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Deoxyribonucleic Acid Dependent Ribonucleic Acid Polymerases from Two T4 Phage-Infected Systems†

Audrey Stevens

ABSTRACT: The properties of DNA-dependent RNA polymerases from two T4 phage-infected systems have been studied and compared with those of RNA polymerase from uninfected *Escherichia coli* (host polymerase). For the T4 phage-infection systems, T4 double amber mutants were used, one being a gene 42⁻Xgene 47⁻ mutant and the other a gene 42⁻Xgene 55⁻ mutant. The contents of the σ subunit and of four new T4-specific polymerase-binding proteins, and the activities of the enzymes, were surveyed at different stages of the purification procedure. In subunit content, the two enzymes from the T4-infected systems differ; the enzyme from the T4 gene 55⁻ system lacks a new binding protein of mol wt 22,000. Considerable loss of both the σ subunit and the small binding protein of mol wt 10,000 (as detected by gel electrophoretic analysis) occurs during the purification of the T4 enzymes. Little loss of the small binding proteins of mol wt 12,000, 15,000, and 22,000 is found during routine purification steps, but chromatography of the enzymes on phosphocellulose results in loss of the mol wt 12,000 protein from the core enzyme fractions and some loss (the amount being dependent on the purification stage at which chromatography is carried out) of the mol wt 22,000 protein. The enzymes from both T4-infected systems have a reduced activity with T4 DNA as compared to host polymerase, and the same activity is sharply reduced by high salt concentration. Results are presented which show that the T4 enzymes contain a material, found in the unadsorbed fraction on phosphocellulose chromatography, which inhibits host σ stimulation of core enzyme activity.

Complex changes occur in the transcription process after T4 phage infection of *Escherichia coli*, and many of the studies of these changes have recently been reviewed (Losick, 1972; Bautz, 1972). The changes accommodate the synthesis of at least four different classes of T4 mRNA and the cessation of *E. coli* RNA synthesis. The studies suggest that changes in the host's transcription enzyme, the DNA-dependent RNA polymerase of *E. coli*, are very important to the new transcription program, although the synthesis of a new transcription enzyme which takes place after T7 and T3 phage in-

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fection (Chamberlin *et al.*, 1970; Dunn *et al.*, 1971) has not been completely excluded for T4 phage infection (Losick, 1972). The synthesis of one class of T4 mRNA, the "late" species, involves modification of the T4 phage DNA at the time of replication (Zograf *et al.*, 1967; Bolle *et al.*, 1968b; Lemback *et al.*, 1969; Riva *et al.*, 1970).

Many changes in *E. coli* RNA polymerase have been found after T4 phage infection. In a process almost coincident with infection, and one not requiring new protein synthesis, the enzyme is altered, as is demonstrated by a decrease in its activity with T4 DNA as a template (Seifert *et al.*, 1969). The activity loss is associated with the purification of a smaller amount of the σ subunit of the polymerase. Walter *et al.* (1968) and Seifert *et al.* (1969, 1971) reported a two-step alteration process involving the α subunits, the first step being independent of new protein synthesis. That the alteration reaction involves incorporation of adenylate-containing residues into the α subunits was first shown by Goff and Weber (1970) and was later also studied by Seifert *et al.* (1971.) Travers (1970b) showed that later in the infection process there is a change in the electrophoretic properties of the β' subunit, and Schachner and Zillig (1971) reported that the β, β', and α subunits are all subject to changes in amino acids, as detected by analyses of tryptic peptides. From isotopic labeling studies of the polymerase following T4 phage infection, we have found that four newly synthesized small proteins are bound to the enzyme, and that they are first detectable at about 5 min after infection (Stevens, 1972; Stevens and Crowder, 1974). Of particular interest was the finding that two of them might be the products of T4 genes 33 and 55, genes that have been shown to be involved in late T4 mRNA synthesis (Bolle *et al.*, 1968b; Pulitzer, 1970; Pulitzer and Geiduschek, 1970; Snyder and Geiduschek, 1968). Horvitz (1973) has recently proved that the mol wt 12,000 protein is coded for by T4 gene 33. Whether these new small binding proteins could be responsible for some of the other late changes in the large host subunits is not yet known. Labeling studies by Goff and Weber (1970) and those we reported (Stevens, 1972; Stevens and Crowder, 1974) show that the β, β', and α subunits of the host enzyme are not exchanged throughout the T4 phage infection cycle.

That the σ activity of RNA polymerase is changed after T4 infection is evident from the decrease in activity of the enzyme with T4 DNA as a template (Seifert *et al.*, 1969; Crouch *et al.*, 1969; Bautz and Dunn, 1969; Travers, 1970b). Whether the decreased activity is the result of less σ purifying with the enzyme because of core enzyme changes or the result of alteration of σ itself has remained unclear, although both possibilities have been suggested (Seifert *et al.*, 1971; Schachner and Seifert, 1971). Various values have been reported for the σ subunit content of RNA polymerase after T4 infection. Seifert *et al.* (1971) showed that enzyme isolated immediately after infection has a reduced amount of σ, and several laboratories reported that enzyme isolated later has none (Seifert *et al.*, 1971; Bautz and Dunn, 1969). More recently, Travers (1970b) and Khesin *et al.* (1972) found substantial amounts of σ on enzyme purified from T4-infected cells. Different purification methods may have caused the different σ values reported. σ can be removed from RNA polymerase holoenzyme by phosphocellulose column chromatography (Burgess *et al.*, 1969; Berg *et al.*, 1971), and σ has been shown to be required for core polymerase to transcribe T4 DNA with the production of early T4 mRNA (Bautz *et al.*, 1969). Travers (1969, 1970b) reported isolating a protein from T4-infected cells which stimulates core enzyme activity but which differs from normal σ in stimulating the synthesis of a different class of early T4 mRNA. An anti-σ activity was reported by Bogdanova *et al.* (1970) and Khesin *et al.* (1972), who found that a supernatant protein fraction partially purified from lysates of T2 or T4 phage-infected *E. coli* inhibits the response of normal *E. coli* core enzyme to σ.

We have been particularly interested in the function of the new small binding proteins, and have examined in detail the DNA-dependent RNA polymerases from two T4 phage-infected systems. Both infection systems involve a T4 double amber mutant, the first being gene 42^-Xgene 47^- and the second being gene 42^-Xgene 55^-. Gene 42 is a DNA synthesis negative (Do) gene, the product of which is deoxycytidylate hydroxymethylase (Wiberg *et al.*, 1962), gene 47 is a delayed DNA synthesis gene, the product of which may be a DNase (Wiberg, 1966), and gene 55 is a maturation-defective gene which, in *vivo*, acts to restrict late T4 mRNA synthesis but not T4 DNA replication (Bolle *et al.*, 1968b). With the 42^-X-47^- mutant, the polymerase isolated contains all four new small binding proteins (Stevens, 1972); with the 42^-X55^- mutant, the resulting polymerase lacks the mol wt 22,000 binding protein because of the gene 55 mutation (Stevens, 1972). We have surveyed the activities of the two enzymes from the infected systems, and enzyme from uninfected cells, in an attempt to answer the following questions. (1) What is the σ content of the enzymes at the different purification stages? (2) What is the small binding protein content at different purification stages? (3) Do the enzymes from the T4-infected cells differ from host polymerase from uninfected *E. coli* cells in any way? (4) Do the enzymes from the two T4-infected systems differ from each other in any way? The last question was of particular interest because of our curiosity about the function of the mol wt 22,000 binding protein. The results of the studies are reported below.

