

Studies with DNA-cellulose Chromatography.

I. DNA-binding Proteins from *Escherichia coli*

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A fascinating variety of proteins must interact with DNA within the cell in order to make possible such basic genetic processes as DNA replication and repair, DNA recombination, selective gene expression, and mRNA transcription. Before gene function can be precisely defined at the molecular level, many such DNA-associated proteins will have to be individually isolated and characterized. We have developed a general method which should facilitate such analyses. This method, which we call 'DNA-cellulose chromatography', relies upon the fact that many of the proteins which function on DNA inside the cell bind tightly to DNA at physiological ionic strengths *in vitro*. At higher salt concentrations these proteins are reversibly released from the DNA, apparently in an undamaged state. Previously characterized proteins with such binding properties include the *E. coli* RNA polymerase (J. R. Richardson, 1966b; Pettijohn and Kamiya, 1967) and the lactose and phage λ repressors (Gilbert and Muller-Hill, 1967; Ptashne, 1967b).

In order to isolate all DNA-binding proteins from an organism, DNA-free crude extracts are prepared and passed through a column consisting of DNA adsorbed onto an inert cellulose matrix ('DNA-cellulose'). This is done at NaCl concentrations not lower than 0.05 M. Subsequent washing of the column with buffer of the same ionic strength leaves bound only proteins with a high affinity for the DNA-cellulose. The NaCl concentration is then increased to elute these proteins from the DNA.

At the salt concentrations used for loading extracts onto DNA-cellulose columns, simple electrostatic complex formation between non-DNA-related basic proteins and DNA should be largely prevented (Hofstee, 1964). In fact, we find that the vast majority of the DNA-binding proteins isolated from bacterial systems are *negatively* charged at the pH used for chromatography. This

appears to indicate that the proteins isolated by our procedure are recognizing DNA as a specific substrate rather than as a mere network of negative charges.

In this report we outline the basic methodology of DNA-cellulose chromatography, including the preparation and properties of the DNA-cellulose matrix. Experimental results which illustrate the potentiality of the method are presented, with special emphasis on the isolation and properties of the bacteriophage T4-induced 'gene 32 protein'. This protein appears to be required both for DNA replication and for genetic recombination in T4 bacteriophage-infected *E. coli* (Epstein et al., 1963; Tomizawa et al., 1966). In addition, preliminary studies are described in which a refinement of DNA-cellulose chromatography has enabled isolation of T4 bacteriophage proteins with a specific affinity for UV-irradiated DNA.

THE DNA-CELLULOSE MATRIX

To make DNA-cellulose, a solution of either native or denatured DNA, at 1–2 mg/ml in a solvent containing 0.01 M Tris-HCl, pH 7.4, plus 0.001 M Na₃EDTA ('Tris-EDTA'), is mixed with clean dry cellulose (Munktell 410, Munktell 400, or Macherey-Nagel 2200 ff) to give a thick paste (~1 g cellulose per 3 ml). After air drying overnight at room temperature, the remaining water is removed by lyophilization. The thoroughly dry powder is suspended in about 20 volumes of Tris-EDTA and left at 4° for a day. After two quick washes to remove free DNA, the DNA-cellulose is stored as a frozen suspension in Tris-EDTA plus 0.15 M NaCl. About one-half of the original DNA is normally bound to the cellulose (500–1500 μ g per packed ml), as determined by DNA released from an aliquot heated at 100° for 10 min in Tris-EDTA buffer. DNA does not bind to the cellulose without drying. For chromatography, enough DNA-cellulose suspension for one packed ml is diluted to 20 ml and left to settle, at room temperature and without de-aeration, into a 5 mm diameter column. Columns are run at 2 ml/hr, which is close to their gravity flow rate. Much larger columns

* This work was initiated while one of us (B. M. A.) held a National Science Foundation Postdoctoral Fellowship (1965–1966) at the Institut de Biologie Moléculaire, Geneva, Switzerland. A preliminary report has been published (Alberts, 1968).

