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Structure of the Poly(G) Polymerase Component of the Bacteriophage f2 Replicase

(protein subunits)

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ABSTRACT **A rifampicin-resistant poly(G) polymerase has been purified from f2 sus 11-infected cells. The poly(G) polymerase is believed to represent part of the f2 replicase on the basis of several criteria. It is present only in infected cells and shares the characteristic rifampicin resistance of crude f2 replicase activity. Partially purified poly(G) polymerase preparations exhibit replicase activity, synthesizing f2 'plus' strand RNA from denatured, partially double-stranded f2 RNA template. Highly purified poly(G) polymerase preparations, although lacking replicase activity, contain a protein which is electrophoretically identical to the protein product of the viral replicase cistron.**

The replicative cycle of RNA bacteriophages, particularly those of the f2 serotype, has been analyzed in some detail (1, 2). The structure of the RNA species produced during the infectious cycle is known, as is the temporal sequence of their synthesis (2). Studies of RNA metabolism in cells infected with various phage mutants indicate that the enzyme or enzyme complex responsible for phage RNA synthesis contains both bacterial and viral components (1, 3). *In vitro* studies of the f2-type phage RNA replicases have, however, been hampered by the instability of these enzymes and the difficulty in obtaining enzyme preparations free of endogenous template (2, 4–6). Thus, almost all of the currently available information on the structure and enzymology of phage replicase has come from studies of the more stable enzyme of the serologically unrelated RNA phage Qβ. The Qβ enzyme appears to be quite complex, requiring several bacterial proteins in addition to a phage-specified protein to carry out net synthesis of phage 'plus'* strand RNA from 'plus' strand template (7–9). Several laboratories have shown that the Qβ replicase contains a simpler 'core' enzyme defined by its ability to utilize phage 'minus'† strands or poly(C) as template and its inability to use phage 'plus' strand template (7–10). Our studies of the f2 replicase were predicated on the assumption that the f2 enzyme is similarly subdivisible and that the 'core' enzyme would prove more amenable to purification and analysis than the complete enzyme.

We report here a purification of the f2 replicase based on its rifampicin-resistant poly(C)-dependent poly(G) polymerase activity.

Abbreviation: RE, replicative ensemble.

* 'Plus' strand denotes the single-stranded RNA occurring in RNA phage.

† 'Minus' strand denotes the complementary copy of 'plus' strand RNA.

MATERIALS

Escherichia coli K 38 (su$^-$) or A 19 (su$^-$*met*$^-$RNase$^-$) served as host bacteria. f2 sus 11 stocks were grown on *E. coli* K 37 (su$^+$). Sus 11 is a double mutant of f2 having amber mutations in the coat and A protein cistrons (11). ATP, CTP, GTP, UTP, and poly(C) were from P-L Biochemicals, Inc.; [^{3}H]-UTP and [^{3}H]GTP from Schwarz/Mann; [^{3}H]poly(C) from Miles Laboratories, Inc.; CF11 cellulose powder and DE 52 DEAE-cellulose from Whatman; Carbowax 6000 from Union Carbide and Dextran T500 from Pharmacia; DPFF DNase I from Worthington Biochemical Corp. and pyruvate kinase from Calbiochem. Canalco products were used for gel electrophoresis. Rifampicin was generously provided by Dr. J. Gelzer of Ciba Pharmaceutical Co. Purified Qβ replicase and factors were kindly supplied by Drs. L. Eoyang and G. H. Kuo; REO virus-infected cells were the gift of Dr. S. Silverstein. Qβ RNA was supplied by Dr. K. Horiuchi and *E. coli* DNA-dependent RNA polymerase by Dr. T. S. Chan, both of this laboratory.

METHODS

Wild-type f2 phage was purified according to Webster *et al.* (12). Sus 11 stocks for replicase preparations were made by the plate-scrape method.