Materials and Methods

Preparation of Phage. The amber mutant phages used were T4 *am* N55-A456 (gene 42^-Xgene 47^-), the gift of Dr. John S. Wiberg, and T4 *am* N122-BL292 (gene 42^-Xgene 55^-), the gift of Dr. Kay Fields. The amber mutant phage stocks were prepared with the su_I^+ host, *E. coli* CR63, by a method described to the author by Dr. G. Gujer-Kellenberger. Cells in M9 medium (Adams, 1959) supplemented with 0.05% casamino acids, 0.2% glucose, and 20 μg/ml of L-typtophan were grown at 30° to a concentration of 2×10^8/ml and then infected with an equal number of phage. After 3–4 hr, the cells were collected, resuspended in a small volume of M9 medium, and lysed by the addition of $^1/_{50}$th its volume of chloroform. After treatment with DNase at 5 μg/ml, the phages were purified by differential centrifugation (Thomas and Abelson, 1966).

Growth of Cells. E. coli B was grown in M9 medium supplemented with 0.2% glucose and 20 μg/ml of L-tryptophan, at 30° in either a 30- or 200-l. fermentor. Normal cells were grown to a cell concentration of 7×10^8/ml, collected, frozen, and stored at −20°. Cells infected with the two amber mutant phages were obtained by growing *E. coli* B to a cell concentration of 3×10^8–4×10^8/ml. Then eight times that amount of phage was added and 25 min later (30°) the cells were collected. Surviving cells were plated 5 min after infection. With the 30-l. fermentor surviving cells were less than 1%, but by use of the 200-l. fermentor the surviving cell count was usually higher, ranging from 1 to 3%. No differences were observed in the enzymes obtained from different batches

of cells. The phage-infected cells were also frozen and stored at −20° prior to use.

Purification of Enzymes. All operations were carried out at 0–4°. Twenty-five grams of cells were ground with alumina and the alumina extract was centrifuged as previously described (Stevens and Henry, 1964). The final supernatant fraction was withdrawn with a pipet, dithiothreitol was added to a concentration of 0.1 mM, and the fraction was stored frozen until the next purification step was done. When assayed for polymerase activity as described below, with calf thymus DNA as a template, the supernatant fractions from uninfected cells contained about 6000 units of activity (average specific activity with calf thymus DNA = 8.0). The supernatant fractions from T4-infected cells were lower in activity and contained about 2000–3000 units (average specific activity with calf thymus DNA = 3.3).

PROTAMINE SULFATE PRECIPITATION. For each T4-enzyme preparation, the total A_{260} units of the supernatant fraction were determined and then NH_4Cl was added to a final concentration of 1 M. About 0.28 ml/100 A_{260} units of protamine sulfate (General Biochemicals) (1%), adjusted to pH 8.0 with 1 N KOH, was added. The solution was stirred for 15 min and then was centrifuged for 10 min at 12,000 rpm (Servall). The supernatant solution was diluted with 2 vol of 10 mM Tris buffer (pH 7.8) containing 10 mM $MgCl_2$, 20 mM NH_4Cl, and 0.1 mM EDTA (TMA buffer)[1] and 0.2 of the original volume of 1% protamine sulfate was added. After stirring and centrifugation as above, the enzyme was precipitated by addition of 0.4 of the original volume of 1% protamine sulfate and collected as described above. The precipitate was suspended in 6 ml of 0.1 saturated ammonium sulfate, pH 8.5. (Saturated ammonium sulfate solution was prepared, adjusted to pH 8.5 by the addition of concentrated NH_4OH, and stored at 4°.) The suspension was centrifuged to remove insoluble material. When assayed for polymerase activity the supernatant solution contained 2000–3000 units of polymerase activity.

For enzyme preparations from uninfected cells the protamine sulfate step was slightly different. The procedure was the same up to the addition of the 2 vol of TMA buffer. Then only TMA buffer was added, and the resulting precipitate was removed by centrifugation. The same volume of TMA buffer and 0.12 of the original volume of 1% protamine sulfate were added. The precipitate was again removed by centrifugation and the enzyme was then precipitated from the supernatant solution by addition of 0.4 of the original volume of protamine sulfate. After collection by centrifugation, it was dissolved in 0.1 saturated ammonium sulfate as described above and centrifuged again to remove insoluble material. The polymerase activity of the supernatant solution was 4000–5000 units.

With each new batch of protamine sulfate used, it was necessary to test the exact requirements for precipitation of the enzyme. This was done by adding different amounts of the protamine sulfate solution and different amounts of TMA buffer until the optimal amounts for precipitation of the enzyme at maximum purity were found.

The protamine sulfate fractions were immediately further fractionated with ammonium sulfate by collecting the 0.4–0.55 saturated fraction as described previously (Stevens and Henry, 1964). Usually, 60–70% of the enzyme activity was recovered. The enzymes were stored at a concentration of about 5 mg/ml in the storage buffer described by Burgess (1969) at −20° until the DEAE-cellulose step.

DEAE-CELLULOSE COLUMN CHROMATOGRAPHY. DE-23 (Whatman) was washed with 0.1 M NaOH by stirring for 30 min at room temperature and then with 0.1 N HCl in the same manner, and stored at 4° in 20 mM Tris buffer (pH 8.0). A small column (0.7 cm × 10 cm) of the material was prepared and washed with about 100 vol of 20 mM Tris buffer (pH 8.0) containing 10% glycerol, 100 mM KCl, 1 mM EDTA, and 0.1 mM dithiothreitol (buffer A). The enzyme sample was dialyzed for 3–4 hr against buffer A prior to chromatography. After application of the sample, the column was rinsed with 1–2 ml of buffer A. The enzyme was then eluted using a 100-ml linear gradient of from 0.1 to 0.4 M KCl in buffer A. Two-milliliter fractions were collected, and the fractions with highest activity were combined and concentrated by precipitation with ammonium sulfate (to 0.6 saturation). The precipitate was dissolved in 0.5 ml of 20 mM Tris buffer (pH 7.8) containing 5% glycerol, 0.4 M KCl, 0.1 mM EDTA, and 0.1 mM dithiothreitol (buffer B). Recovery of activity from the DEAE-cellulose column ranged from 60 to 90% for all the enzymes.

DENSITY-GRADIENT CENTRIFUGATION. The enzyme sample was dialyzed for 3–4 hr against buffer B, and then 0.2-ml aliquots of the sample were layered on 4.6-ml linear gradient solutions (12.5–30% glycerol in buffer B) and centrifuged for about 14 hr at 32,000 rpm (SW39 head, Spinco L2). Fractions (0.15 ml) of the gradients were collected and assayed. The recovery of activity from the gradients was 60–90%. The gradient fractions were concentrated by precipitaton with ammonium sulfate to 0.6 saturation and either stored in Burgess storage buffer (Burgess, 1960) or prepared for a second gradient centrifugation by dialysis as described above. The second gradient centrifugation was carried out in the same manner, or with solutions containing 0.05 M KCl rather than 0.4 M KCl. After the first gradient centrifugation, the enzyme was 95–98% pure as analyzed by gel electrophoresis. A second gradient centrifugation at either low or high salt reduced the amount of the unidentified impurity bands found on gel electrophoresis, but did not eliminate any one completely. The overall recovery of enzyme activity was 20–35%.

Contaminating Enzyme Activities. DNase activity of the enzyme preparations was measured by incubating the enzymes with [^{3}H]thymidine-labeled T4 DNA under the polymerase assay conditions described below except that 2-mercaptoethanol was omitted from the reaction mixtures. Sarkosyl (to 1%) was added to terminate the reactions and the [^{3}H]DNA was then sedimented in alkaline sucrose gradients. No alteration of the sedimentation rate of the DNA was found after incubation with enzymes which had been purified by centrifugation in the high salt-containing gradient solutions. Enzymes at prior purification steps all contained DNase activity.