TABLE 1. STABILITY OF NATIVE DNA-CELLULOSE

Temperature	Time for loss of one-half of adsorbed DNA	
	0.001 M Na ₃ EDTA 0.01 M sodium phosphate, pH 7.4	0.20 M NaCl 0.001 M Na ₃ EDTA 0.01 M sodium phosphate, pH 7.4
37°	400 hr	400 hr
50°	110 hr	120 hr
70°	11 hr	20 hr
90°	<0.08 hr	0.3 hr

Calf thymus DNA (Worthington Biochemicals) was adsorbed to Munktell 410 cellulose (Bio-Rad Laboratories). The resulting DNA-cellulose (560 μ g/packed ml) was washed and resuspended in the buffer indicated. After various times of incubation, the DNA released into the supernatant of a low speed spin was determined by absorbance measurements. Estimated initial rates are shown. The rate of DNA loss decreases with increasing incubation times, indicating that individual DNA molecules are bound to the cellulose with varying affinities.

are feasible, but they were not required in the experiments described below.

In preparing DNA-cellulose, DNA of mol wt about 10×10^6 daltons is probably optimal, since in this case the viscosity is low enough to permit quite concentrated solutions of DNA to be dried with cellulose. DNA-cellulose with a high DNA content may also be obtained using dilute DNA solutions, but repeated cycles of drying are then necessary.

The complex formed by drying DNA with cellulose is slowly reversible in aqueous suspension, and at equilibrium very little DNA remains adsorbed. However, the rate of desorption at low temperature is insignificant compared to the duration of a typical chromatographic experiment. This is shown in the table, where the time required for loss of one-half of adsorbed native calf thymus DNA from DNA-cellulose is listed as a function of temperature. Note that the half-time of adsorbed DNA at 37° is about 400 hr, whereas column fractionations require less than 12 hr at 4°. The stability of this native DNA-cellulose at 37° was relatively insensitive to changes in NaCl concentration (0.01 M to 5 M) and pH in the range pH 5 to pH 9.

Similar complexes between high molecular weight denatured DNA and cellulose are quite stable, while R₁₇ RNA and sonicated native DNA complexes with cellulose break down more rapidly. Drying tRNA with cellulose does not produce any detectable complex. It appears that a weak adsorption normally occurs at several sites on a single nucleic acid molecule, and that the cumulative effect of many such interactions holds long nucleic acid molecules to the cellulose more firmly than shorter molecules which have fewer sites of interaction.

In order for nucleic acid-cellulose complexes to be useful in the fractionation of proteins, the adsorbed nucleic acid must be accessible for protein

binding. Since a brief pancreatic DNase or RNase treatment suffices to remove all adsorbed DNA and RNA, respectively, from cellulose, we conclude that nucleic acids adsorbed to cellulose do in fact remain freely accessible to proteins in solution.

A potential difficulty is that drying double-helical DNA with cellulose might lead to its

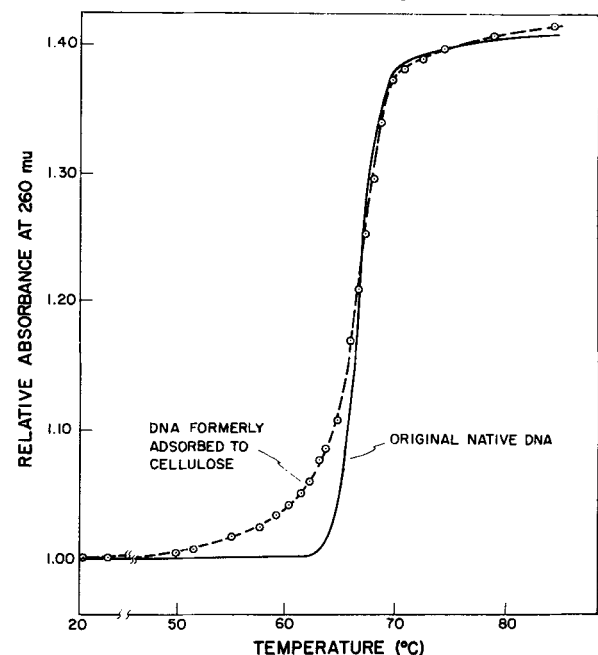


FIGURE 1. Absorbance-temperature profile of native T4 bacteriophage DNA removed from DNA-cellulose by agitation. Native T4 DNA-cellulose, prepared as described previously, was washed and resuspended in 0.01 M potassium phosphate, pH 7.4. Four ml of this suspension was homogenized for 30 min at 10,000 rpm in the microcup of a Virtis homogenizer (23°), releasing 50% of the bound DNA. A sample of this released DNA, at 10 μ g/ml in a sealed cuvette, was heated at 22°/hr in the thermostated compartment of a Gilford Spectrophotometer. The absorbance and temperature were automatically recorded. A native T4 DNA standard was simultaneously examined at the same concentration and in the same buffer. Similar results were obtained for native calf-thymus DNA which had been removed from cellulose.