Wild-type f2 RNA was purified from phage by the method of Webster *et al.* (12). Partially double-stranded RNA ['replicative ensemble' or RE (13)] was purified from f2-infected cells as described by Robertson and Zinder (14). RE was denatured by heating and quick-cooling in 0.5 mM EDTA as described by Weissmann *et al.* (15). Double-stranded REO virus RNA was purified from infected mouse L2 cells as described by Schonberg *et al.* (16).

RNA-cellulose

Purified single-stranded f2 RNA in 35% ethanol–TSE buffer (35 ml of absolute ethanol and 65 ml of TSE buffer: 0.05 M Tris (pH 6.85)–0.1 M NaCl–0.001 M EDTA) was mixed with 75–150 mg of ethanol-washed CF11 cellulose/mg of RNA. The slurry was allowed to stand for 1 hr at room temperature, diluted 50-fold with cold absolute ethanol, filtered under suction through Whatman No. 3 paper, and allowed to dry. The powder was suspended in 20 ml of absolute ethanol/500 mg of cellulose, made 10 mM in magnesium acetate, and UV-irradiated for 30 min as described by Litman (17). The ethanol was removed by filtration and the powder was dried at room temperature. Unbound RNA was removed

by washing with cold TSE buffer. RNA-cellulose used for chromatography contained 4–12 mg of bound f2 RNA/g cellulose.

Assays

Reaction mixtures for the poly(G) polymerase assay contained 50 mM Tris (pH 7.5), 1 mM EDTA, 10 mM magnesium acetate, 10% glycerol, 1 mM phosphoenolpyruvate, 5 μg/ml pyruvate kinase, 0.5 mM dithiothreitol, 0.2 mM GTP, and [^{3}H]GTP, 28 cpm/pmol. Rifampicin and poly(C) were used at 2.5 and 5 μg/ml, respectively. In the assay for poly(G) polymerase, 1–20 μl of enzyme was incubated in 0.1–0.2 ml of the assay mixture at 30°C for 10 min. The reaction was terminated by addition of 3 ml of 5% Cl_3CCOOH containing 1 M NaCl and 0.01 M sodium pyrophosphate; precipitates were collected on Millipore filters, washed with the Cl_3CCOOH–NaCl–pyrophosphate solution, dried and counted. Activity is expressed as picomoles of GTP converted to acid-insoluble GMP-equivalents by the enzyme aliquot assayed (5–10 μl, 1–120 μg). Assays for ribonuclease activity were performed under the poly(G) polymerase assay conditions. The reaction mixture was modified by the omission of [^{3}H]GTP and unlabeled poly(C) and addition of 0.1 μg/ml [^{3}H]poly(C) (approximately 3000 cpm acid-precipitable). Complete reaction mixtures for measuring endogenous activity in crude extracts contained 0.2 mM UTP, CTP, and ATP in addition to the GTP. Reaction conditions were as above. When the phage-specific component of total NTP-incorporation was to be measured, both rifampicin and DNase (50 μg/ml) were present. Assays for template-dependent activity generally included a 10-min prior incubation at 30°C of the enzyme in 25–50 μl of SB20 [standard buffer: 50 mM Tris (pH 7.5)–5 mM magnesium acetate–1 mM EDTA–0.1 mM dithiothreitol–20% glycerol], and the reaction mixture contained [^{3}H]GTP and [^{3}H]UTP (both at 28 cpm/pmol).

Gel electrophoresis

Proteins were analyzed on 10% acrylamide sodium dodecyl sulfate gels as described by Weber and Osborn (18). The gels were stained with Coomassie Brilliant Blue (18), destained in the dye solvent solution, transferred to 5% methanol–7.5% acetic acid, and scanned with a Gilford spectrophotometer equipped with a linear transport. Gels containing labeled proteins were also frozen, sliced, and prepared for counting as described by Fromageot and Zinder (19), except that the slices were first dried to remove acetic acid.

Storage of enzyme

Enzyme preparations in SB50 (standard buffer containing 50% glycerol) were stored at −13°C or quick-frozen in Dry Ice–ethanol and stored in liquid nitrogen. The half-life of partially purified enzyme is 12 hr at 0°C, 1 month at −13°C, and indefinite in liquid nitrogen. Highly purified enzyme has been stored successfully only in liquid nitrogen.

