*REPLICATION OF RNA VIRUSES, IV. INITIATION OF RNA SYNTHESIS BY THE Qβ RNA POLYMERASE**

By A. K. Banerjee, Lillian Eoyang, K. Hori, and J. T. August†

DEPARTMENT OF MOLECULAR BIOLOGY, ALBERT EINSTEIN COLLEGE OF MEDICINE, NEW YORK

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Investigation of the mechanism of viral RNA synthesis has been greatly facilitated by use of the RNA-dependent RNA polymerase isolated from cells infected with the single-stranded RNA phage Qβ. In the reaction catalyzed by this enzyme, Qβ RNA and ribonucleoside triphosphates are required. The RNA product has a molecular weight of approximately 1×10^6 daltons, corresponding to that of Qβ RNA, and may be produced in manyfold excess of the amount added to the reaction.[1, 2]

As part of our studies of the enzyme, we have investigated the initiation of RNA synthesis. In general, synthesis of polynucleotide chains is thought to involve a nucleophilic attack by the 3′-hydroxyl group of the polynucleotide on the α-phosphate of the entering nucleoside triphosphate.[3–5] By this mechanism, newly synthesized RNA could contain a nucleoside triphosphate as one of the termini of the molecule. Maitra and Hurwitz[5] and Bremer *et al.*[6] have described the presence of such termini in the RNA product of the DNA-dependent RNA polymerase reaction.

Methods.—γ-P^{32}-adenosine 5′-triphosphate (ATP), γ-P^{32}-guanosine 5′-triphosphate (GTP), and γ-P^{32}-uridine triphosphate (UTP) were prepared by the exchange of P^{32} with the corresponding ribonucleoside triphosphate by the method of Penefsky *et al.*[7] The reaction was allowed to proceed until all of the P_i^{32} was converted into a charcoal-adsorbable form. The γ-P^{32}-nucleoside triphosphates were then purified by chromatography on Dowex-1-Cl^-. γ-P^{32}-cytidine 5′-triphosphate (CTP) was prepared from γ-P^{32}-ATP and cytidine 5′-diphosphate (CDP) in the reaction catalyzed by nucleoside diphosphokinase[8] and was purified by chromatography on Dowex-1-Cl^-. These substrates were all free of P^{32} in the α-position as shown by the complete loss of charcoal-adsorbable radioactivity after incubation in 1 *N* HCl for 10 min at 100°C.

The Qβ RNA polymerase was purified from extracts of Qβ[9] infected *E. coli* Q13[10] by fractionation with ammonium sulfate, followed by *O*-(diethylaminoethyl) (DEAE) and hydroxylapatite chromatography, as described elsewhere.[11] The enzyme fraction used in these studies was 500- to 1000-fold purified, with a specific activity of 1000–2000 mμmoles guanosine 5′-phosphate (GMP) incorporated per milligram protein per 20 min. The addition of a factor of as yet undetermined composition, isolated during enzyme purification by DEAE and hydroxylapatite chromatography, was necessary for maximal activity. The DNA-dependent RNA polymerase was isolated by the same purification procedure as a fraction from the DEAE column and was further purified by DEAE-Sephadex chromatography.[11]

Enzyme assays: Qβ RNA polymerase, γ-P^{32}-ribonucleoside triphosphate incorporation: The formation of triphosphate termini was assayed by the incorporation of acid-soluble γ-P^{32}-labeled substrates into acid-insoluble material. Reaction mixtures (0.35 ml) contained 35 μmoles Tris-HCl buffer, pH 7.6, 3 μmoles $MgCl_2$, 1 μmole 2-mercaptoethanol, 100 mμmoles each ATP, GTP, UTP, and CTP, one labeled with P^{32} in the γ-phosphate position with a specific activity of $1–2 \times 10^6$ cpm/mμmole, 5.0 mμmoles Qβ RNA,[12] 2–12 μg (dry weight) factor, and 1–3 μg Qβ RNA polymerase of the hydroxylapatite fraction. The mixture was incubated at 37° for 30 min and the acid-insoluble material isolated according to the procedure of Maitra and Hurwitz.[5] Control tubes were those in which RNA or enzyme were omitted or to which acid was added before incubation.