denaturation. In order to test this possibility, an absorbance-temperature profile was measured on native T4 bacteriophage DNA which had first been adsorbed to cellulose and then removed by violent agitation. The results are shown in Fig. 1. Comparison with the original native DNA indicates that while the desorbed DNA may have accumulated some imperfections, its degree of hypochromicity is unchanged. It is therefore concluded that native DNA remains largely double helical when adsorbed to the cellulose. However, since regions of DNA which are locally denatured as a result of direct contact with cellulose might renature when the cellulose is removed, the DNA-cellulose could contain considerably more exposed single strands than are revealed by this technique.

PURIFICATION OF KNOWN DNA-ASSOCIATED ENZYMES ON DNA-CELLULOSE

In initial experiments with DNA-cellulose columns, we investigated the chromatographic behavior of DNA and RNA polymerase enzymatic activities from crude extracts of *E. coli*. By maximizing the recovery of these labile DNA-binding enzymes, we derived optimal chromatographic procedures for subsequent studies with DNA-binding proteins of unknown function. Typical results obtained with two main variants of the resulting procedure are illustrated in Fig. 2 and 3; the two experiments differ chiefly in the method used for removing DNA from the crude protein extract applied to the column. In one method, the cell sonicate to be chromatographed is treated with pancreatic DNase, centrifuged at high speed to eliminate particulate matter, dialyzed against an EDTA-containing buffer to inactivate DNase, and then passed through a native T4 DNA-cellulose column. As shown in Fig. 2A, both DNA and RNA polymerase activities in such a DNA-free extract are bound to the column and can be recovered approximately 100-fold purified by raising the NaCl concentration from 0.15 M to 0.60 M. No binding to a control, DNA-free cellulose column is detected. Pancreatic DNase itself does not bind to DNA-cellulose under these conditions (<0.05%), so that it does not contaminate the purified polymerases.

Without DNase pretreatment, a major portion of both DNA and RNA polymerase activities washes through the DNA-cellulose column, bound to the endogenous DNA of the extract, as shown in Fig. 2B. DNA-free crude extracts are thus highly desirable. An alternative method of preparing such extracts is to partition the crude lysate in a polyethylene glycol-dextran, aqueous two-phase system in the presence of 5 M NaCl (Albertsson, 1960; Okazaki and Kornberg, 1964; Alberts, 1967). This

removes ribosomes as well as nucleic acids from the protein-containing, polyethylene glycol-rich top phase. The NaCl is removed by dialysis, and the polyethylene glycol passes through the DNA-cellulose column. The chromatographic purification of RNA polymerase from such extracts, using both step and continuous gradient NaCl elutions, is shown in Fig. 3. Recovery of the RNA-polymerase activity applied was quantitative. Moreover, 5 ml of crude extract, obtained from 2 g of wet cells, was applied to a one ml column; the polymerase recovered was thereby concentrated 5–10-fold. At a flow rate of 2 ml/hr, the entire experiment required 5 hr. We are indebted to Mr. Larry Owens for the results of this experiment.

By either of the above methods, we have been able to obtain DNA-free crude extracts with high RNA polymerase activity only from RNase I deficient *E. coli* strains [strain RNase₁₀ (Gesteland, 1966) or strain MRE600 (Cammack and Wade, 1965)]. Wild type *E. coli* K12 and *E. coli* B show a pronounced, apparently irreversible, loss of RNA polymerase activity upon DNA removal. This effect is dominant in mixed extracts. The activity of DNA polymerase in DNA-free extracts is not reduced in RNase I positive strains. We tentatively attribute the unusual inactivation of RNA polymerase to the known inhibitory complex which the free enzyme forms with RNA (Tissières et al., 1963; Bremer et al., 1966) and assume that RNA fragments generated from RNase I digestion are primarily involved.

ISOLATION OF TOTAL DNA-BINDING PROTEINS FROM T4 BACTERIOPHAGE-INFECTED AND UNINFECTED *E. coli* WITH DNA-CELLULOSE

The experiments with RNA and DNA polymerase (Fig. 2 and 3) indicate that DNA-cellulose chromatography is a mild and effective technique for the isolation of functional DNA-binding proteins. By chromatography of radio-isotopically labeled protein extracts, followed by polyacrylamide gel electrophoresis and autoradiography of the dry sliced gels (Fairbanks et al., 1965), DNA-binding proteins whose function on DNA is unknown can be recognized and cataloged according to their electrophoretic mobilities.

