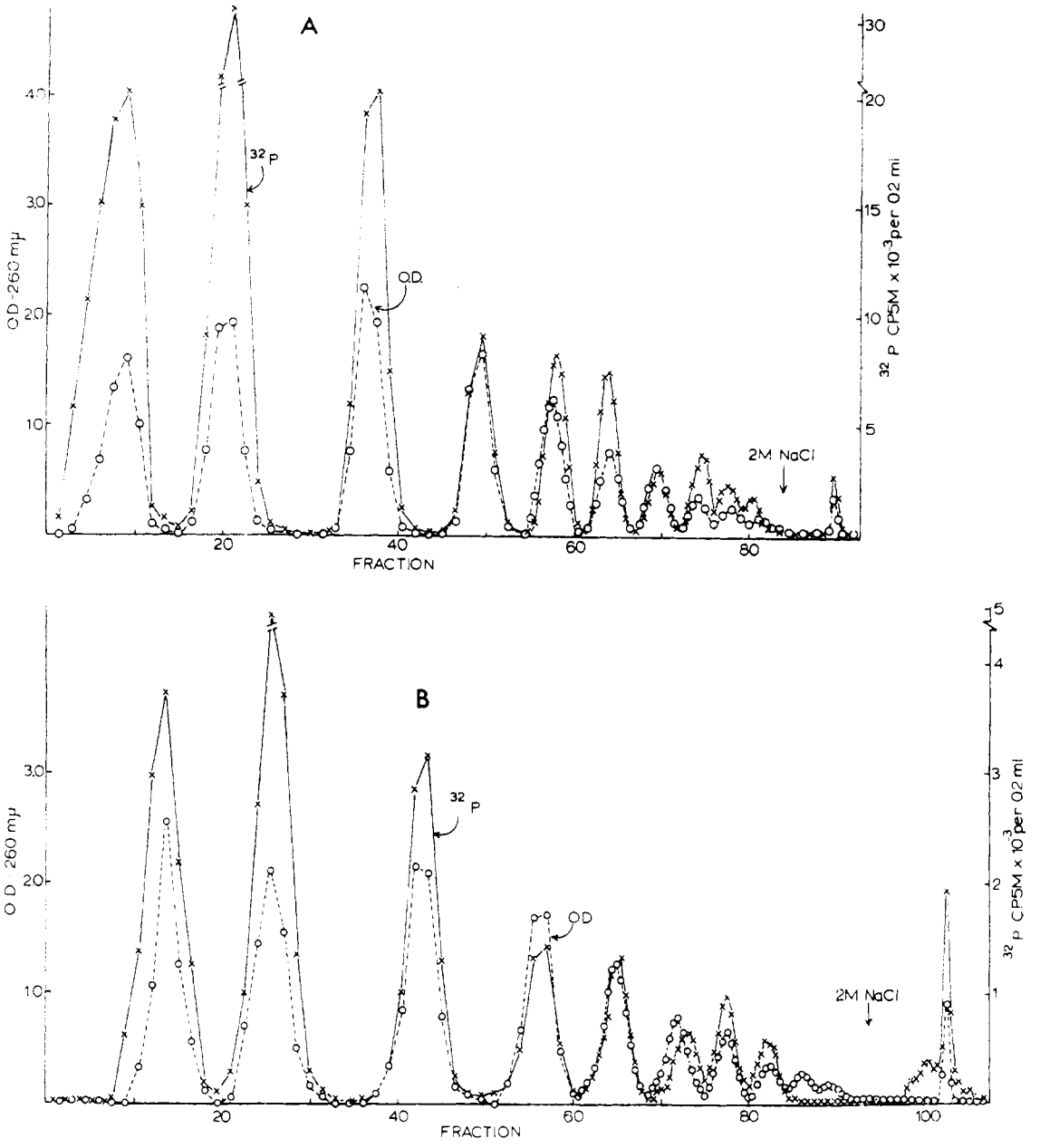

FIGURE 3: DEAE chromatography of pancreatic ribonuclease digests of [α-^{32}P]ATP-, [α-^{32}P]UTP-, [α-^{32}P]GTP-, and [α-^{32}P]CTP-labeled variant *plus* and *minus* strand RNA. The preparation of variant plus (A) or minus (B) strand RNA labeled with all four riboside triphosphates, pancreatic ribonuclease digestion, and separation into oligonucleotides on DEAE is identical with that described in Figures 1 and 2.

noticeable that the septanucleotides were not labeled by [α-^{32}P]CTP while the unique 5'-terminal oligonucleotide was not labeled by [α-^{32}P]UTP.