Qβ RNA polymerase, α-P^{32}-ribonucleotide incorporation: The reaction mixture was identical to that above except that α-P^{32}-GTP ($1–2 \times 10^3$ cpm/mμmole) served as the labeled substrate.

The reaction was terminated and the acid-insoluble material washed and collected on membrane filters as described previously.[13]

DNA-dependent RNA polymerase: RNA synthesis and the formation of triphosphate termini were assayed as described by Maitra and Hurwitz.[5]

Materials.—α-P^{32}- and C^{14}-labeled ribonucleotides were obtained from Schwarz BioResearch, Orangeburg, N. Y.; unlabeled ribonucleotides from Mann Research Laboratories, New York, N. Y.; pancreatic DNase I electrophoretically purified free of RNase, pancreatic RNase A, alkaline phosphatase (BAPF), and calf thymus DNA from Worthington Biochemical Corporation, Freehold, N. J.; adenosine 5'-tetraphosphate from Sigma Chemical Co., St. Louis, Missouri; and P_i^{32} from International Chemical Nuclear Co., City of Industry, Calif. Qβ RNA was prepared by phenol extraction of Qβ phage purified by liquid polymer phase fractionation and density equilibrium centrifugation in CsCl.[14] P^{32}-labeled Qβ RNA and bacterial ribosomal RNA were kindly provided by Drs. M. Watanabe and I. Smith, respectively, of this institution. Purified human prostatic phosphomonoesterase was a generous gift from Dr. J. Hurwitz of this institution. Nucleoside diphosphokinase was purified from rabbit muscle by the method of Bessman.[15]

Results.—Incorporation of P^{32} with γ-P^{32}-ribonucleoside triphosphates: Incorporation of P^{32} into acid-insoluble material was detected in the reaction catalyzed by the Qβ RNA polymerase when γ-P^{32}-GTP was used as substrate (Table 1). None of the other γ-P^{32}-labeled substrates was incorporated to an extent comparable to that of GTP incorporation. The level of acid-precipitable radioactivity in control reactions, omitting enzyme or treating with acid immediately after the addition of enzyme, was equivalent to approximately 0.2 $\mu\mu$moles of γ-P^{32}-labeled substrate. A slight amount of acid-precipitable radioactivity in excess of the control value was consistently observed with γ-P^{32}-ATP. The product synthesized with γ-P^{32}-ATP has not yet been characterized, however, and the possible incorporation of this material must be studied further under conditions of greater sensitivity. No incorporation of γ-P^{32}-UTP or γ-P^{32}-CTP was observed, although both of these substrates were found to support the incorporation of other ribonucleoside triphosphates to the same extent as authentic UTP and CTP. In all experiments, synthesis of RNA was two- to threefold in excess of added Qβ RNA.

Requirements for incorporation of γ-P^{32}-GTP: The incorporation of γ-P^{32}-GTP was dependent on the presence of Mg^{++}, ribonucleoside triphosphates, and Qβ RNA (Table 2). These same additions to the reaction mixture were necessary for total RNA synthesis as assayed with α-P^{32}-GTP as substrate. The reaction with either substrate was completely inhibited by 1 μg of RNase, whereas the addition of 10 μg of DNase had no effect.

Characterization of the guanosine triphosphate terminus: The P^{32} incorporated when γ-P^{32}-GTP was used as substrate was shown to be in a terminal position in the

TABLE 1

INCORPORATION OF γ-P^{32}-RIBONUCLEOSIDE TRIPHOSPHATE INTO RNA

Labeled substrate	Nucleotide incorporation ($\mu\mu$moles)
γ-P^{32}-ATP	0.24
γ-P^{32}-GTP	3.7
γ-P^{32}-UTP	<0.1
γ-P^{32}-CTP	<0.1
α-P^{32}-GTP	3200

The conditions for nucleotide incorporation are described in the *Methods* section. The specific activities of γ-P^{32} substrates were (10^6 cpm/mμmole): 1.9, 2.0, 1.4, and 1.0 for ATP, GTP, UTP, and CTP, respectively; and of α-P^{32}-GTP, 950 cpm/mμmole. The reaction mixtures were incubated at 37° for 30 min with 2.5 μg protein of the Qβ RNA polymerase hydroxylapatite fraction. The incorporation shown is that of the labeled substrate, not total nucleotide incorporation.