Chromatographic profiles. DNA-free crude extracts of ³⁵S-labeled bacteria were dialyzed against a Mg⁺⁺-free, EDTA-containing buffer and then passed through a DNA-cellulose column in the presence of 0.05 M NaCl. After an extensive wash with a special 'rinse buffer' containing 0.05 M NaCl, the ³⁵S-labeled DNA-binding proteins were eluted by raising the NaCl concentration in three separate steps of 0.15 M NaCl, 0.60 M NaCl, and 2.0 M NaCl. Details of the procedure and a typical elution

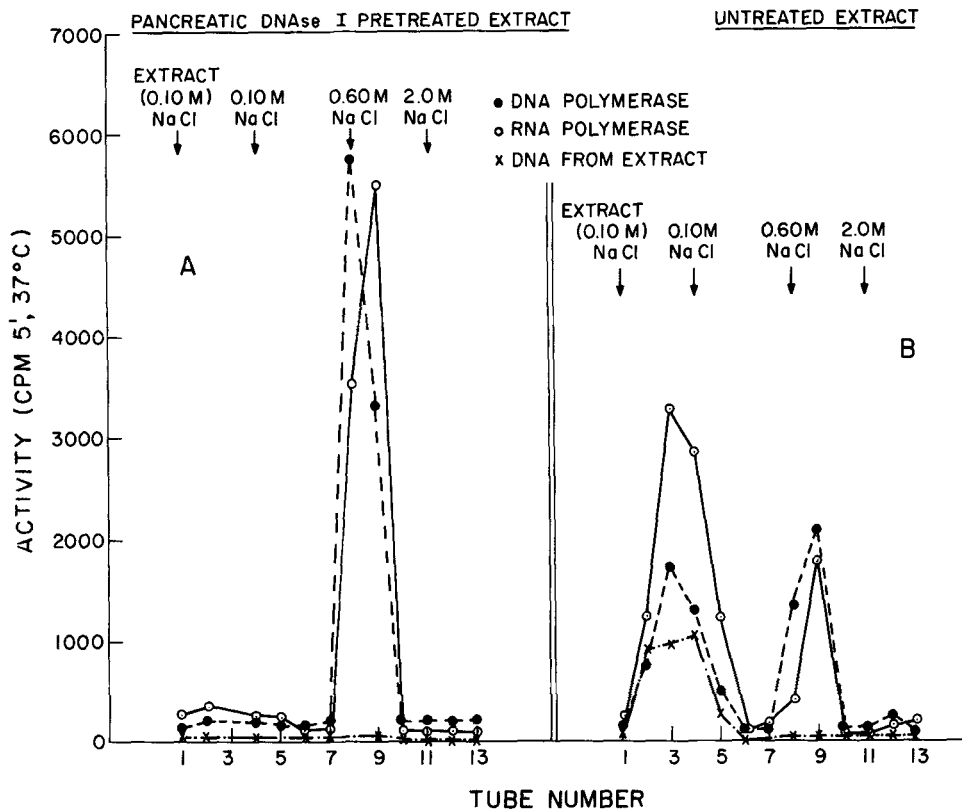


FIGURE 2. Behavior of *E. coli* RNA and DNA polymerases on columns of T4 native DNA-cellulose. Cells of *E. coli* K12 strain RNase₁₀ (Gesteland, 1966), growing in M9 minimal medium (Champe and Benzer, 1962) were lightly labeled with ¹⁴C-thymidine and then harvested. For one wet gram of cells, 4.0 ml of 'sonication buffer' (0.01 M MgCl₂, 1 mM mercaptoethanol, 0.2 mM Na₃EDTA, and 0.04 M Tris-HCl, pH 8.1) was added; the cells were broken by sonication and centrifuged at high speed to remove particulate matter (38,000 rpm for 1 hr in the Spinco 40 rotor). An aliquot of the supernatant was treated for 15 min at 23° with 20 µg/ml of pancreatic DNase I (Worthington Biochemical). Both DNase-treated and untreated extracts were dialyzed against two 7 hr changes of 'dialysis buffer' (0.10 M NaCl, 0.001 M mercaptoethanol, 0.001 M Na₃EDTA, 0.02 M Tris-HCl, pH 8.1), clarified for 10 min at 10,000 × *g*, and diluted with two volumes of 'rinse buffer' [dialysis buffer containing 10% glycerol and 500 µg/ml bovine serum albumin (BSA, Calbiochem, A grade)]. A volume of 1.5 ml of each of these extracts was loaded at 2 ml/hr onto a T4 native DNA-cellulose column (0.75 ml packed volume), which had been prewashed for several hours at 4° with the rinse buffer. After loading, the column was washed again with this buffer, and bound proteins were then eluted by increasing the NaCl concentration in the rinse buffer in the steps shown. All fractions were 0.5 ml, collected once every 15 min. Unless noted, all operations were at 4°.