Replicase purification

Bacterial cultures in enriched medium (32 g of tryptone, 20 g of yeast extract, and 5 g of NaCl/liter of medium) were infected at a multiplicity of 4–5 sus 11 phage per bacterium, grown for 1.5 hr at 37°C, chilled by pouring over frozen, crushed medium, and harvested by centrifugation. Frozen cell pellets (20–40 g) were combined with 2.5 g of washed glass beads and 1 ml of SB20 per g of cells, in the large container of a Virtis homogenizer. The vessel was placed in an ice-bath and the cells were ground at medium speed for 15 min, interspersing 3-min grinding intervals with 1-min intervals for chilling. Grinding and all subsequent operations were performed at 4°C. Glass beads were removed as described by Burgess (20) and the homogenate was clarified by centrifugation at 8000 rpm for 10 min. The supernatant was subjected to liquid-polymer phase partitioning (9, 10). The combined top phases were dialyzed against SB20 without Mg^{++} for 6–7 hr until the salt concentration had been reduced to 0.05 M NaCl or less (checked by conductivity).

DEAE-cellulose chromatography

The dialysate was applied to DEAE-cellulose (4 × 30 cm column for extract from 40 g of cells) equilibrated with SB20 (no Mg^{++}) containing 0.05 M NaCl. The column was washed with 1–2 column volumes of the same buffer and developed with a 0.08–0.25 M NaCl gradient in SB20 (no Mg^{++}). Peak fractions of poly(G) polymerase activity were dialyzed against SB20 (no Mg^{++}) for 1 hr and then against SB50 (no Mg^{++}) for 3 hr.

RNA-cellulose chromatography

RNA-cellulose (0.35–1.0 g) was suspended in SB50 (no Mg^{++}) containing 0.05 M NaCl and poured into a small column (0.5–0.9 cm in diameter). The column was washed free of UV-absorbing material with the same buffer at a flow rate of about 4 ml/hr. An aliquot of dialyzed DEAE-enzyme (35–40 mg) was applied at a flow rate of 1–2 ml/hr. The flow rate was restored and the column was washed with the 0.05 M NaCl loading buffer until the A_{280} of the eluate was 0.05 or less, then it was washed with 1–2 column volumes of 0.1 M NaCl in the same buffer. Poly(G) polymerase activity was eluted with 0.2 M NaCl in SB 50 (no Mg^{++}).

Glycerol gradient centrifugation

Active fractions from the RNA column were dialyzed for 2 hr against low glycerol–high salt standard buffer (standard buffer containing 4% glycerol and 1 M NaCl). Glycerol gradients (7–25%) were made in the same high-salt buffer. Dialyzed RNA column enzyme was layered on 13-ml gradients and centrifuged at 40,000 rpm for 24 hr at 2°C in the SW 40 rotor of the Beckman L2-65B ultracentrifuge. The gradients were collected in 0.5-ml fractions and assayed for poly(G) polymerase and RNase activity.

RESULTS AND DISCUSSION

The appearance of a new, DNase-resistant, ribonucleoside triphosphate-incorporating activity upon infection of *E. coli* with bacteriophage f2 or the f2 mutant sus 11 is well established (11, 21). Extracts of sus 11 mutant-infected cells have 10–20 times the wild-type level of replicase activity (11, 21, 22). Replicase activity in crude cell extracts is insensitive to the addition of template RNA (21). To test the possibility that the f2 replicase has a poly(C)-dependent poly(G) polymerase analogous to that of the Qβ replicase, we had first to free the enzyme from endogenous template, which we did by liquid-polymer phase partitioning (9). Table 1 shows that this procedure does indeed abolish template-independent GTP incorporation by extracts of sus 11-infected cells. Upon addition of poly(C) template, infected cell extracts stimulate polymerization of about 5 times as much GTP as uninfected