Since *E. coli* polynucleotide phosphorylase has been shown to break down to a 95,000 mol wt subunit, which migrates similarly to the σ subunit of RNA polymerase on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Lehrach *et al.*, 1971), the different enzyme fractions were assayed for polynucleotide phosphorylase activity by the polymerization assay of Williams and Grunberg-Manago (1964). Enzymes at the protamine sulfate stage of purification contained about 0.03 unit/mg and no activity was detectable with the DEAE-cellulose and gradient-purified fractions.

Phosphocellulose Column Chromatography. Phosphocellulose (P11, Whatman) column chromatography was usually carried out as described by Burgess *et al.* (1969), using 0.7 × 5 cm

[1] Abbreviation used is: TMA buffer, 10 mM Tris–10 mM $MgCl_2$–20 mM NH_4Cl–0.1 mM EDTA.

TABLE I: Specific Activities of Host Enzyme, Enz 42⁻X47⁻, and Enz 42⁻X55⁻ with T4 DNA and Calf Thymus DNA at Different Purification Stages.

Purification Stage	Host		Enz 42⁻X47⁻		Enz 42⁻X55⁻	
	Calf Thymus	T4	Calf Thymus	T4	Calf Thymus	T4
Protamine sulfate	220	220	215	100	205	120
DEAE-cellulose	250	250	240	90	210	120
Density gradient 1	400	400[a]	420	105[a]	400	140[a]
Density gradient 2	450	450[a]	450	105[a]	450	140[a]

[a] There was more variation in these values with the different enzyme preparations, probably dependent upon the amount of σ lost on centrifugation.

columns. Approximately 1-mg samples of the enzymes were applied. Most of the assays of the phosphocellulose column fractions were done the same day, or after storage overnight in ice. Freezing of the fractions resulted in losses of activity, but similar results were obtained with the less-active fractions.

Assay of Polymerase Activity. For assay of the enzyme fractions, the reaction mixtures (0.2 ml) contained [^{14}C]ATP (Schwarz BioResearch, Inc.) (0.25 mM, 3400 cpm/nmol), UTP, CTP, and GTP (P-L Biochemicals, Inc.) (0.25 mM each), Tris buffer (pH 7.7) (20 mM), $MgCl_2$ (10 mM), 2-mercaptoethanol (10 mM), T4 DNA (isolated from T4D phage according to the procedure of Thomas and Abelson, 1966) (8–10 μg) or calf thymus DNA (Worthington Biochemicals Corp.) (10 μg), and enzyme. Incubation was for 10 min at 37°, and determination of radioactivity incorporated into RNA was carried out as previously described (Stevens and Henry, 1964). Specific activity is expressed as nanomoles of [^{14}C]AMP incorporated/milligram of protein under the assay conditions.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis, using 12% acrylamide gels, and staining and scanning the gels were carried out as previously described (Stevens, 1972). Urea–sodium dodecyl sulfate–polyacrylamide gel electrophoresis with 15 × 0.6 cm columns was carried out as described by Swank and Munkres (1971). The 10% acrylamide gels were 10 cm long.

Other Methods. Protein concentration was determined by the method of Lowry *et al.* (1951). Competition hybridization measurement of late T4 mRNA and RNA asymmetry measurements were carried out according to the procedures of Bolle *et al.* (1968a) and Brody *et al.* (1970), by use of 5- and 20-min T4 RNA samples prepared as described by these authors.

Results

Designations Used for Enzymes and New Binding Proteins. The following designations for the enzymes and the new small binding proteins are used: T4 enzyme, enzyme from T4 phage-infected cells; host enzyme, enzyme from uninfected cells; Enz 42⁻X47⁻, enzyme from the system infected by T4 phage *am* gene 42⁻X47⁻; Enz 42⁻X55⁻, enzyme from the system infected by T4 *am* gene 42⁻Xgene 55⁻. The small binding proteins are designated on the basis of their molecular weights of 22, 15, 12, and 10 (each $X10^3$) as "22," "15," "12," and "10," respectively (Stevens, 1972).

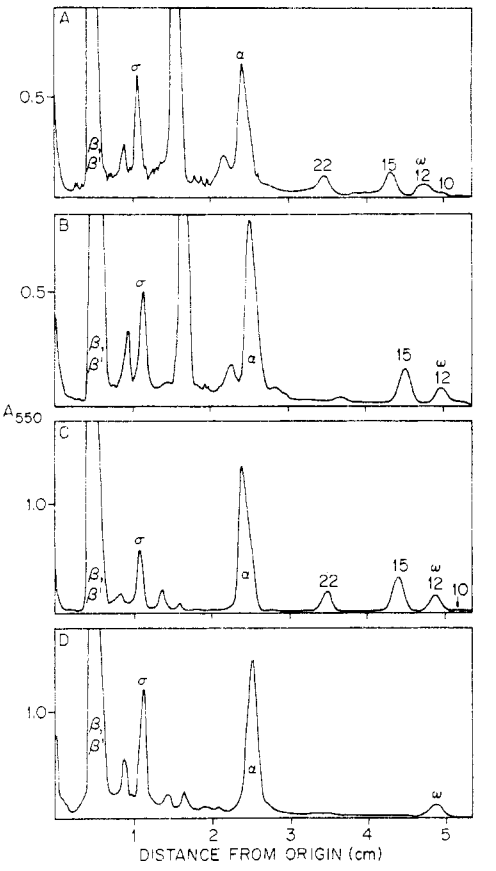

FIGURE 1: Densitometer scans of stained gels of the T4 enzymes at different purification stages and of gradient-purified host enzyme: (A) 10 μg of Enz 42⁻X47⁻ purified by DEAE-cellulose chromatography; (B) 15 μg of Enz 42⁻X55⁻ purified by DEAE-cellulose chromatography; (C) 12 μg of Enz 42⁻X47⁻ purified further by a high salt gradient centrifugation step; (D) 15 μg of host enzyme purified by gradient centrifugation. The 0.1% sodium dodecyl sulfate–12% polyacrylamide gels were run, stained, and scanned as described under Materials and Methods. The small band observed in B, that migrates slightly faster than "22," was present in even higher concentration on gels of the protamine sulfate Enz 42⁻X55⁻ fraction. It is possible that it is an amber peptide of "22," but it has not been investigated further. It was never observed after gradient centrifugation of Enz 42⁻X55⁻.

Specific Activities and Subunit Content of Enzymes at Different Purification Stages. The specific activities of the two T4 enzymes have been compared with host enzyme with both calf thymus DNA and T4 DNA used as templates as shown in Table I. The values given are an average obtained with several preparations of each enzyme. The three enzymes are similar in their specific activities with calf thymus DNA as template at each purification stage. Calf thymus DNA has been found to be a template, the activity of which is little influenced by the σ content of RNA polymerase (Bautz and Dunn, 1969). The specific activity of host enzyme with T4 DNA is similar to that with calf thymus DNA at each purification stage. The activity of the T4 enzymes with T4 DNA at any one of the purification stages was sigmoidal with enzyme concentration at concentrations below 0.5 unit/0.2 ml of reaction mixture. (Figure 4 below shows an enzyme concentration curve under the low salt assay conditions.) The activity with calf thymus DNA was linear at all enzyme concentrations. The values (Table I) for the specific activities of the T4 enzymes with T4 DNA are given for enzyme concentrations of 0.5–1.0 unit/0.2 ml of reaction mixture. With Enz 42⁻X47⁻ and T4 DNA the specific activity at the first

TABLE II: σ and Small Binding Protein Content (Equivalents/Enzyme Molecule) of Enz 42⁻X47⁻ and Enz 42⁻X55⁻ at Different Purification Stages.[a]