Unfortunately, quantitation of the relative numbers of oligonucleotides or determination of the molecular weight of the species from the integrals of the high oligonucleotides is complicated by our observation that pancreatic ribonuclease can, to varying degrees depending upon the conditions employed, hydrolyze oligonucleotides at adenosine sites. Both for the variant species and Qβ viral RNA some 10–20% higher values of the molecular weight are obtained by integral calculations than are realized by calculation from the relative [β,γ-^{32}P]GTP to total [^{3}H]ATP labels (D. Mills D. H. L. Bishop, and S. Spiegelman, unpublished data). Evidence for digestion at adenosine residues has also been obtained by examining pancreatic digestion of [^{3}H]ATP-labeled RNA and finding adenosine monophosphate in the mononucleotide isopleth (D. Mills, D. H. L. Bishop, and S. Spiegelman, unpublished work) in agreement with the demonstration of pancreatic ribonuclease digestion of poly A by Beers (1960) and Gabbay and Shimshak (1968).

Length of the 5'-Pancreatic Oligonucleotide Isolated by DEAE Chromatography. It has been suggested that the basis of chromatographic separation of

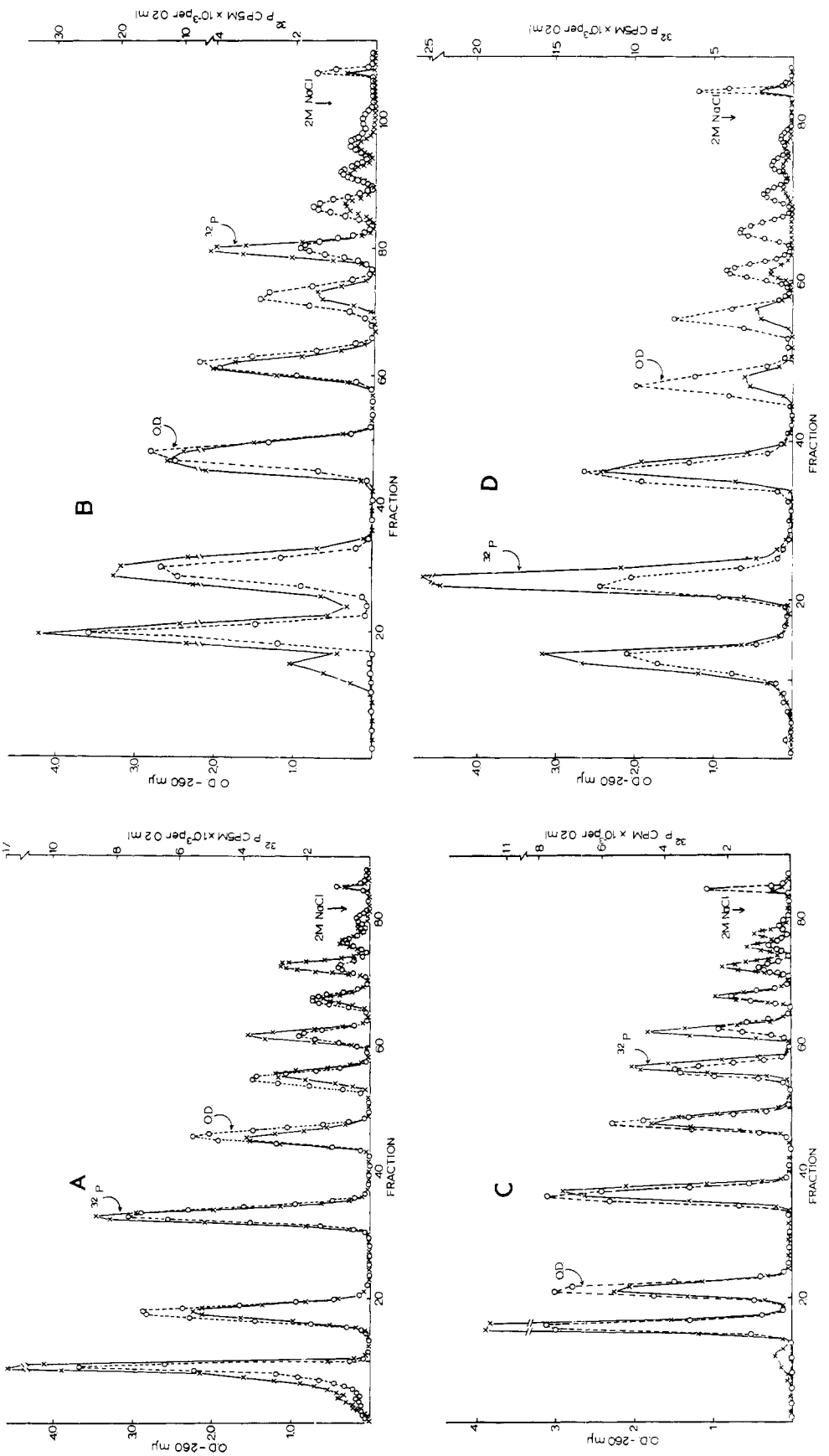

FIGURE 4: DEAE chromatography of pancreatic ribonuclease digests of [α-^{32}P]ATP-, [α-^{32}P]UTP-, [α-^{32}P]GTP-, or [α-^{32}P]CTP-labeled variant *plus* strand RNA. RNA preparation, ribonuclease digestion, DEAE chromatography of the digests and monitoring of the eluents are identical with that described in Figures 1 and 2. Plus strand variant RNA was labeled with either (A) [α-^{32}P]ATP, (B) [α-^{32}P]UTP, (C) [α-^{32}P]GTP, or (D) [α-^{32}P]CTP.

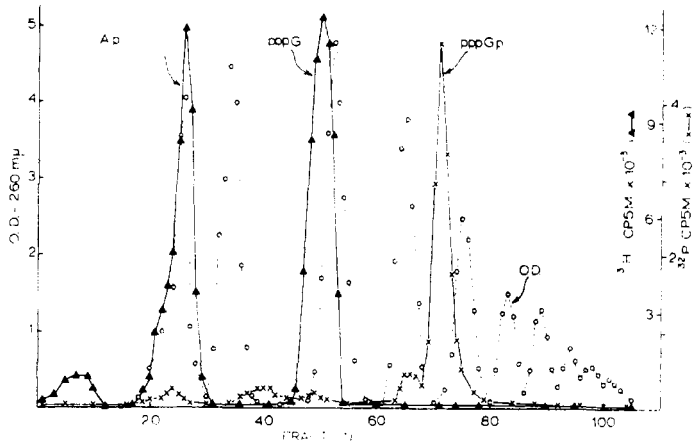

FIGURE 5: DEAE chromatography of an alkaline digest of [^{3}H]ATP- and [β,γ-^{32}P]GTP-labeled variant plus RNA mixed with [^{3}H]GTP. Variant plus strand RNA labeled with [^{3}H]ATP and [β,γ-^{32}P]GTP were digested with alkali, mixed with [^{3}H]GTP (pppG), and a pancreatic ribonuclease digest of *E. coli* RNA, then resolved by DEAE chromatography as described in the legends to Figures 1 and 2 and the Experimental Section. The [β,γ-^{32}P]GTP label was eluted as guanosine tetraphosphate (pppGp) just in front of the pentanucleotides, while the 2′,3′-adenosine monophosphate ^{3}H label was recovered in the mononucleotide region.