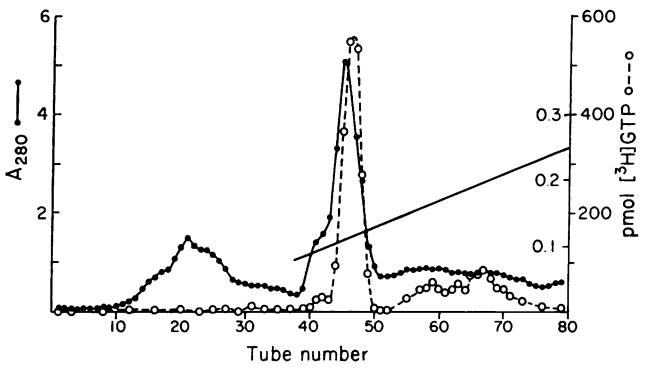

FIG. 1. DEAE-cellulose chromatography of rifampicin-resistant poly(G) polymerase activity. The solid line represents the eluting NaCl gradient (0.08–0.25 M). A 10-μl aliquot of each 10-ml fraction was assayed for poly(G) polymerase activity in the presence of rifampicin (O- - -O); UV absorbance was read at 280 nm (●——●).

cell extracts. Furthermore, 80% of the activity in infected-cell extracts is resistant to rifampicin, in contrast to the complete drug-sensitivity of uninfected extract activity. Thus infected cells contain, in addition to the rifampicin-sensitive synthetic polymer activity of the DNA-dependent RNA polymerase (10, 23), a rifampicin-resistant poly(G) polymerase induced or greatly augmented by phage infection. Since replicase activity in crude cell extracts is similarly resistant to rifampicin (Table 1), it appears likely that this poly(G) polymerase activity is associated with the f2 replicase.

DEAE-cellulose chromatography of rifampicin-resistant poly(G) polymerase is shown in Fig. 1. The phage-induced activity is eluted by 0.10–0.12 M NaCl, a salt concentration at which most of the DNA-dependent RNA polymerase remains bound (20). The small amount of activity that is eluted behind the main peak is not template-dependent and may be residual template-bound enzyme. Table 2 summarizes

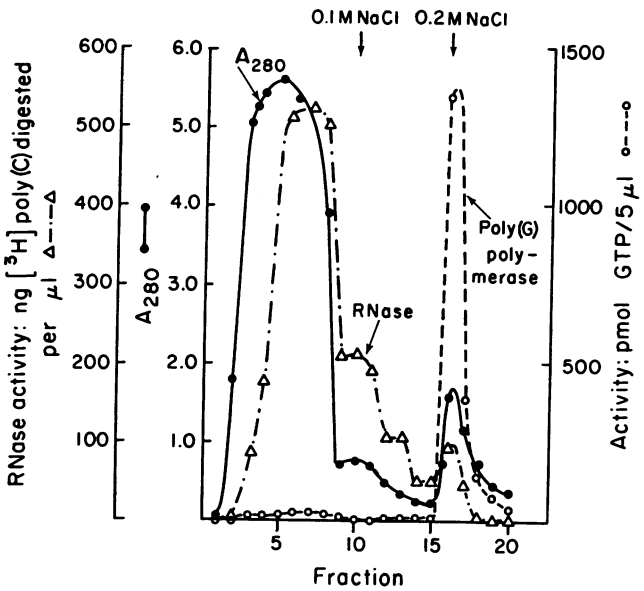

FIG. 2. RNA-cellulose chromatography of DEAE-cellulose enzyme. A 1-μl aliquot of each 1-ml fraction was assayed for RNase activity (Δ-·-Δ), a 5-μl aliquot was assayed for rifampicin-resistant poly(G) polymerase activity (O- - -O), and the UV absorbance was read at 280 nm (●——●).

TABLE 1. *Poly(G) polymerase activity in infected and uninfected extracts*

	Additions			GTP incorporation*	
	DNase	Rifampicin	Poly(C)	Infected	Uninfected
Crude extract	+	+	−	501	13.5
Extract after phase partitioning	+	−	−	1.7	7.0
	+	−	+	389	84.0
	+	+	+	311	6.7

Assays for crude, template-independent activity and for poly-(G) polymerase activity were as described in *Methods*.