Purification Stage	Enz 42⁻X47⁻					Enz 42⁻X55⁻				
	σ	"22"	"15"	"12"	"10"	σ	"22"	"15"	"12"	"10"
Protamine sulfate[b]	0.4 ± 0.1	0.6 ± 0.1	1.0 ± 0.1	0.2 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	ND[d]	1.0 ± 0.1	0.2 ± 0.1	0.5 ± 0.1
DEAE-cellulose	0.35 ± 0.1	0.6 ± 0.1	1.0 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	ND[d]	1.0 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Density gradient 1	0.17 ± 0.05	0.5 ± 0.1	1.0 ± 0.1	0.2 ± 0.1	*c*	0.15 ± 0.05	ND[d]	1.0 ± 0.1	0.2 ± 0.1	*c*
Density gradient 2	0.17 ± 0.05	0.5 ± 0.1	1.0 ± 0.1	0.2 ± 0.1	*c*	0.15 ± 0.05	ND[d]	1.0 ± 0.1	0.2 ± 0.1	*c*

[a] The amounts of each subunit per enzyme molecule were calculated from the scans of the subunit peaks on gels as shown in Figures 1 and 2. The amount of the α subunit was used as the measure of the number of enzyme molecules. Molecular weights of 90,000 for the σ subunit and 40,000 for the α subunit (Losick, 1972) were used in the calculations. [b] The values of all the subunits were difficult to determine with accuracy because of impurity bands on the gels. [c] The values of "10" were usually too low to be estimated with accuracy when 20 μg of enzyme was applied to the gels. Enz 42⁻X47⁻ after gradient centrifugation had an amount of "10" which was visible on gels, while Enz 42⁻X55⁻ usually did not. [d] ND = not detectable.

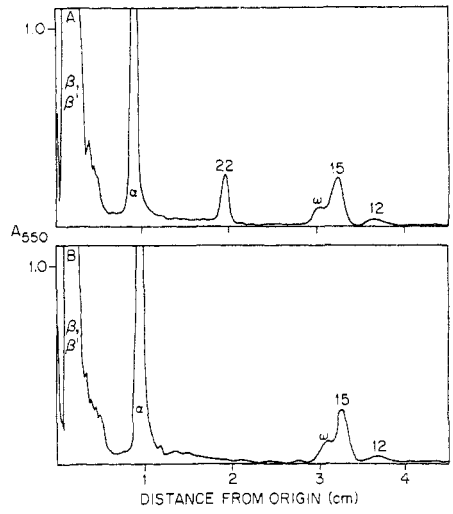

FIGURE 2: Densitometer scans of stained urea–sodium dodecyl sulfate–polyacrylamide gels of the two gradient-purified T4 enzymes: (A) 20 μg of Enz 42⁻X47⁻; (B) 20 μg of Enz 42⁻X55⁻. The urea–sodium dodecyl sulfate gels were run as described under Materials and Methods.

two purification stages is about 0.4 that of host enzyme, and after gradient centrifugation it is about 0.25 that of host enzyme. With Enz 42⁻X55⁻, the values of specific activity with T4 DNA are slightly higher than those for Enz 42⁻X47⁻. The reactions of all three enzymes are linear with time up to 10 min. No major differences in DNA concentration curves have been found.

Host enzyme contains about 0.6 equiv of σ at the protamine sulfate and DEAE-cellulose stages of purification and somewhat less (0.4 ± 0.1 equiv) after gradient centrifugation. The contents of the σ band and the new small binding proteins of Enz 42⁻X47⁻ and Enz 42⁻X55⁻ at the different purification stages are shown in Table II. Densitometer scans of typical gels from which the measurements were made are shown in Figures 1 and 2. There was no change in the content of β, β', α, and ω (the conserved host subunits) of T4 enzymes (Stevens, 1972). As shown in Table II, both T4 enzymes at the protamine sulfate step contain, per enzyme molecule, 0.4 ± 0.1 equiv of a protein migrating like σ on gels. In spite of some losses, the DEAE-cellulose purified enzymes still contain substantial amounts of a σ band (Figures 1A and 1B). The activities of both T4 enzymes with T4 DNA (Table I) are lower than that of host enzyme even though they contain substantial amounts of a σ band. About one-half of the σ band of both T4 enzymes is lost on density-gradient centrifugation (see Enz 42⁻X47⁻ in Figure 1C).

Of the new small binding proteins, only "10" is lost on purification of the T4 enzymes. Very small amounts of "22" are lost (from Enz 42⁻X47⁻) on gradient centrifugation, but "15" and "12" remain the same in amount throughout the purification steps as determined by urea–sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 2). The loss of "10" suggests that it binds poorly to the T4 enzymes and evidence has been presented previously (Stevens and Crowder, 1974) that it binds better to host enzyme, thus accounting for its detection in the labeling experiments in which the small binding proteins were first found (Stevens, 1972).

The activities and subunit content of different fractions of Enz 42⁻X47⁻ and Enz 42⁻X55⁻ from the DEAE-cellulose column have been determined. The ratio of calf thymus

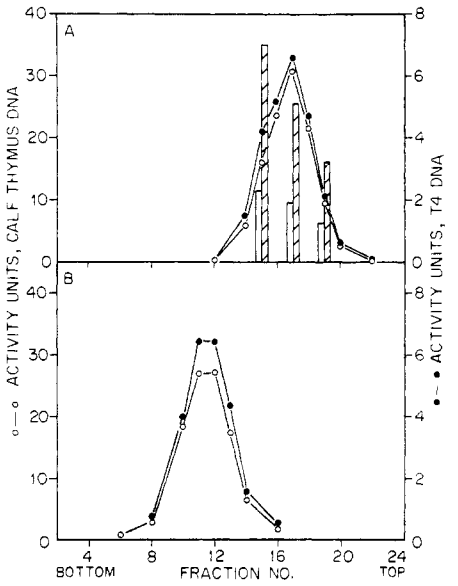

FIGURE 3: Sedimentation profile of Enz 42^{-}X47^{-} activities with calf thymus DNA and T4 DNA in a high-salt gradient (A) or a low-salt gradient (B). The centrifugations were carried out as described under Materials and Methods and aliquots of each fraction were assayed. Aliquots containing 5 units of activity (with calf thymus DNA) of gradient fractions 15, 17, and 19 of A were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the amounts of the different subunits were determined from densitometer scans of the stained gels. In Figure 1A, the amounts of σ band and "22" in each of the three fractions are shown by the open columns and the shaded columns, respectively. The amount of σ in fraction 15 was 0.23 equiv/enzyme molecule, and the amount of "22" was 0.70 equiv/enzyme. The values in fractions 17 and 19 are plotted relative to values in fraction 15.

DNA/T4 DNA activity remains the same through the enzyme peak, and no significant separation of enzyme fractions containing different small proteins was found. Measurements of the activity of Enz 42^{-}X47^{-} density-gradient fractions with both calf thymus DNA and T4 DNA are shown in Figure 3. Figure 3A is a 0.4 M KCl gradient and Figure 3B is a 0.05 M KCl gradient. While the high salt gradient showed no significant variation in the ratio of calf-thymus DNA activity/T4 DNA activity through the enzyme peak, both the σ band and "22" protein are concentrated toward the heavier side of the enzyme peak (Figure 3A). The low salt gradient showed no separation of activities, but it did show that Enz 42^{-}X47^{-} dimerizes in the low salt solution as does host enzyme (Richardson, 1966; Stevens *et al.*, 1966).