For RNA polymerase, the assay reaction mixture contained, per ml, 0.04 mmoles Tris-HCl, pH 8.1, 0.065 mmoles KCl, 0.25 µmoles Na₃EDTA, 0.005 mmoles MgCl₂, 0.004 mmoles mercaptoethanol, 500 µg BSA, 160 µg calf thymus DNA, 0.25 µmoles each CTP, GTP, and UTP and 0.07 µmoles ATP including 16 µC of ³H-ATP. For DNA polymerase, the assay reaction mixture contained, per ml, 0.04 mmoles Tris-HCl, pH 7.4, 0.25 µmoles Na₃EDTA, 0.007 mmoles MgCl₂, 0.001 mmoles mercaptoethanol, 500 µg BSA, 160 µg native calf thymus DNA, 0.04 µmoles each of dCTP, dGTP and dTTP, and 0.03 µmoles dATP including 10 µC ³H-dATP. In both cases, the count/min shown include a blank of about 150 count/min and represent TCA-insoluble incorporation catalyzed by 5 µl of enzyme in a 50 µl reaction mixture (10 µl in 100 µl for RNA polymerase) after 5 min at 37°, counted at 30% efficiency. Conversion of count/min to mµmoles of product is difficult because of the significant but undetermined extent of radiation-induced breakdown of the ³H-triphosphates used. The presence of pancreatic DNase in fractions 1-4 of column A has no appreciable effect on the RNA polymerase activity measured, but it depresses potential DNA polymerase incorporation 5-fold.

pattern resulting from chromatography of a normal *E. coli* extract on native T4 DNA-cellulose are given in Fig. 4, along with the results of an identical experiment performed with an extract labeled at early times after T4-bacteriophage infection. In both cases, the majority of DNA-binding proteins elute in the first 0.60 M NaCl fraction, although there is also an appreciable component at 0.15 M NaCl and a minor one at 2.0 M NaCl. A striking difference between the two chromatographic separations is that only about 1%

of the total soluble protein from uninfected cells binds to DNA, as against 8% of the soluble 'early' phage-induced protein. This difference suggests that, as expected, an unusually large fraction of the bacteriophage protein made early after infection is involved in DNA-related functions.

Additional experiments in which T4-induced proteins were labeled *late* in infection reveal that about 3% of the soluble 'late' phage protein binds to DNA; these proteins may function in DNA