* Picomoles of GTP converted to acid-insoluble GMP equivalents by 5 μl of extract.

some of the properties of the DEAE-purified enzyme. Under standard assay conditions, the enzyme shows good template dependence. However, just as with the partially purified Qβ replicase, prolonged incubation results in the unprimed incorporation of nucleotides (24). Of the template RNAs tested, f2 DEAE-enzyme will efficiently utilize only denatured f2 RE and poly(C). A striking feature of the reaction with phage RNA template is the 10-fold stimulation observed upon incubation of the enzyme in buffer at 30°C prior to the addition of substrate and template. The product of the reaction with denatured f2 RE template has been analyzed by annealing and found to consist exclusively of f2 'plus' strand RNA (to be published). Thus, the partially purified rifampicin-resistant poly(G) polymerase preparation contains phage replicase activity. Its inability to utilize phage 'plus' strand RNA as template suggests that some part of the

TABLE 2. *Template activity of DEAE-cellulose enzyme*

Template*	Preincubation (min)	Incubation (min)	Incubation† GTP	Incubation† GTP + UTP
None	—	10	8.2	—
Poly(C)	—	10	443.6	—
None	10	10	—	4.3
f2 single-stranded RNA	10	10	—	11.2
Qβ single-stranded RNA	10	10	—	0.0
E. coli RNA‡	10	10	—	0.0
Native f2 RE	—	10	—	8.3
Denatured f2 RE	—	10	—	35.3
Native f2 RE	10	10	—	16.5
Denatured f2 RE	10	10	—	383.5
None	10	20	—	56.7
None	10	40	—	122.0
Native REO RNA	10	10	—	11.7
Denatured REO RNA	10	10	—	15.1

The composition of the reaction mixtures is given in *Methods*, as are the assay conditions.

* Template concentrations per 0.1 ml were: 1 μg of poly(C); 2 μg of f2 and Qβ single-stranded RNAs and *E. coli* RNA; 1.75 μg of f2 native and denatured RE; 1.6 μg of native and denatured REO RNA.

† Picomoles of GTP or GTP + UTP converted to acid-insoluble nucleoside monophosphate equivalents by 120 μg of enzyme.

‡ Total cellular RNA prepared by phenol extraction and DNase treatment of *E. coli* nucleic acids.

TABLE 3. *Template activity of RNA-column enzyme*

Template*	Preincubation (min)	Incubation (min)	Incorporation† GTP	Incorporation† GTP + UTP
None	—	10	0.4	—
Poly(C)	—	10	363.8	—
Native f2 RE	10	20	—	0.0
Denatured f2 RE	10	20	—	0.4

Composition of reaction mixtures and reaction conditions as described in *Methods*.

* Poly(C) was present at 1 μg and f2 RE at 1.75 μg/0.1 ml.

† Picomoles of GTP or GTP + UTP converted to acid-insoluble NMP equivalents by 1 μg of enzyme.

holoenzyme has already been lost during purification or that the holoenzyme has dissociated irreversibly under the assay conditions used.

The phage poly(G) polymerase activity was further purified by affinity chromatography on CF11 cellulose containing bound f2 RNA. Fig. 2 shows the elution profile of such a column. About 75% of the input poly(G) polymerase activity is recovered from the column. Since the half-life of the enzyme at 0°C is 12 hr, the 25% loss of activity is almost all attributable to enzyme inactivation at 4°C during the 5- to 6-hr column run. However, prolonged washing of the column in an attempt to remove all RNase activity prior to elution of the enzyme results in much greater loss of enzyme activity. Template properties of the poly(G) polymerase after RNA-cellulose chromatography are given in Table 3. Poly(G) polymerase of high specific activity is recovered from the column. However, the poly(G) polymerase fraction no longer displays activity with denatured replicative ensemble (RE) template.