TABLE 2

REQUIREMENTS FOR INCORPORATION OF P^{32} FROM γ-P^{32}-GTP AND α-P^{32}-GTP

Reaction mixture	Radioactivity Incorporated from γ-P^{32}-GTP ($\mu\mu$moles)	Radioactivity Incorporated from α-P^{32}-GTP ($\mu\mu$moles)
Complete	3.6	3100
Omit Mg^{++}	0.3	2
Omit ATP, UTP, CTP	0.4	4
Omit Qβ RNA	0.3	1
Complete plus DNase (10 μg)	3.6	3090
Complete plus RNase (1 μg)	0.2	2

The complete reaction mixture (0.35 ml) contained 35 μmoles Tris-HCl buffer, pH 7.6, 3 μmoles $MgCl_2$, 1 μmole 2-mercaptoethanol, 100 mμmoles each ATP, CTP, and UTP, 100 mμmoles γ-P^{32}-GTP (2.0×10^6 cpm/mμmole) or α-P^{32}-GTP (950 cpm/mμmole), 5.0 mμmoles Qβ RNA, 12 μg (dry weight) factor, and 2.5 μg protein of the hydroxylapatite fraction. Additions and omissions were made as indicated. The reaction mixtures were incubated at 37° for 30 min and treated as described in the *Methods* section.

RNA product by a variety of chemical and enzymic procedures (Table 3). Incorporation of P^{32} into RNA was indicated by resistance to DNase and by the effect of RNase and alkali in converting the acid-insoluble material into acid-soluble but charcoal-adsorbable products. The terminal position of the P^{32} was indicated by the susceptibility of the phosphate linkage to acid hydrolysis and to treatment with alkaline phosphatase.

The foregoing evidence suggested that the P^{32} was present in a terminal portion of the RNA molecule, presumably as p^{32}ppGpRp - - -, and not in internucleotide linkage, as - -Rp^{32}GpRp - - - ("R" being an unspecified ribonucleoside). Alkaline hydrolysis should thus have released the P^{32} as the tetraphosphate, p^{32}ppGp, rather than as a 2′,3′-monophosphate. The results obtained by high-voltage electrophoresis were in accord with this expectation. Following alkaline hydrolysis the electrophoretic mobility at pH 3.5 of the P^{32}-labeled material was more rapid than that of adenosine tetraphosphate and identical to that of guanosine tetraphosphate isolated from the RNA product of the DNA-dependent RNA polymerase reaction[5] (Fig. 1). There was no loss of radioactivity during electrophoresis and none of the P^{32} was associated with the 2′,3′-ribonucleoside monophosphates, as would have occurred if any of the substrate had been labeled as α-P^{32}-GTP.

Additional experiments were carried out to eliminate the possibility that the rapidly moving material was GTP that remained despite the extensive washing procedure used to remove substrate from the acid-insoluble RNA product. In this

TABLE 3

CHARACTERIZATION OF P^{32}-RNA SYNTHESIZED WITH γ-P^{32}-GTP

Treatment	Acid-insoluble radioactivity (cpm)	Charcoal-adsorbable radioactivity (cpm)
None	748	782
Alkaline phosphatase (5 units)	82	55
RNase (10 μg)	80	665
DNase (10 μg)	690	680
0.3 *M* KOH (18 hr, 37°)	81	673
1.0 *N* HCl (10 min, 100°)	—	52

The γ-P^{32}-GTP-labeled RNA was prepared as described in the text. Alkaline phosphatase treatment was carried out in Tris-HCl buffer, pH 8.4, with 5 units of enzyme (1 unit = 1 μmole of *p*-nitrophenol/min at 25°). The tubes were incubated at 37° for 30 min. Acid-precipitable radioactivity was isolated by the addition of approximately 4 ml of cold 5% trichloroacetic acid (TCA) and filtration through a Millipore HA filter; the filters were then washed 4 times with 5% TCA, dried, and the radioactivity counted in a windowless gas-flow counter. Charcoal-adsorbable radioactivity was isolated by the addition of 0.3 ml of 7% $HClO_4$ and 0.05 ml of a 20% charcoal suspension (Norit) to the reaction mixtures, after which the tubes were shaken well and filtered through a Millipore HA filter; the filters were washed several times with 0.01 *M* H_3PO_4, dried, and the radioactivity counted in a windowless gas-flow counter.