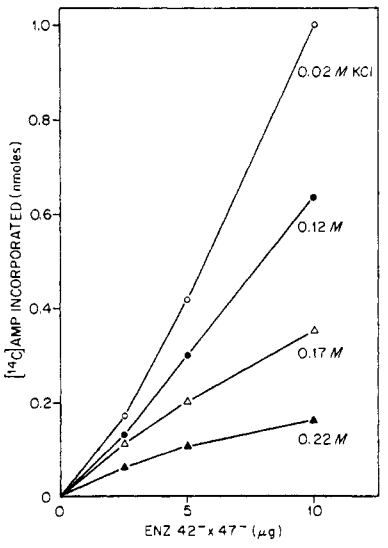

FIGURE 4: Enzyme concentration curves of Enz 42^{-}X47^{-} with T4 DNA at different KCl concentrations. The reaction mixtures were as described under Materials and Methods and contained the concentration of KCl shown.

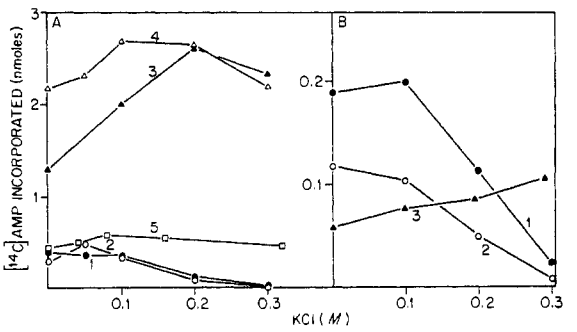

FIGURE 5: Effect of KCl concentration on the activities of different enzymes. The reaction mixtures were as described under Materials and Methods; T4 DNA was used, except in A, curve 4, which was done with calf thymus DNA. KCl was present in the reaction mixtures before enzyme addition except in the case of □. In the latter case, KCl and [^{14}C]ATP were added after a 2-min incubation period with the four unlabeled nucleotides: (A) (●) Enz 42^{-}X47^{-}, 4 μg; (○) Enz 42^{-}X55^{-}, 4 μg; (▲) host enzyme, 2.5 μg; (△) Enz 42^{-}X47^{-}, 4 μg; (□) Enz 42^{-}X47^{-}, 5 μg; (B) (●) Enz 42^{-}X47^{-} core enzyme, 4 μg; (○) Enz 42^{-}X55^{-} core enzyme, 4 μg; (▲) host core enzyme, 2.5 μg.

Effect of Salt (KCl) Concentration on the Activities of the Enzymes. KCl, at concentrations from 0.1 to 0.25 M, stimulates the reaction of host enzyme with T4 DNA as a template. Its effects have been shown to involve both the initiation and termination steps of RNA synthesis and have recently been studied by Schäfer *et al.* (1973), Schäfer and Zillig (1973), and Matsukage (1972), who also review previous studies of others. In sharp contrast to the salt stimulation of host enzyme with T4 DNA, the T4 enzymes are inhibited by high KCl concentration. In Figure 4 it can be seen that, at all enzyme concentrations, Enz 42^{-}X47^{-} is inhibited by KCl concentrations above 0.1 M. The effect is more pronounced at higher enzyme concentrations. Figure 5A, curves 1–3, shows the effect of KCl concentration on the activities of the three enzymes with T4 DNA. Host enzyme is stimulated by KCl concentrations up to 0.2 M and slightly inhibited at 0.3 M. Enz 42^{-}X47^{-} and Enz 42^{-}X55^{-}, at the enzyme concentrations shown in Figure 5A, are little affected by 0.1 M KCl and inhibited about 70% by 0.2 M and >90% by 0.3 M. The effect of KCl on Enz 42^{-}X47^{-} activity with calf thymus DNA is shown in Figure 5A, curve 4. The activity is slightly stimulated at 0.1 and 0.2 M, and reduced slightly at 0.3 M. Curve 5 of Figure 5A shows that when KCl at 0.3 M is added to an Enz 42^{-}X47^{-} reaction mixture 2 min after the start of the reaction, it does not inhibit the reaction. This result suggests that KCl inhibits at the initiation step, but certainly further studies are necessary to clarify its inhibitory activity. The effect of salt on the three core enzymes obtained by phosphocellulose chromatography, described below, is shown in Figure 5B. The same inhibition is found as with the holoenzymes.

Phosphocellulose Column Chromatography. SPECIFIC ACTIVITIES AND SUBUNIT CONTENT OF THE CORE ENZYMES. A summary of the activities and subunit content of the enzymes before and after phosphocellulose column chromatography is presented in Table III. The results with host enzyme are

TABLE III: Activities and Small Binding Protein Content of Enzymes before and after Phosphocellulose Chromatography.

Enzyme		Sp Act. with Calf Thymus DNA	Sp Act. with T4 DNA	Subunit Content[a]				
				σ	"22"	"15"	"12"	"10"
Host, gradient purified	Before	450	450	0.5 ± 0.1				
	After	250	30	<0.05				
Enz 42$^-$X47$^-$, gradient purified	Before	450	105	0.17 ± 0.05	0.5 ± 0.1	1 ± 0.1	0.2 ± 0.1	<0.05
	After	450	50	<0.05	0.4 ± 0.1	1 ± 0.1	*b*	*b*
Enz 42$^-$X55$^-$, gradient purified	Before	450	140	0.15 ± 0.05		1 ± 0.1	0.2 ± 0.1	*b*
	After	400	30	<0.05		1 ± 0.1	*b*	*b*
Enz 42$^-$X47$^-$, protamine sulfate	Before	215	100	0.4 ± 0.1	0.6 ± 0.1	1 ± 0.1	0.2 ± 0.1	0.5 ± 0.1
	After	200	50	<0.05	0.2 ± 0.1	1 ± 0.1	*b*	*b*

[a] The subunit content is expressed as equivalents per enzyme molecule and was determined as described in Table II. [b] Not detectable on gels of 20 μg of enzyme.

similar to those described by others (Burgess *et al.*, 1969; Berg *et al.*, 1971). σ is found in the unadsorbed fraction, and the core enzyme, eluted with 0.4 M KCl, has about 60% residual activity with calf thymus DNA and less than 10% with T4 DNA. With gradient-purified T4 enzymes, the major portions of σ, "12," and residual "10" are found in the unadsorbed fraction. "15" and most of "22" (in the case of Enz 42$^-$X47$^-$) remain bound to the core enzymes. When Enz 42$^-$X47$^-$ is chromatographed after either the protamine sulfate step or the DEAE-cellulose step, most of "22" is also found in the flow-through fraction. The different affinity of "22" for core enzyme at the different purification stages may be related to the σ or "10" content of the holoenzyme. Enz 42$^-$X47$^-$ core enzyme with calf thymus DNA as template has a specific activity similar to that of the holoenzyme and 50% residual activity with T4 DNA. Enz 42$^-$X55$^-$ loses little activity with calf thymus DNA, but its specific activity with T4 DNA is reduced to the level of host core enzyme. Enz 42$^-$X47$^-$ core enzyme consistently has a 1.5- to 2-fold higher specific activity with T4 DNA than do host or Enz 42$^-$X55$^-$ core enzymes.

ANALYSES OF RNA PRODUCTS MADE WITH HOLOENZYME AND CORE ENZYME FRACTIONS. Previous studies by other investigators showed that host holoenzyme with mature T4 phage DNA as a template synthesizes asymmetric early T4 mRNA (Geiduschek *et al.*, 1966; Bautz *et al.*, 1969; Brody *et al.*, 1970) transcribed from the l strand of T4 DNA (Guha *et al.*, 1971). With host core enzyme, symmetric RNA is the product because loss of the initiation specificity factor, σ, results in random initiation on both strands of the T4 DNA molecule (Bautz *et al.*, 1969). Studies on the type of RNA product formed with T4 enzymes have also been reported by Bautz *et al.* (1969) and Hall *et al.* (1970). Using T4 enzymes which contained no detectable σ activity, but which presumably contained the small binding proteins that were later described, they found that the RNA products produced with T4 DNA as a template were quite symmetric. On the other hand, Travers (1970b), using a T4 enzyme which contained some σ, as detected by gel electrophoresis, found that the RNA product formed was principally asymmetric early T4 mRNA.