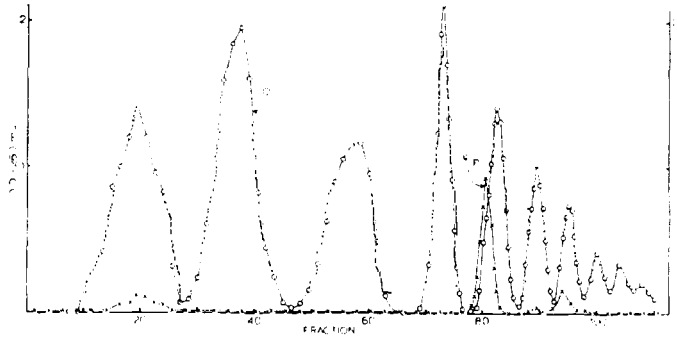

FIGURE 6: Phosphatase-treated variant plus strand 5′-oligonucleotide labeled with all four riboside triphosphates. The unique 5′-oligonucleotide labeled with all four [α-^{32}P]riboside triphosphates and recovered between the nona- and decanucleotides of the *E. coli* digest (Figure 3A) was purified from urea and salt, then treated with alkaline phosphatase as described in the text and Experimental Section. After removal of divalent ions from the phosphatase, the material was mixed with a pancreatic digest of *E. coli* RNA and subjected to DEAE chromatography as described in the legends to Figures 1 and 2.

pancreatic oligonucleotides on DEAE in 7 M urea at pH 7.8 is due to the net phosphate charge of the nucleotides (Tomlinson and Tener, 1963). GTP, however, is eluted slightly ahead of the trinucleotides (both having a net phosphate charge of -4), and its 3′-phosphate analog, guanosine tetraphosphate (pppGp), is recovered *between* the tetra- and pentanucleotides although it has the same (-6) net phosphate charge as the pentanucleotides (Figure 5). Consequently, although the [β,γ-^{32}P]GTP-labeled oligonucleotide from the 5′ terminus of the variant plus RNA is recovered between the nona- and decanucleotides, this is no assurance that it has a net -10 or -11 phosphate charge.

To determine the number of nucleotides present in the plus strand 5′-terminal oligonucleotide, the effect of removing terminal phosphates with alkaline phosphatase was examined. A pancreatic RNase digest obtained of variant *plus* strand RNA labeled with all four [^{32}P]riboside triphosphates was resolved on DEAE. The 5′-terminal oligonucleotide isolated from the DEAE chromatogram (Figure 3) was freed from urea and NaCl as described in the Experimental Section. After conversion into the sodium salt, the oligonucleotide was incubated in 1.0 ml of 0.10 M Tris-HCl buffer (pH 8.0)–0.01 M $MgCl_2$ with 15 μg of alkaline phosphatase for 3 hr at 37°. It was then made 0.15 M with respect to EDTA and brought to pH 2.0 with 1 N HCl to remove divalent cations from the phosphatase as described by Weith and Gilham (1967). After 60 min at room temperature, the sample was brought to pH 7 with 0.1 M NaOH, diluted tenfold with 7 M urea, 0.003 M EDTA, and 0.01 M Tris-HCl buffer (pH 7.8), and loaded on a DEAE column with a pancreatic digest of 10 mg of *E. coli* bulk RNA. The subsequent fractionation of oligonucleotides is shown in Figure 6. The dephosphorylated oligonucleotide was eluted on the leading side of the pentanucleotides indicating a net -6 phosphate charge and hence a *septanucleotide* composition. Free phosphate was evident in the mononucleotide peak and the small amount of radioactivity observed in the septanucleotides presumably represents dephosphorylated nonanucleotides contaminating the original sample.

Composition of the 5′-Terminal Oligonucleotide from

TABLE I: Base Composition of [α-^{32}P]Ribose Triphosphate Labeled 5′-Oligonucleotide from the Variant *Plus* Strand RNA.[a]

Label Nucleotide	I [α-^{32}P]ATP		II [α-^{32}P]CTP		III [β-^{32}P]GTP		IV [α-^{32}P]4XTP	
	Counts/5 min	Counts Ratio	Counts/5 min	Counts Ratio	Counts/5 min	Counts Ratio	Counts/5 min	Counts Ratio
Cp	89	0	3410	1	80	0	1812	1
Ap	2728	1	3750	1	309	0	3816	2
Gp	2930	1	33	0	5400	1	5826	3
Up	100	0	106	0	0	0	315	0
pppGp	103	0	0	0	4880	1[b]	4200	2[b]