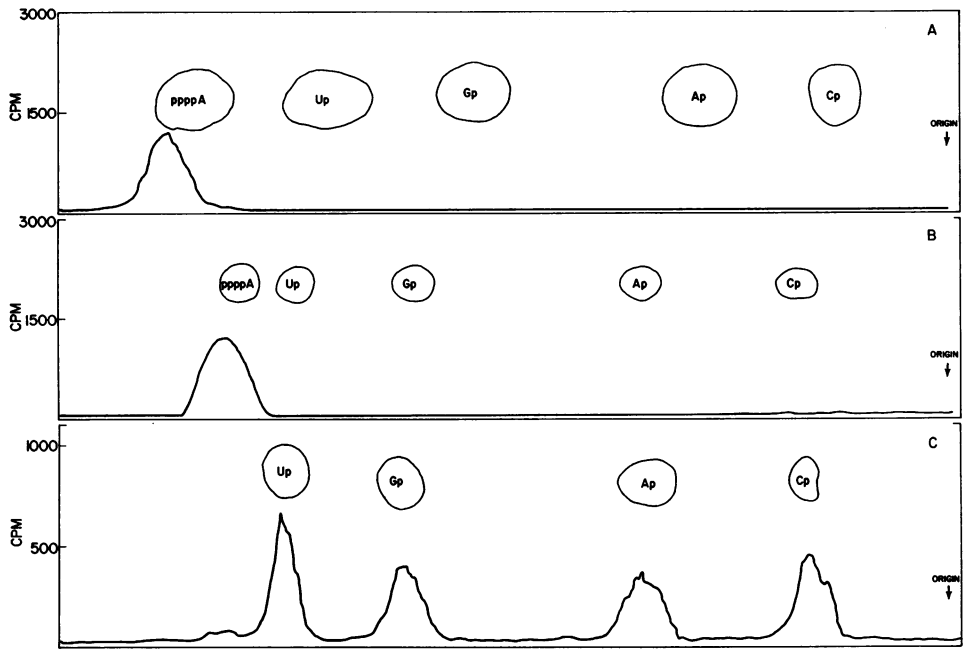

FIG. 1.—Distribution of P^{32}, following alkaline hydrolysis and electrophoresis of RNA synthesized with γ-P^{32}-GTP or α-P^{32}-GTP as substrate. The P^{32}-labeled RNA was prepared as described in the text with (*A*) $Q\beta$ RNA polymerase and γ-P^{32}-GTP, (*B*) DNA-dependent RNA polymerase and γ-P^{32}-GTP, and (*C*) $Q\beta$ RNA polymerase and α-P^{32}-GTP. The acid-precipitable product of each reaction was then hydrolyzed with 0.3 *M* KOH at 37° for 18 hr. The 2′-3′ nucleoside monophosphates were added as markers to each tube as well as adenosine 5′-tetraphosphate to tubes A and B. The hydrolysates were neutralized to pH 7.0 with Dowex-50 (H^+), after which the resin was removed by filtration through glass wool and the effluent concentrated and spotted on Whatman no. 3MM paper. Electrophoresis was carried out in pyridine, acetic acid, water (1:10:89), pH 3.5, at 5000 for 2 hr. The markers were visualized by ultraviolet light and the radioactivity scanned in a Vanguard 880 strip scanner.

experiment, the RNA product was prepared with H^3-GTP as substrate. After alkaline hydrolysis, α-P^{32}-GTP was added to provide an internal radioactive marker in order to monitor for the presence of GTP. Electrophoresis was carried out at pH 4.0, by which complete separation of pppGp from pppG could be obtained. The radioactivity was clearly distributed into four major fractions: (*A*) H^3-labeled material remaining near the origin, (*B*) H^3-2′,3′-GMP present as the major labeled component after alkaline hydrolysis, as expected, (*C*) the P^{32}-GTP marker, and (*D*) H^3-labeled material migrating as would pppGp (Fig. 2). H^3-GTP was only barely detectable, indicating the efficiency of the washing procedure. The ratio of radioactivity in peak *B* (Gp) to that in peak *D* (pppGp) was 412,000 cpm to 630 cpm, close to that expected, as discussed below.