When RNA-column enzyme was centrifuged through a high-salt glycerol gradient, poly(G) polymerase was detected as a single 6.6 S peak (Fig. 3). Pooled active fractions from the glycerol gradient were analyzed on sodium dodecyl sulfate–polyacrylamide gels. The tracing in Fig. 4 shows that the glycerol-gradient enzyme contains five major proteins and a number of minor components. To determine which of the proteins purified with poly(G) polymerase activity, we compared glycerol-gradient enzyme with RNA column enzyme (inset to Fig. 4). The relative concentrations of four bands (labeled I, II, III, and IV) were enhanced by sedimentation of the RNA column enzyme through glycerol. The relative amounts of all other proteins decreased. Bands I–IV were therefore tentatively identified as poly(G) polymerase-associated proteins. Approximate molecular-weight estimates based on electrophoretic mobility are 75,000, 63,000, 46,000, and 33,000 for protein I–IV, respectively. About 80% of the protein on gel *b* is present in bands I–IV. At this stage of purification the enzyme is extremely unstable; attempts to obtain active preparations of greater purity have not yet been successful. Thus evidence that proteins I–IV are subunits of the poly(G) polymerase is, of necessity, indirect.

To determine whether the product of the viral replicase cistron is represented in the poly(G) polymerase preparation, we coelectrophoresed the glycerol-gradient enzyme with *in vivo* viral proteins. Sus 11-infected cells, treated with rifampicin and labeled with ^{14}C-labeled amino acids, were used as a source of *in vivo* viral replicase protein. The identity

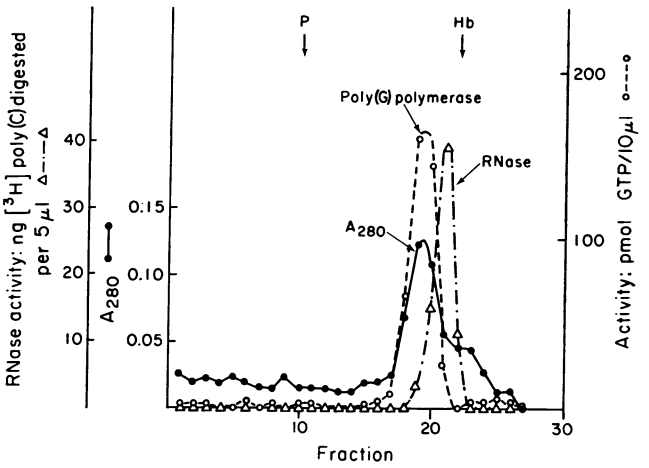

FIG. 3. Glycerol-gradient centrifugation of f2 poly(G) polymerase. Hemoglobin (Hb, 4.3 S) and *E. coli* DNA-dependent RNA polymerase (P, 13–15S) were used as sedimentation markers. A 10-μl aliquot of each 0.5-ml fraction was assayed for poly(G) polymerase activity (O- - -O), a 5-μl aliquot was assayed for RNase activity (Δ—·—Δ), and UV absorbance was read at 280 nm (●—●).

and electrophoretic behavior of the f2 and MS2 proteins has received much attention (19, 25, 26). Of the three known viral proteins, the largest has been identified as the product of the replicase cistron and is known to be hyperproduced by coat mutants of MS2 and f2 (25, 27). The amber mutations in the sus 11 coat and A protein cistrons make sus 11-infected cells an excellent source of replicase protein. The replicase protein, which appears in slices 14 and 15 of the gel shown in Fig. 5, is the only protein which is labeled in sus 11-infected