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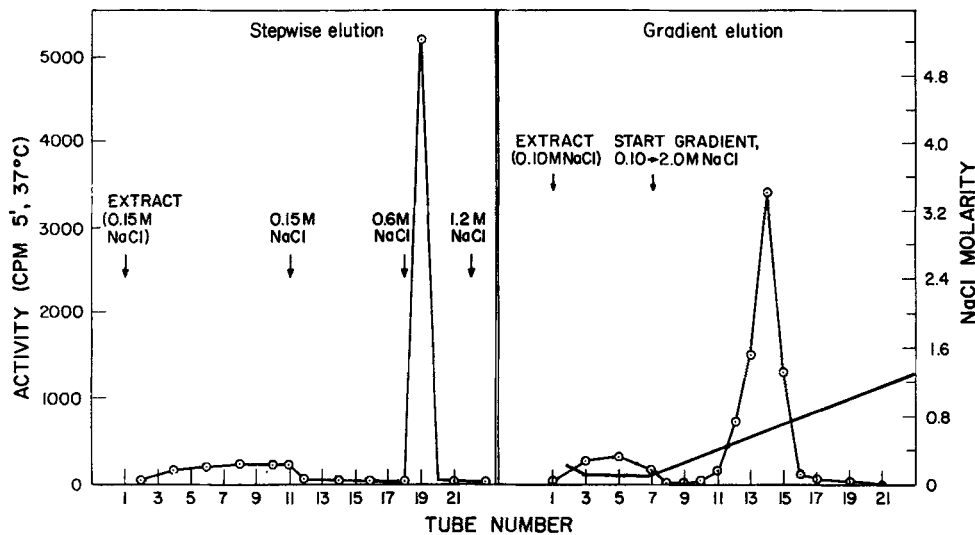
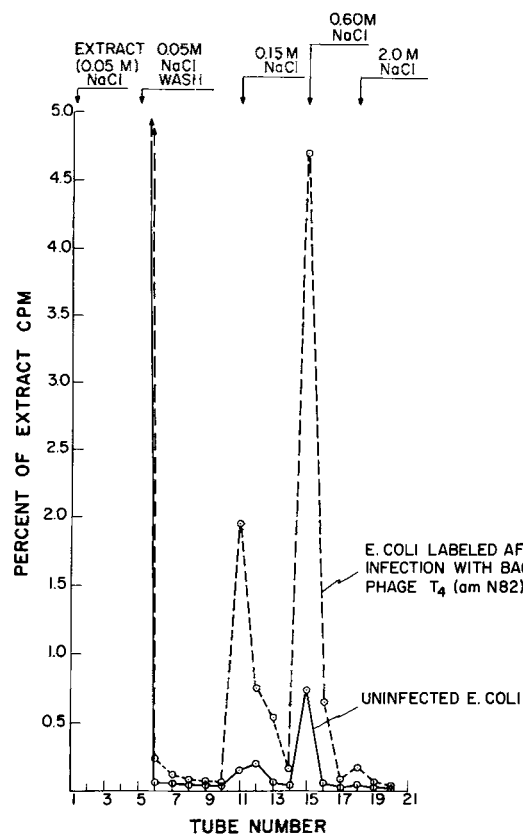


FIGURE 3. Purification of *E. coli* RNA polymerase on calf-thymus native DNA-cellulose. After sonication of 2 g of RNase⁻ cells as described in Fig. 2, the sonicate was made 5 M in NaCl and centrifuged at low speed. To a total volume of 10 ml of supernatant, 0.6 g powdered Carbowax 6000 (Union Carbide Chemicals) and 0.4 g Dextran 500 (Pharmacia) were added. The polymers were dissolved by mixing at 4°, and two phases of approximately equal volume were separated by a 10 min centrifugation at 10,000 × *g*. The polyethylene glycol-rich top phase was withdrawn and dialyzed. All subsequent details were identical to those in Fig. 2, except that the 5 ml of dialyzed extract (~8 mg/ml protein) was chromatographed without further dilution on a 1 ml DNA-cellulose column containing 0.56 mg of native calf thymus DNA (Worthington Biochemical). Fractions of 0.5 ml were collected at 15 min intervals. The count/min given are for 1 μl of enzyme in 50 μl of reaction mixture (Fig. 2), with incubation for 5 min at 37°. A variable fraction of the RNA polymerase activity precipitates in the phase system; consequently, somewhat less than one-half of the initial sonicate activity is normally recovered by this procedure. In preliminary studies of 0.60 M NaCl eluates from similar columns, nearly complete binding of DNA methylase activity from strain MRE600 has been observed using the assay of Gold and Hurwitz (1964). Substantial *E. coli* deoxy-ribonuclease activity is also bound, although the major portion of this activity passes through the column (B. Alberts, unpubl. experiments).

maturation or in the packaging of DNA into phage heads.

Only about 0.1% of the total labeled protein applied to a control, DNA-free cellulose column appears in column eluates. Therefore, the binding of proteins to DNA-cellulose represents almost exclusively binding to DNA, despite the fact that, on a weight basis, the cellulose is present in about a 200-fold excess over DNA. To obtain this low blank value, it is necessary to use a highly purified 'cotton linters' grade of cellulose, to include 10% glycerol in column buffers, and to remove particulate matter completely from the extracts to be chromatographed.