Material isolated as p^{32}ppGp after alkaline hydrolysis was further examined and compared with $ppp^{32}G$ and Gp^{32} by other chemical and enzymic procedures. The labeled compounds were all isolated by elution with water from paper electropherograms. As would be expected, the radioactivity of each, measured by adsorption to charcoal, was sensitive to alkaline phosphatase, whereas only the phosphomonoester of Gp^{32} was sensitive to prostatic phosphomonoesterase, and only of p^{32}ppGp was the P^{32} completely removed by acid hydrolysis (Table 4).

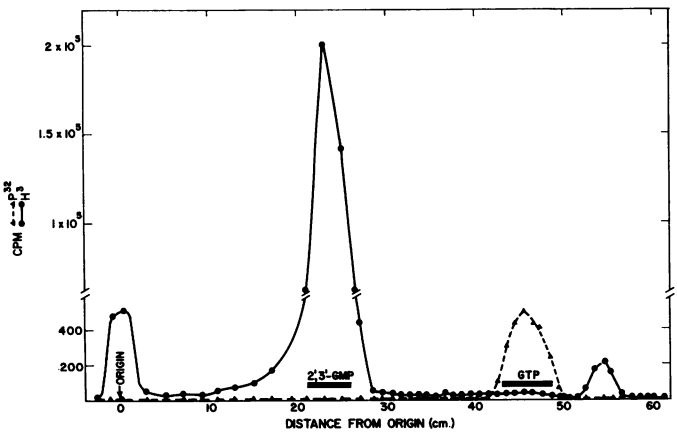

FIG. 2.—Separation of α-P^{32}-GTP and H^3-labeled fractions after alkaline hydrolysis of RNA synthesized with H^3-GTP as substrate. The H^3-labeled RNA was synthesized in a reaction mixture (0.20 ml) containing 25 μmoles Tris-HCl buffer, pH 7.6, 2.5 μmoles $MgCl_2$, 1 μmole 2-mercaptoethanol, 200 μmoles each ATP, UTP, and CTP, 60 mμmoles H^3-GTP (2.7×10^6 dpm/mμmole), 0.8 mμmoles Qβ RNA, 2 μg factor, and 2 μg Qβ RNA polymerase. After incubation at 37° for 20 min, the acid-insoluble material was isolated as described in *Methods* for assay of γ-P^{32}-nucleoside triphosphate incorporation. The final precipitate was dissolved in 1.0 ml 0.3 *M* KOH at 37° for 18 hr. 2′,3′-GMP and GTP were added as reference markers and α-P^{32}-GTP (2720 cpm) as a radioactive marker. The hydrolysate was then further treated as described in Fig. 1. Electrophoresis was carried out in 0.04 *M* sodium citrate buffer pH 4.0, at 5000 v for 2 hr. The reference markers were visualized by ultraviolet light and the paper then cut into 1 × 2 cm strips. The strips were folded and placed in scintillation vials containing phosphor (3 gm of 2,5-diphenyloxazole and 100 mg of 1,4-bis-2(5-phenyloxazolyl)-benzene per liter of reagent grade toluene) and counted in a liquid-scintillation spectrometer using separate channels to discriminate P^{32} from H^3 radioactivity. The efficiency of detection for H^3 radioactivity was 4.3%.

Correlation of the extent of γ-P^{32}-GTP incorporation with the size of the RNA product: If a single species of RNA the same size as that of Qβ RNA was synthesized in the Qβ RNA polymerase reaction, there would be an expected incorporation of one equivalent of an initiating nucleotide to approximately 3500 equivalents of total nucleotide. A greater ratio of γ-P^{32} incorporation would suggest the presence of incomplete chains or multiple initiation points. Conversely, fewer triphosphate

TABLE 4

PROPERTIES OF THE γ-P^{32}-GTP-LABELED FRACTION AFTER ALKALINE HYDROLYSIS

Treatment	Labeled Material (cpm charcoal adsorbable)		
	$p^{32}ppGp$	$ppp^{32}G$	Gp^{32}
None	400	500	216
Alkaline phosphatase (10 units)	28	22	12
Phosphomonoesterase (6.0 units)	350	450	12
Acid hydrolysis (1 *N* HCl, 100°, 10 min)	40	500	192