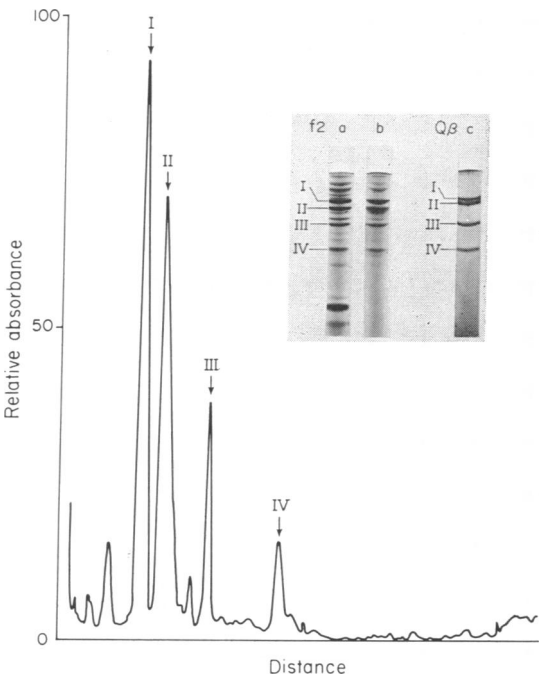

FIG. 4. Sodium dodecyl sulfate–polyacrylamide gels of poly(G) polymerase. Stained gels were scanned at 600 nm. Bands identified as poly(G) polymerase subunits are marked I, II, III, and IV. *Inset: a*, f2 RNA-column enzyme; *b*, f2 glycerol-gradient enzyme; *c*, purified Qβ replicase

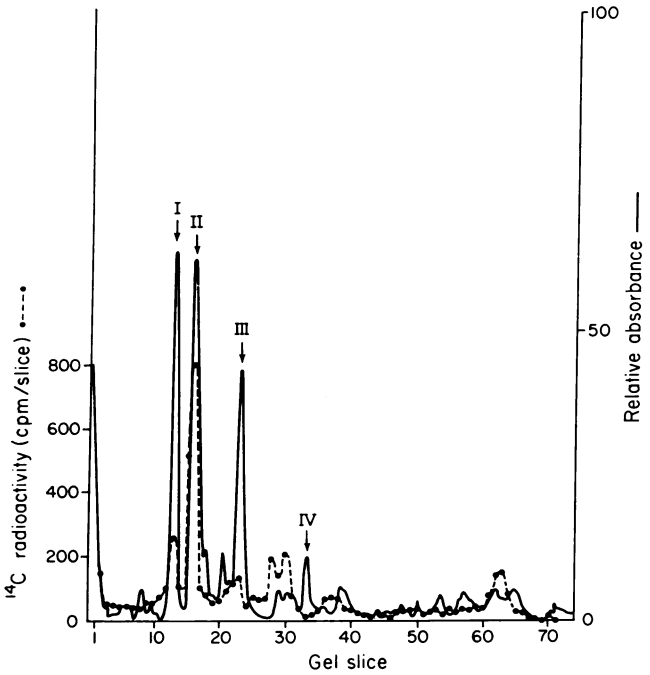

FIG. 5. Coelectrophoresis of *in vivo* [^{14}C]proteins from sus 11-infected cells with purified poly(G) polymerase. Proteins were extracted from infected cells treated with rifampicin at 20 min and labeled from 30 to 60 min after infection, with 5 μCi each of ^{14}C-labeled lysine, leucine, phenylalanine, and arginine as described by Fromageot and Zinder (19). Gels were stained and scanned for poly(G) polymerase proteins (——), then sliced and counted for radioactive proteins (●- - -●).