[a] Variant *plus* strand RNA labeled with (I) [α-^{32}P]ATP, (II) [α-^{32}P]CTP, (III) [α-^{32}P]GTP, or (IV) all four [α-^{32}P]-riboside triphosphates (XTP) was digested with pancreatic ribonuclease, and the oligonucleotides were resolved on DEAE (Figure 4) and purified from urea and salt as described in the Experimental Section. After alkaline hydrolysis, the 2′,3′-ribonucleoside monophosphates and guanosine tetraphosphate (pppGp) were resolved by pH 3.5 paper electrophoresis (Sanger *et al.*, 1965), and the nucleotides were detected by radioautography (Sanger *et al.*, 1965), cut out, and counted. The counts ratios are ratios to the lowest significant value in each column rounded off to the nearest integer. [b] Note that the counts for pppGp in columns III and IV represent 2 equiv of ^{32}P, 1 from its neighbor and 1 of its own, indicating a molar equivalence of 1 for the tetraphosphate. Further, the Gp in column III is represented by 2 equiv of ^{32}P although 3 molar equiv are present, one having received an unlabeled P from its non-G neighbor.

Variant Plus RNA. Knowledge of the base composition and nearest-neighbor frequency of each component should provide the information required to deduce the sequence of the 5′ fragment. The base composition can be obtained by running a synthesis with all four riboside triphosphates labeled with ^{32}P in the α-phosphorous at equal specific activities. The distribution of ^{32}P in the nucleotides of an alkaline hydrolysate yields the base composition. Recall the fact that on alkali digestion, the α-phosphorous of the original riboside triphosphate is transferred to its neighboring nucleotide. Consequently, the nearest-neighbor frequency to each donor nucleotide can be determined by examining the distribution of ^{32}P in the nucleotides of alkaline digests of a series of syntheses in which *each* synthesis contains *one* of the riboside triphosphates carrying a ^{32}P in the α position. Accordingly, the 5′-pancreatic oligonucleotide from the variant plus strand RNA labeled with either [α-^{32}P]CTP, [α-^{32}P]ATP, [α-^{32}P]-GTP, or all four [α-^{32}P]riboside triphosphates was isolated as described in the Experimental Section and digested with alkali. The 2′,3′-mononucleotides and guanosine tetraphosphate derivatives were resolved by paper electrophoresis at pH 3.5 as described by Sanger *et al.* (1965).

The relative distribution of ^{32}P in the components are given in Table I. The last column (IV) lists the outcome of the synthesis carried out with all four α-^{32}P-labeled riboside triphosphates. In agreement with the [α-^{32}P]UTP exponent of Figure 3 we find no evidence for U residues in the 5′ fragment. The ^{32}P counts found in the various components indicate that the molar ratio of Cp:Ap:Gp:pppGp is 1:2:3:1. Note that pppGp, the 5′ terminus, carries 2 equiv of ^{32}P as it should; 1 of its own and 1 from its neighbor. From column III we can deduce that every G has G as its neighbor and hence that all four are clustered at the 5′ end with no other residues intervening. From the synthesis with [α-^{32}P]ATP (column I) we find that A has A and G as neighbors with equal frequency. Finally, from column II we infer that C has A and C as neighbors, again with equal frequency. Finding C as a neighbor to the only C in the septanucleotide allows us to specify C as the base not recovered in the ribonuclease-resistant 5′ fragment; a unique advantage conferred by the possibility of performing a nearest-neighbor analysis. All of these facts leads us to write pppGpGpGpGp-ApApCpCp as the unique sequence of the 5′ end of the variant plus strand.

Confirmation of the correctness of the assigned order of the residues can be obtained by examining RNase T_1 digests of [β,γ-^{32}P]GTP-, [α-^{32}P]GTP-, and [α-^{32}]CTP-labeled fragments. The results of such experiments are given in Figure 7 where a comparison is made of the electrophoretic behavior on DEAE paper in 7% formic acid of (1) an alkali digest of [β,γ-^{32}P]GTP-labeled variant plus strand RNA, showing the expected liberation of pppGp; (2) the control undigested [α-^{32}P]GTP-labeled 5′-oligonucleotide; (3) an RNase T_1 digest of the [α-^{32}P]GTP-labeled 5′-pancreatic oligonucleotide showing the predicted occurrence of Gp and pppGp; (4) an RNase T_1 digest of ^{32}P-labeled Qβ viral RNA to identify Gp; (5) a pancreatic digest of ^{32}P-labeled Qβ viral RNA to identify ApApCp; (6) an RNase T_1 digest of the [α-^{32}P]CTP-labeled 5′ pancreatic oligonucleotide exhibiting the recovery of the ApApCp expected from an RNase T_1 cleavage of a GpApApCp sequence; (7) another RNase T_1 digest of the ^{32}P-labeled Qβ viral RNA to identify Gp RNA. It is evident that the RNase T_1 digests of the appropriately labeled 5′