RNA synthesized with γ-P^{32}-GTP or α-P^{32}-GTP as substrate was hydrolyzed with alkali and the degradation products isolated by high-voltage electrophoresis as described in Fig. 1. Radioactive fractions corresponding to $p^{32}ppGp$ and Gp^{32} were eluted from the paper with water. Authentic $ppp^{32}G$ was also subjected to paper electrophoresis and recovered in the same manner. Prostatic phosphomonoesterase treatment was carried out in sodium acetate buffer, pH 4, with 6 units of enzyme (1 unit = 1 μmole *p*-nitrophenol/min at 37°). The alkaline phosphatase reaction and assay for charcoal-adsorbable radioactivity were performed as described in Table 3.

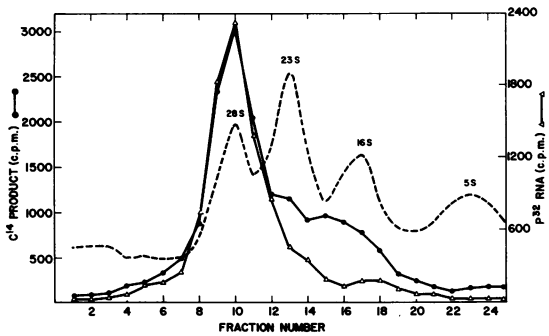

FIG. 3.—Sucrose gradient centrifugation of the RNA product. Thirty mμmoles (total nucleotide) of RNA were synthesized in a reaction mixture (0.25 ml) containing 25 mμmoles Tris-HCl, pH 7.6, 3 μmoles $MgCl_2$, 1 μmole 2-mercaptoethanol, 150 mμmoles C^{14}-GTP (1.8×10^3 cpm/mμmole), 200 mμmoles each of ATP, CTP, UTP, 1.2 mμmoles Qβ RNA, 12 μg (dry weight) factor, and 0.8 μg enzyme protein of the hydroxylapatite fraction. After incubation at 37° for 20 min, the reaction was terminated by the addition of 0.2 ml of a solution containing 0.5% sodium lauryl sulfate, 0.05 *M* Tris-HCl, pH 7.6, 0.01 *M* EDTA, and 0.1 *M* NaCl. P^{32}-Qβ RNA (220 mμmoles, 7000 cpm) was then added and the entire mixture (0.50 ml) was layered on a solution as above containing a sucrose gradient of 15–30%. Ribosomal RNA (250 mμmoles) and Qβ RNA (125 mμmoles) were added as reference markers to an otherwise identical tube. The preparations were centrifuged in a SW25.1 Spinco rotor at 23,500 rpm for 16 hr at 15°. Fractions were collected and assayed for absorbancy and for acid-precipitable radioactivity. The radioactivity of the material collected on Millipore filters was measured in a liquid scintillation spectrometer using separate channels to discriminate P^{32} and C^{14}.

ends would suggest the synthesis of chains containing more than 3500 bases or the presence of undetected initiation sites.

In the experiments shown in Tables 1 and 2, 3.6–3.7 μμmoles of γ-P^{32}-GTP were incorporated as compared to 3,100–3,200 μμmoles of α-P^{32}-GMP incorporation. Since GMP comprises 23.7 moles per cent of the nucleotide content of Qβ RNA,[16] the total nucleotide incorporation in these experiments by calculation was about 13,100–13,500 μμmoles. The actual experimental value obtained by measuring the incorporation of each of the four C^{14}-labeled ribonucleoside triphosphates was 13,600. Therefore, the average chain length of the RNA molecules synthesized in these experiments, calculated from the ratio of γ-P^{32}-GTP nucleotides to total internal nucleotides, was about 3,600.

This evidence that the RNA product was composed predominantly of molecules of similar size to Qβ RNA was confirmed by other studies of the physical properties of the RNA product (Fig. 3). In sucrose gradient centrifugation, the main component of the *in vitro* product sedimented as 28*S* RNA, together with Qβ RNA. A small fraction of the RNA product sedimented as 16–23*S* material. In addition, the elution profile of the RNA product on methylated albumin kieselguhr columns was identical to that of Qβ RNA (Fig. 4).