FIGURE 4. Chromatography of ³⁵S-labeled crude extracts on T4 native DNA-cellulose. *E. coli* B were grown to 3 × 10⁸ cells/ml in modified M9 minimal medium containing 5 μg/ml Na₂SO₄ as the sole source of sulfur. Two aliquots of 150 ml each were cooled to 25° and labeled for 14 min by addition of 0.5 mc carrier-free H₂³⁵SO₄; in one aliquot the label was added 4 min after the cells had been infected with T4 amN82 phage (m.o.i. = 5), and in the other the cells were not infected. Each culture was washed and resuspended in 2 ml of sonication buffer. Subsequent steps were identical to those described in Fig. 2, except that DNase treatment was for 1 hr at 10°. Approximately 40 μc of extract were applied to each column. Fraction of 0.5 ml were collected at 15 min intervals, and 20 μl aliquots were counted in Brays-Cab-O-Sil scintillation fluid (Bray, 1960; Ott et al., 1959). After the 2.0 M NaCl elution, only about 0.2% of the original radioactive protein remains adsorbed to the DNA-cellulose.



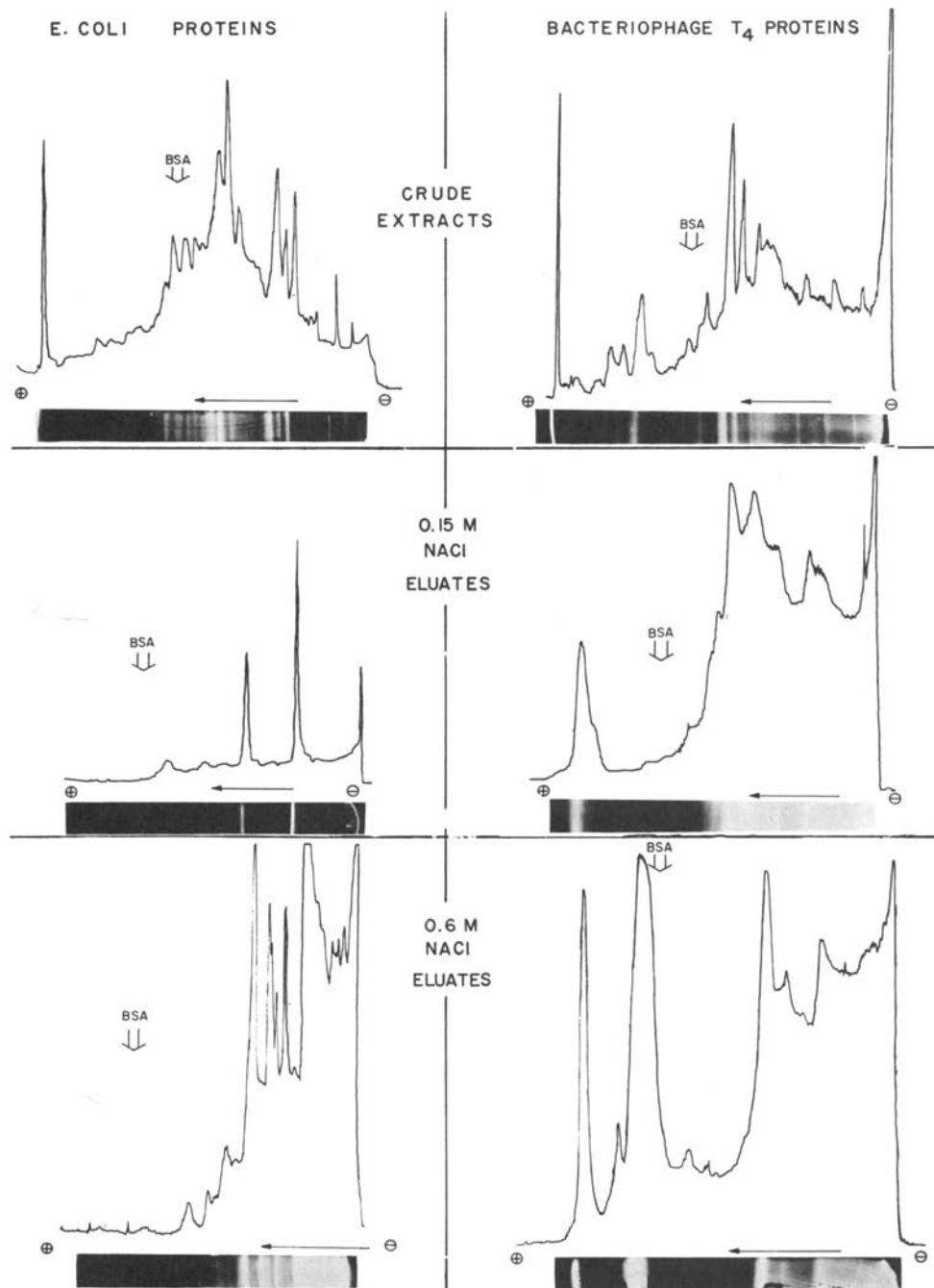


FIGURE 5. Polyacrylamide gel electrophoresis of ^{35}S -labeled DNA-binding proteins. Samples of 0.10 ml of the peak fractions from Fig. 4 and an aliquot of dialyzed extract were analyzed in the pH 8 gel system of Jovin et al. (1964), using 5 mm I.D. $\times$ 7 cm gels. To facilitate subsequent drying, the bottom gel composition was changed to 7.5% acrylamide, 0.10% bisacrylamide. Gels were fixed and stained for 24 hr in 10% TCA plus 1/20th saturated Coomassie Brilliant Blue R250 (Colab Laboratories, Chicago). Less than 0.5 μg of protein per band could be readily seen, even without destaining. The gels were then dried and autoradiographed (Fairbanks et al., 1965). Joyce-Loebl tracings of over-exposed films are shown in order to maximize detail; tracings of less exposed films are given in Fig. 6. Exposure times were 1 to 5 days. Band patterns which are very similar, but not identical, to those above were obtained in experiments in which the T4 native DNA-cellulose was replaced by either calf thymus native or denatured DNA-cellulose, and when phase-extracted extracts (Fig. 3) were chromatographed in the presence of 0.01 M MgCl_2 . It is therefore clear that the majority of DNA-binding proteins neither show an absolute specificity for a particular species of DNA, nor require Mg^{++} for their binding.