Studies that we have done using different T4 enzyme preparations show that the symmetry of the RNA product is influenced by the σ-band content of the preparations. Enz 42$^-$X47$^-$ preparations usually yield symmetric RNA products unless the σ-band content is high. Some of these results have been reported previously (Stevens and Crowder, 1974). Results of RNA product analyses are presented in Table IV for three holoenzyme preparations and three core enzyme preparations. The T4 holoenzymes each contained about 0.15 equiv of σ as shown by gel electrophoretic analyses. Both the percentage of late T4 mRNA, determined by competition hybridization analyses, and the antimessenger content (symmetry) of the RNA products were determined. The results show that Enz 42$^-$X47$^-$, as compared to host holoenzyme, yields an RNA product which is quite symmetric, *i.e.*, high in antimessenger content. Enz 42$^-$X55$^-$ yields a product with

TABLE IV: Analyses of RNA Products for Late T4 mRNA and for Antimessenger Content.[a]

Enzyme	Late T4 mRNA (%)	Antimessenger [5-min T4 RNA] (%)	Antimessenger [20-min T4 RNA] (%)
Host holoenzyme	2	2	11
Enz 42$^-$X47$^-$ holoenzyme	25	31	38
Enz 42$^-$X55$^-$ holoenzyme	7	10	15
Host core enzyme	17	35	45
Enz 42$^-$X47$^-$ core enzyme	22	35	41
Enz 42$^-$X55$^-$ core enzyme	19	38	48

[a] [^{14}C]RNA products were prepared by use of reaction mixtures containing the following components: [^{14}C]ATP, 0.25 mM (30,000 cpm/nmol); UTP, CTP, and GTP, 0.25 mM each; Tris buffer (pH 7.8), 20 mM; $MgCl_2$, 10 mM; 2-mercaptoethanol, 10 mM; T4 DNA, 22 μg; and 8 μg of each holoenzyme or 15 μg of each core enzyme in a final volume of 0.5 ml. After incubation for 15 min at 37°, the labeled RNA products were isolated as described by Bolle *et al.* (1968a) and the percentages of late T4 mRNA and of antimessenger content were determined as described under Materials and Methods.

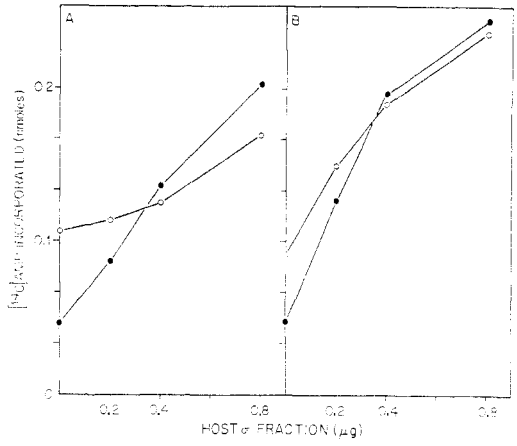

FIGURE 6: Effect of the σ-containing fraction of host enzyme on the activities of Enz 42⁻X47⁻ and Enz 42⁻X55⁻ holoenzymes. The reaction mixtures were as described under Materials and Methods. The σ-band content of the fraction used here was about 70%, as determined by gel electrophoresis: (A) Enz 42⁻X47⁻, 1.4 μg, with T4 DNA; (○) 0.02 M KCl; (●) 0.15 M KCl; (B) Enz 42⁻X55⁻, 1.2 μg, with T4 DNA; (○) 0.02 M KCl; (●) 0.15 M KCl.

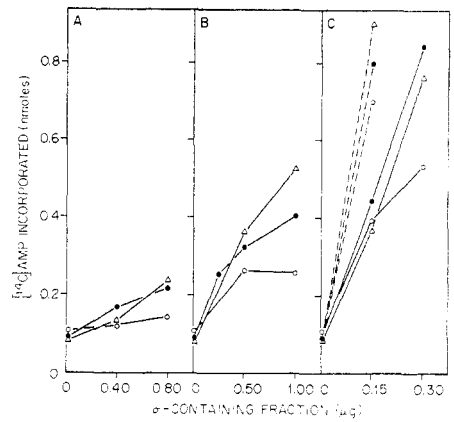

FIGURE 7: (A) Effect of the unadsorbed fraction of Enz 42⁻X47⁻ on the activities of Enz 42⁻X47⁻, Enz 42⁻X55⁻, and host core enzymes. The reaction mixtures were as described under Materials and Methods, with T4 DNA and 0.03 M KCl. The σ content of the unadsorbed fraction was about 40%, as determined by gel electrophoresis: (○–○) Enz 42⁻X47⁻ core enzyme, 3 μg; (●–●) Enz 42⁻X55⁻ core enzyme, 4 μg; (Δ–Δ) host core enzyme, 4 μg. (B) Effect of the unadsorbed fraction of Enz 42⁻X55⁻ on the activities of Enz 42⁻X47⁻, Enz 42⁻X55⁻, and host core enzymes. The reaction mixtures and designations are the same as in A. The σ content of the unadsorbed fraction was about 40%, as determined by gel electrophoresis. (C) Effect of the unadsorbed fraction of host enzyme on the activities of Enz 42⁻X47⁻, Enz 42⁻X55⁻, and host core enzymes. The reaction mixtures and designations for the assays at 0.03 M KCl concentration are the same as in A. Reaction mixtures with 0.15 M KCl are designated as follows: (○---○) Enz 42⁻X47⁻ core enzyme; (●---●) Enz 42⁻X55⁻ core enzyme; (Δ---Δ) host core enzyme. The σ content of the unadsorbed fraction was about 70%, as determined by gel electrophoresis.

more symmetry than that made by host enzyme, but considerably less than that made by Enz 42⁻X47⁻. A very symmetric product is obtained with all three core enzymes (Table IV). The percentage of late T4 mRNA content of the RNA products was in all cases less than the percentage of anti-messenger RNA competing with 5-min T4 mRNA. Since both would be formed by transcription of the r strand of T4 DNA (Guha *et al.*, 1971), random initiation events leading to the production of the late T4 RNA cannot be distinguished from specific initiation events. The results showed that Enz 42⁻X47⁻ is more corelike in activity than Enz 42⁻X55⁻ with respect to the RNA product made, which suggests that, compared to Enz 42⁻X55⁻, the activity of σ is reduced on Enz 42⁻X47⁻.

EVIDENCE FOR AN ALTERED σ ACTIVITY OF THE T4 ENZYMES. That the σ activity of the T4 enzymes might be altered was suggested by their low activities with T4 DNA, even at early purification stages when their σ-band content appeared high on gels. Also, the corelike activity of Enz 42⁻X47⁻ (Table IV) suggested a modified σ activity. Some studies demonstrating an altered σ function are described here.

Assays of σ-containing (unadsorbed) fractions and core enzyme fractions of gradient-purified host enzyme, Enz 42⁻X47⁻, and Enz 42⁻X55⁻ were carried out. First, the effect of host σ on the T4 holoenzymes was tested. The results, shown in Figure 6A for Enz 42⁻X47⁻, show that the enzyme is quite resistant to stimulation by low concentrations of σ at low salt, while stimulation is found with low concentration at high salt. Enz 42⁻X55⁻, under the same conditions (Figure 6B), is stimulated by low concentrations of host σ at both low and high salt concentration.