Discussion.—The RNA product of the Qβ RNA polymerase reaction was found to contain guanosine triphosphate at the 5′-terminus, as the structure pppGpRp - - -. Experiments are currently in progress to identify the unspecified ribonucleoside, "R." Quantitatively, this structure was, at the least, the highly preferred 5′-terminal form since (*a*) γ-P^{32}-GTP incorporation was at least 90–95 per cent of

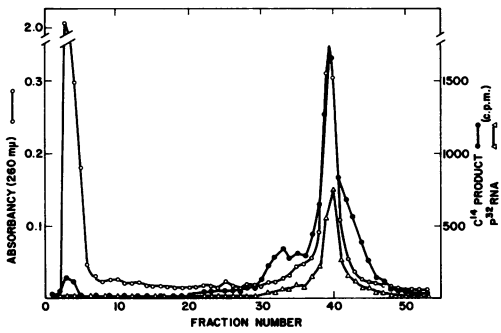

FIG. 4.—Chromatography of the RNA product on a methylated albumin kieselguhr (MAK) column. RNA, 8.4 mμmoles total nucleotide, was synthesized in a reaction mixture containing C^{14}-GTP (2.2×10^3 cpm/mμmole), 1.5 μg enzyme protein of the hydroxylapatite fraction, and other additions as described in Fig. 3. The reaction was terminated by chilling, 0.03 ml of P^{32}-Qβ RNA (80 μg, 1800 cpm) was added and the volume adjusted to 1.0 ml with 0.1 *M* NaCl in 0.02 *M* potassium phosphate buffer, pH 6.8. The solution was applied to a 1×10 cm MAK column and eluted with a linear gradient of NaCl, 0.5–1.2 *M*, in 100 ml of 0.02 *M* potassium phosphate buffer, pH 6.8.[17] Fractions of 2.0 ml were collected and analyzed for absorbancy and acid-precipitable radioactivity as described in Fig. 3.

total γ-P^{32}-labeled nucleoside triphosphate incorporation, and (*b*) the size of the product as determined by sedimentation analysis and chromatography in methylated albumin kieselguhr corresponded closely with that calculated from the ratio of GTP termini to total nucleotide incorporation. From this ratio, a chain length of approximately 3600 nucleotides was estimated for the RNA product, the bulk of which sedimented together with Qβ RNA. It is yet to be determined how these findings correlate with the structure of the 5′-terminus of RNA isolated from Qβ phage.

From these results a distinction can be made between the Qβ RNA polymerase and several enzymes where synthesis preferentially begins by addition to a 3′-hydroxyl terminus, as with the sRNA adenylate (cytidylate) pyrophosphorylase,[3] DNA polymerase,[18] polynucleotide phosphorylase,[19] and polyriboadenylate polymerase.[20] On this basis the Qβ enzyme more closely resembles the DNA-dependent RNA polymerase which also has been shown to initiate synthesis with ribonucleoside triphosphates.[5] With the bacterial DNA-dependent RNA polymerase, however, initiation occurs with either ATP or GTP and there appear to be many initiation sites per molecule of DNA.

The predominance of a single terminal nucleotide, in this case GTP, provides a partial explanation for the specificity of the Qβ RNA polymerase reaction. Moreover, the results are in accord with the concept of a single initiation site per molecule of template RNA, presumably at one of the termini. However, despite the finding that this enzyme demonstrates a remarkable preference for Qβ RNA among a variety of RNA preparations tested as template,[1, 21] it has been found that polycytidylic acid will also support the reaction as will many ribocopolymers of widely varying base composition, provided they contain cytosine as one of the bases.[21] With these synthetic polymers, regardless of base composition, initiation also occurs predominantly with GTP. Thus, it appears that one of the important features regulating the activity of the Qβ RNA polymerase is the initiation of synthesis.

Summary.—A RNA product of an *in vitro* reaction with the phage Qβ RNA polymerase has been found to terminate with guanosine 5′-triphosphate. The size of the RNA product calculated from the ratio of initial to total nucleotides corresponds to that of Qβ RNA.

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