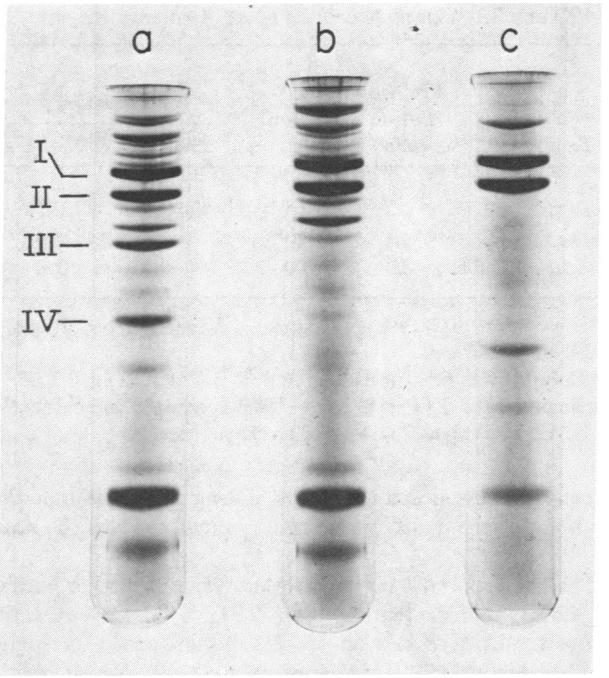

FIG. 6. Sodium dodecyl sulfate–poly acrylamide gels of RNA-cellulose enzyme.

cells, but not in uninfected cells after actinomycin (25) or rifampicin treatment (P. Model, N. Fedoroff, unpublished observations). Fig. 4 shows that the replicase protein labeled *in vivo* coelectrophoreses with the 63,000-dalton protein of the purified poly(G) polymerase. Similar results were obtained when viral proteins made *in vitro* using wild-type f2 RNA template were coelectrophoresed with the purified poly(G) polymerase (not shown). It appears likely, therefore, that protein II of the poly(G) polymerase is the viral replicase protein.

The significance of bands I, III, and IV is less clear. There is some evidence that bands III and IV are necessary for poly(G) polymerase activity. In Fig. 6, gels *a* and *b* show the poly(G) polymerase peak proteins from two RNA columns of aliquots from the same DEAE-cellulose preparation. Several weeks of storage intervened between column runs, and the specific activity of the loading enzyme was considerably lower in the case of the second column. In addition, the second column was subjected to more extensive washing prior to elution of enzyme than the first. The specific activities of enzyme from the two columns were 250 and 40 (as nanomoles GTP/mg per 10 min), respectively. A careful comparison of the two gels shows that all proteins appear in similar amounts in both preparations, with the exception of bands III and IV, which are barely detectable in gel *b*. Rechromatography of the preparation shown in gel *b* on a second RNA column further decreased the specific activity to 20 and virtually eliminated bands III and IV (gel *c*). This observation suggests that bands III and IV are required for poly(G) polymerase activity.

A gel of the Qβ replicase, electrophoresed under the same conditions as the f2 poly(G) polymerase, is shown in Fig. 3, gel *c*. The subunits are identified according to Kamen's designation (10). Qβ subunits I, III, and IV appear to correspond to bands I, III, and IV of the f2 poly(G) polymerase. Only the phage-specified subunit II shows a marked difference in electrophoretic mobility. Despite their apparent structural similarity, the Qβ and f2 enzymes differ significantly in stability and template activity. If subunits I, III, and IV of the two enzymes represent the same bacterial proteins, then the difference in stability must be attributable to the structure of the phage-specific subunit. A more fundamental difference between the enzymes lies in their abilities to use phage template RNA. Qβ poly(G) polymerase will use Qβ 'minus' strand template (10). Addition of the factors described by August and coworkers (factors I and II) to the Qβ enzyme of gel *c* (Fig. 4) restores its capacity to synthesize Qβ 'plus' strands from 'plus' strand template (7). Neither August's factors, nor various other bacterial protein preparations, has yet been found to restore either activity to the f2 poly(G) polymerase (unpublished observations).

Large amounts of protein I and the phage-specific protein II have invariably been recovered from RNA cellulose. The observation of Kondo *et al.* (28) that only the two largest subunits of the Qβ replicase could be recovered from a replicase–Qβ RNA complex may be pertinent in this connection. Quite probably, proteins I and II of the f2 replicase, like those of the Qβ enzyme, contain the RNA-binding site. These subunits would, therefore, bind to the RNA column whether associated with III and IV or not. The smaller components, on the other hand, may be retained on the RNA-cellulose only when bound to I and II. Thus, the poly(G) polymerase eluted from RNA-cellulose may be enriched for inactive subunits I and II.

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