The effects of all three unadsorbed fractions on the activities of the three core enzyme fractions with T4 DNA as template were examined. Some of the results with the σ-containing fractions of Enz 42⁻X47⁻ and host enzyme have been described previously (Stevens, 1973). At low salt (0.03 M KCl), the stimulations of core enzyme activity obtained with the unadsorbed fractions of Enz 42⁻X47⁻, Enz 42⁻X55⁻, and host enzyme are shown in Figures 7A, 7B, and 7C, respectively. The unadsorbed fraction of Enz 42⁻X47⁻ was the least stimulatory to all three core enzymes and the unadsorbed fraction of host enzyme was the most stimulatory. The unadsorbed fractions alone had negligible activity. In the presence of higher KCl (0.15 M), the fold stimulation of activity obtained with Enz 42⁻X47⁻ and Enz 42⁻X55⁻ unadsorbed fractions remained about the same, but the host σ fraction stimulated all three core enzymes better at 0.15 M KCl (Figure 7C, dashed lines).

Enz 42⁻X47⁻ and Enz 42⁻X55⁻ unadsorbed fractions were also added to reaction mixtures of the core enzymes with host σ. At low salt (0.03 M KCl), Enz 42⁻X47⁻ unadsorbed fraction was inhibitory to the host σ-stimulated activity of both T4 core enzymes, with Enz 42⁻X47⁻ being inhibited slightly more (Figure 8A). The unadsorbed fraction of Enz 42⁻X55⁻ also inhibited the host σ-stimulated activity of Enz 42⁻X47⁻, and from a comparison of its effects on Enz 42⁻X55⁻ core enzyme and host core enzyme, it is also inhibitory to Enz 42⁻X55⁻ core enzyme (Figure 8B). At 0.15 M KCl, the host σ-stimulated activity of all three core enzymes was inhibited by small amounts of the unadsorbed fraction of Enz 42⁻X47⁻ (Figure 8C). At the higher salt concentration, the unadsorbed fraction of Enz 42⁻X55⁻ also inhibited the activity of all three core enzymes in the presence of host σ. All the studies shown in Figures 7 and 8 were done with less than a stoichiometric amount of σ being used. The percentages of inhibitions shown in Figure 8 were reduced by about one-third when the host σ concentration was doubled. Using higher concentrations of both host σ fraction and Enz 42⁻X47⁻ unadsorbed fraction, preincubation (for 2–3 min at 37°) of core enzymes with host σ in reaction mixtures lacking CTP was found to decrease the percentage of inhibition more than one-half. More extensive kinetic studies of the inhibition have not yet been done.

Phosphocellulose unadsorbed fractions of the T4 enzymes at the protamine sulfate or the DEAE-cellulose stage of purification also contained the inhibitory material. The inhibitor

sedimented to the same position as σ upon gradient centrifugation, suggesting that it is an altered σ, or that it binds to σ, but further studies of its nature are needed. With most enzyme preparations that have been examined, the unadsorbed fraction of Enz 42$^-$X47$^-$ contained more of the inhibitory material than did that of Enz 42$^-$X55$^-$. Enz 42$^-$X47$^-$ core enzyme appeared to be slightly more sensitive to the inhibitor also, particularly at low salt (Figure 8A) so it is possible that Enz 42$^-$X47$^-$ has a greater affinity for the inhibitory material.

Discussion

Answering the questions asked in the introductory statement will best serve to summarize the many types of results presented here.

(1) What is the σ-band content of the enzymes at the different purification stages? Host enzyme has 0.4–0.6 equiv of σ/enzyme molecule, with small amounts being lost on gradient centrifugation, as has also been found by Berg *et al.* (1971). Both of the T4 enzymes described here contain a protein that migrates similarly to σ on gel electrophoresis, and that, upon gradient centrifugation, is reduced in content by about one-half.

(2) What is the small binding protein content at the different purification stages? "15" is present on both T4 enzymes at a concentration of approximately 1 equiv/enzyme molecule at all the purification stages, and it remains bound to the core enzymes on phosphocellulose chromatography. "12" is present on both enzymes at an approximate concentration of 0.2 equiv/enzyme molecule at all the purification stages, and is found in the unadsorbed fraction on phosphocellulose chromatography. "10" is lost drastically from both enzymes during the purification procedure, particularly on density gradient centrifugation. "22," which is found only on Enz 42$^-$X47$^-$ (and not on Enz 42$^-$X55$^-$), is present at a concentration of ~0.6 equiv/enzyme molecule, and most of "22" remains bound to the core enzyme on phosphocellulose chromatography of gradient-purified Enz 42$^-$X47$^-$. On phosphocellulose chromatography of Enz 42$^-$X47$^-$ at the protamine sulfate or DEAE-cellulose steps of purification, a higher percentage (>50%) of "22" is found in the unadsorbed fraction. It is possible that the binding of "22" is influenced by the amount of σ and/or "10" on the enzyme.

(3) Do the T4 enzymes differ from host enzyme in any way? In contrast to host enzyme, the T4 enzymes have a consistently lower activity with T4 DNA than with calf thymus DNA, even at early purification stages when their σ band content appears to be almost as high as that of host enzyme. The σ-containing fractions obtained by phosphocellulose chromatography of the T4 enzymes are less stimulatory to core enzyme fractions than host σ, and can be shown to inhibit the host σ stimulation of core enzyme activity. The activities of the T4 enzymes with T4 DNA are inhibited by KCl concentrations above 0.1 M while host enzyme is stimulated by KCl up to a concentration of at least 0.2 M. Crouch *et al.* (1969) reported that a polymerase fraction from T4-infected cells was inhibited by 0.2 M KCl. The salt sensitivity and sigmoidal concentration curves of the T4 enzymes suggest that the enzymes are more stable or more active with T4 DNA as template under conditions favoring dimer formation.

(4) Do the two T4 enzymes differ from each other in any way? The significance of the differences between the two T4 enzymes described here cannot really be evaluated until more T4-infected systems are analyzed, or until the proteins involved in making the enzymes different are purified and analyzed. We find that Enz 42$^-$X47$^-$ differs slightly from Enz 42$^-$X55$^-$ in the content of the inhibitor of host σ-stimulated core enzyme activity, and in its response to the inhibitor. The greater inhibition of Enz 42$^-$X47$^-$ is apparently reflected in its lower activity with T4 DNA, in its lower degree of stimulation by host σ, and in its more symmetric RNA product made with T4 DNA as template.

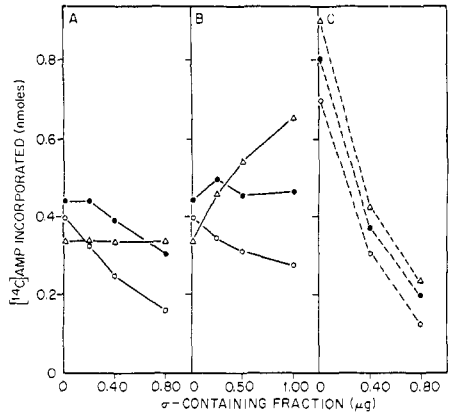

FIGURE 8: (A) Effect of the unadsorbed fraction of Enz 42$^-$X47$^-$ on the host σ-stimulated activity of the three core enzymes at 0.03 M KCl. The reaction mixtures were as described above. Host σ fraction, 0.15 μg, was present. Other details and designations are similar to those given in Figure 7A. (B) Effect of the unadsorbed fraction of Enz 42$^-$X55$^-$ on the host σ-stimulated activity of the three core enzymes at 0.03 M KCl. Reaction mixtures and designations are as described under Figure 7B. Host σ fraction, 0.15 μg, was present in each reaction mixture. (C) As in A, but using 0.15 M KCl in the reaction mixtures.