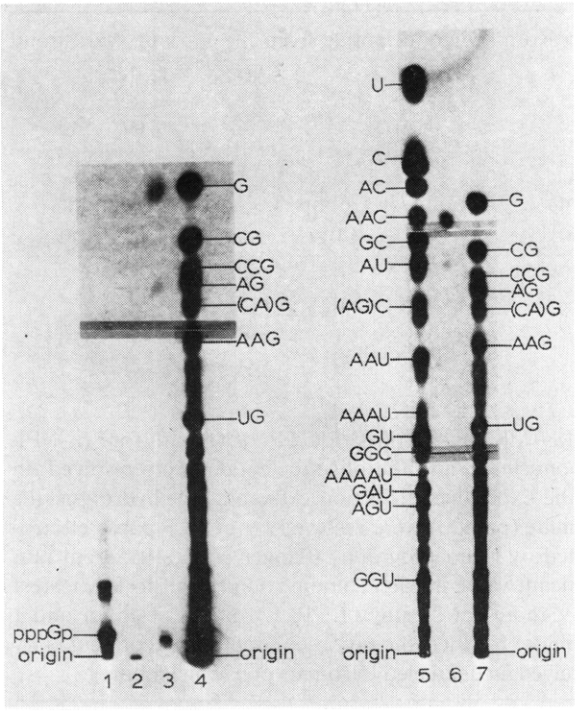

FIGURE 7: Paper electrophoresis of digests of the 5′-terminal oligonucleotide from variant plus strand RNA. [β,γ-^{32}P]GTP- and [^{3}H]ATP-labeled variant plus strand RNA and the unique 5′-terminal oligonucleotide of the variant plus strand RNA labeled with [α-^{32}P]GTP or [α-^{32}P]CTP (Figure 4) were prepared and isolated as described in the Experimental Section. [^{32}P]Qβ viral RNA was obtained from labeled virus particles as described by Bishop *et al.* (1967c). Alkali, pancreatic, or T_1 ribonuclease digestion and electrophoresis in 7% formic acid are as described by Sanger *et al.* (1965). The assignment of oligonucleotides resolved from the digests by paper electrophoresis is based on the elegant analyses of Sanger *et al.* (1965). A comparison is made by electrophoresis between (1) an alkali digest of [β,γ-^{32}P]GTP, [^{3}H]ATP-labeled variant *plus* strand RNA; (2) the [α-^{32}P]GTP-labeled variant *plus* strand 5′-terminal oligonucleotide (see Figure 4C); (3) a ribonuclease T_1 digest of the [α-^{32}P]GTP variant *plus* strand 5′ fragment; (4) a ribonuclease T_1 of [^{32}P]Qβ viral RNA; (5) a pancreatic ribonuclease digest of [^{32}P]Qβ viral RNA; (6) a ribonuclease T_1 digest of the [α-^{32}P]CTP-labeled 5′-terminal fragment from the variant *plus* strand RNA (Figure 4D); and (7) a ribonuclease T_1 digest of [^{32}P]Qβ-RNA.

fragment yielded in all instances the components predicted from the sequence deduced from the base composition and nearest-neighbor analysis.

Discussion

Although still fragmentary, the information on sequence presented here illuminates certain chemical features of the replication mechanism. Before considering these, it is useful to summarize some of the relevant findings with the Qβ replicase. Feix *et al.* (1968) demonstrated that *minus* strands of Qβ are excellent templates for the generation of plus strands by Qβ replicase. Mills *et al.* (1968) found that this was also true for the minus strands of the V2 variant of Qβ. They further showed that when variant *minus* strands are employed as template, polymerization of the product *plus* strands proceeds in a 5′ to 3′ direction, a feature also observed (N. R. Pace, K. H. Huang, and S. Spiegelman, unpublished observations) with free *minus* of parental Qβ-RNA. The evidence (Bishop *et al.*, 1967b) for 5′ termini in FS structures containing all four residues requires further examination for its relevance to replication. Finally, it has been demonstrated (D. Mills, D. H. L. Bishop, and S. Spiegelman, unpublished) in reactions templated with either *variant* plus strands or with Qβ plus strands (Bishop *et al.*, 1967b) that polymerization of *minus* strands proceeds in a 5′ to 3′ direction.