In looking at the overall results, the following fact is evident. The σ subunit of *E. coli* RNA polymerase has such a dramatic effect on the activity of the enzyme with T4 DNA as a template that almost all the results one obtains with σ-containing enzyme systems can be related in some manner to σ activity. The finding of varying amounts of σ on polymerase fractions isolated from different T4 phage-infected systems, as described in the introductory statement, is most probably related to the different purification techniques used. The results presented here suggest that σ is not bound as tightly to the T4 enzymes since more of it is lost on density gradient centrifugation. The inhibitor of host σ activity is being purified and, until it is identified, it is difficult to evaluate some of the results presented here. The results suggest that the inhibitor may be an altered σ, perhaps because of binding of some material. That the presence of the material causes no new initiation specificity with mature T4 phage DNA as a template, but inhibits σ-promoted early T4 mRNA formation, is suggested by the type of RNA products made with the T4 enzymes. It is possible that a σ alteration could result from *in vitro* handling of the T4 enzymes. Even if it is an artefact of purification, it is still of interest because of its influence on studies of the T4 enzymes. The inhibitor may be similar to the anti-σ activity reported in T2 or T4 phage-infected systems by Bogdanova *et al.* (1970) and by Khesin *et al.* (1972). They found that the polymerase-free protein fraction containing anti-σ activity rendered host core enzyme a less effective competitor of host holoenzyme for T4 DNA binding sites. They have suggested that the anti-σ activity may play an important role in the phage-infection cycle by inhibiting the host σ stimulation of early T4 mRNA synthesis, thus allowing the polymerase or a fraction of it to participate in the formation of late T4 mRNA. Khesin *et al.* (1962), Sköld and Buchanan (1964), and Furth

and Pizer (1966) have all reported the presence of an inhibitor of RNA polymerase activity in crude extracts of T4-infected cells. Mahadik *et al.* (1972) have described an anti-σ activity in T3 phage-infected extracts.

The complex changes that occur in the host polymerase after T4 infection make analyses of the functions of the new small proteins difficult. It is possible that some of the changes may be interrelated, *e.g.*, the small proteins may act as transpeptidases to modify the larger host subunits. A possible binding interaction of "22" and the σ band of Enz $42^{-}X47^{-}$ is suggested by the findings that on gradient centrifugation of Enz $42^{-}X47^{-}$ the faster sedimenting enzyme fractions contain both σ and "22," that "22" is removed along with σ from core enzyme on phosphocellulose chromatography of enzyme fractions that contain substantial amounts of σ, and that the content of inhibitory material sedimenting like σ is higher in concentration and more active on the enzyme containing "22."

Of particular interest is the question of whether the highly modified T4 polymerase does transcribe late T4 mRNA. Certainly, that it binds the gene 33 and probably the gene 55 products (Stevens, 1972; Horvitz, 1973), both implicated in late T4 mRNA synthesis *in vivo* (Bolle *et al.*, 1968b; Pulitzer, 1970; Pulitzer and Geiduschek, 1970), suggests that the enzyme is involved. Only with crude lysate fractions from T4-infected cells, similar to the fractions described by Snyder and Geiduschek (1968), have we been able to demonstrate a stimulation of asymmetric late T4 mRNA formation using the T4 enzyme. These results will be reported elsewhere.

Acknowledgments

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Physical Identity of the SV40 Deoxyribonucleic Acid Sequence Recognized by the *Eco* RI Restriction Endonuclease and Modification Methylase†

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ABSTRACT: The *Eco* RI modification methylase introduces two methyl groups into one SV40 DNA molecule. The only base methylated has been identified as N^6-methyladenine. Both of the methyl groups are introduced into the same fragment (designated F, about 400 base pairs long) of a *Hin*$_d$ II endonuclease digest of SV40 DNA. The *Eco* RI endonuclease makes one double-strand cleavage in SV40 DNA. The site of this cleavage is also contained within the F fragment. Analysis of dinucleoside monophosphates, trinucleoside diphosphates, and tetranucleoside triphosphates generated by partial digestion of the methylated DNA with pancreatic DNase I gives the following sequence of nucleotides at the site of methylation by the *Eco* RI methylase: GpApm^{6}ApTpTpC. This sequence (with A in place of m^{6}A) is also found at the site of phosphodiester-bond cleavage by the *Eco* RI restriction endonuclease. Using [γ-^{32}P]rATP and polynucleotide kinase, SV40 DNA has been labeled in each strand with ^{32}P specifically at the phosphodiester bonds cleaved by the *Eco* RI endonuclease. The DNA was polymerized at 4° by hydrogen bonding of the cohesive termini of the *Eco* RI endonuclease break. The labeled 5′-monophosphates at the staggered single-strand breaks were esterified with the adjacent 3′-hydroxyl groups by polynucleotide ligase at low temperature, and the covalently polymerized DNA was methylated by the *Eco* RI modification methylase using *S*-adenosyl-L-[*methyl*-^{3}H]methionine. Analysis of the radioactive labels in the mono- and dinucleotides from a partial digest of this double-labeled DNA identifies physically the same sequence of base pairs in SV40 DNA as the substrate site for the *Eco* RI endonuclease and for the *Eco* RI modification methylase.

Restriction endonucleases and their related modification methylases are enzymes that recognize and react only within well-defined base sequences in double-stranded DNA molecules (Kelly and Smith, 1970; Hedgpeth *et al.*, 1972; Boyer *et al.*, 1973). It has been postulated that the two enzymes react with the same substrate site (Arber and Linn, 1969; Boyer, 1971; Meselson *et al.*, 1972) although there is some suggestion that this might not be true for all restriction enzymes (Horiuchi and Zinder, 1972). Similarities between the DNA sequence cleaved by the *Eco* RII endonuclease[1] and methylated by the *Eco* RII methylase have been reported recently (Boyer *et al.*, 1973).

In SV40 DNA, the *Eco* RI endonuclease makes only one double-stranded break (Morrow and Berg, 1972; Mulder and Delius, 1972) which is staggered in such a way that cohesive termini are formed (Mertz and Davis, 1972). The sequence of DNA base pairs adjacent to the phosphodiester bonds cleaved by this enzyme has been determined (Hedgpeth *et al.*, 1972). Here we present analysis of the sequence of nucleotides adjacent to the adenosine methylated by the *Eco* RI modification methylase. This sequence is found to be chemically identical with the previously determined sequence recognized and cleaved by the *Eco* RI endonuclease (Hedgpeth *et al.*, 1972).

A DNA molecule may be a substrate either for a restriction endonuclease or modification methylase but not for both, since treatment with one enzyme renders the DNA insensitive to the other. Since the single-stranded termini of *Eco* RI restricted SV40 DNA have complementary base sequences, the linear molecules produced by digestion of closed circular SV40 DNA will reassociate under appropriate conditions, forming circular molecules or linear concatemers that will be substrates for polynucleotide ligase, an enzyme that catalyzes the esterification of single-stranded phosphodiester-bond scissions in a bihelical DNA molecule (Weiss *et al.*, 1968a). Using this property, we have produced a DNA molecule that has been used as a substrate by both the *Eco* RI endonuclease and methylase. This DNA was used to demonstrate that the two enzymes recognize a single site in the SV40 genome.

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[1] The *Eco* RI enzymes are controlled by an *fi*$^+$ R factor in *Escherichia coli* and the *Eco* RII enzymes by an *fi*$^-$ R factor. The *Hin*$_d$ II restriction endonuclease is the enzyme from *Hemophilus influenzae* isolated as described by Smith and Wilcox (1970). The abbreviations of species names are those proposed by Danna *et al.* (1973). The symbols A, T, G, and C without a prefix are deoxynucleotides; m^{6}A is N^6-methyldeoxyadenosine. Short DNA sequences containing a radioactive phosphorus will be represented by placing the atomic mass number (32) not at the atomic symbol (P) but rather directly at the symbol (p). For example, pG^{32}pA is a dinucleoside diphosphate having a ^{32}P-labeled phosphate at the internal position.