It would appear then that whenever single strands are used as templates, whether variant or Qβ-RNA, a 5′ to 3′ direction is employed in the synthesis of both plus and minus strands. This requires that the complementary copying begin in all cases at the 3′ end of the template. It is, therefore, of central interest to examine the 3′ ends of plus and minus templates for common features.

The demonstration that the 5′-terminal sequence of the variant plus strand is pppGpGpGpGpApApCpCp-.... implies that the complementary variant *minus* strand has ....pGpGpUpUpCpCpCpC as its 3′-terminal sequence. We have further seen that the variant *minus* strand has a 5′-terminal sequence rich in purines and begins with GTP. This, in turn, implies that the 3′ end of the plus is rich in uridine and cytosine and *also* terminates in *cytosine*.

Similarly, one can infer a terminal cytosine at the 3′-replicative ends of the plus and minus Qβ-RNA from the demonstration that both contain pppGp as the 5′ terminus (Bishop *et al.*, 1967b). Thus, the 3′ ends of all plus and minus templates which function with Qβ replicase terminate in cytosine. More details on the sequence at the 3′ ends will be required to reveal how much further the similarity extends.

It seems likely that the initiation of polymerization is related to the ability of Qβ replicase to carry out a poly C dependent poly G synthesis (Hori *et al.*, 1967; Eikhom and Spiegelman, 1967). The latter demonstrated that Qβ replicase can be separated into two protein components of differing molecular weights and that the heavy component possessed the poly G polymerase activity. Eikhom *et al.* (1968) found that the heavy component was unique to cells infected with Qβ virus; the lighter protein component has been isolated from uninfected cells.

The demonstration that the 5′ termini of plus and minus strands differ and can be readily separated furnishes an excellent quantitative tool for determining nascent plus and minus strands in the different structures which accumulate during the *in vitro* synthesis (Bishop *et al.*, 1967a). The resulting information should permit a more definitive delineation of these structures and their role as intermediates in the replicative process.

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Amino Acid Transfer Factors from Yeast. II. Interaction of Three Partially Purified Protein Fractions with Guanosine Triphosphate*

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ABSTRACT: Two protein fractions (1-P and 2-P) isolated from yeast supernatant fluid exhibited two kinds of enzymatic activity when combined: they catalyzed (1) peptide chain elongation and (2) ribosome-dependent guanosine triphosphate hydrolysis. 1-P was able to form a complex with guanosine triphosphate. Further purification of 1-P and 2-P by adsorption chromatography and gel filtration yielded T_1 and T_2 still complementary in guanosine triphosphate splitting but without any transfer effect. Purified T_1 had lost most of the guanosine triphosphate binding activity.

The T factors showed great heat lability. By contrast, a further ribosome-dependent guanosine triphosphatase was obtained from the supernatant fluid, which was more heat stable and had a higher molecular size than T_1 and T_2. This stable guanosine triphosphatase did not complete the T factors in amino acid polymerization; however, the transfer effect of an additionally isolated protein fraction was enhanced twofold by the combined T factors.

For some years it has been known that peptide chain elongation with purified ribosomes needs more than one component from the soluble fraction of the cell. Three enzyme factors isolated from bacterial sources (Lucas-Lenard and Lipmann, 1966) possessed amino acid incorporation activity only when combined, hence their functions in the elongation process must be different. One factor (G) was shown to contain a ribosome-dependent GTPase activity (Nishizuka and Lipmann, 1966) and a second (T_u) was found to bind GTP (Gordon, 1967; Allende *et al.*, 1967; Ravel *et al.*, 1967; Lucas-Lenard and Haenni, 1968). In mammalian systems, however, only two complementary transfer fractions could be separated up to now (Arlinghaus *et al.*, 1963; Gasior and Moldave, 1965; Klink *et al.*, 1967a). One of these (Klink *et al.*, 1966; Arlinghaus *et al.*, 1964) or both (Rao and Moldave, 1967; Ibuki and Moldave, 1968) were reported to interact with GTP.

In contrast to bacterial enzymes, transfer factors from

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