Polyacrylamide gel electrophoresis of DNA-binding proteins. As a first step in the characterization of the ^{35}S -labeled DNA-binding proteins whose isolation was described in Fig. 4, each peak fraction was subjected to electrophoresis through a polyacrylamide gel. Autoradiographs of the band patterns obtained are presented in Fig. 5. For both the *E. coli* and the T4 phage experiments, the electrophoretic patterns of DNA-binding proteins can be seen to be much simpler than that of the corresponding crude extract. DNA-cellulose chromatography therefore removes only a select group of proteins from the extract. It is also clear that those proteins eluted at a given salt concentration are by and large completely eluted at that point, since the 0.15 M NaCl and the 0.60 M NaCl gel patterns in Fig. 5 are quite different. It should be noted that *E. coli* proteins binding to DNA do not appear among DNA-binding proteins labeled during T4 bacteriophage infection, because host protein synthesis is rapidly turned off by this phage (Levinthal et al., 1967).

Like the previously characterized *E. coli* RNA polymerase, DNA polymerase, and bacteriophage lambda repressor proteins (J. R. Richardson, 1966a; C. C. Richardson et al., 1964; Ptashne, 1967a), all of the proteins in the electrophoretic diagrams of Fig. 5 carry a net *negative* charge at pH 8. Tests show that more than 90% of the total protein isolated by DNA-cellulose chromatography shares this property, and yet these proteins bind quite strongly to the polyanionic DNA. This fact suggests that nonspecific associations between DNA and protein have been largely eliminated under our conditions. We believe therefore that most, if not all, of the DNA-binding proteins seen as bands in Fig. 5 actually have a DNA-related function.

THE ISOLATION AND CHARACTERIZATION OF A PROTEIN REQUIRED FOR BOTH DNA RECOMBINATION AND REPLICATION IN T4 BACTERIOPHAGE-INFECTED CELLS

Identification of the gene 32 protein. The foregoing experimental work provides a new general method for biochemical analysis of genetic systems which we are currently exploiting in several ways. In particular, radioisotopically labeled DNA-binding proteins have been isolated from non-permissive *E. coli* B cells infected with various amber mutants of bacteriophage T4 (Epstein et al., 1963). Since amber mutants produce only an incomplete fragment of the protein corresponding to the mutated gene (Stretton and Brenner, 1965), the presence of an amber mutation in a gene which codes for a DNA-binding protein would normally be expected to eliminate the ability of that protein

to bind to DNA. Analysis by polyacrylamide gel electrophoresis of DNA-binding proteins isolated from T4 amber mutant infections should thus enable some of the T4 protein bands in Fig. 5 to be assigned to known bacteriophage genes. The bacteriophage T4 system is particularly well suited for this type of analysis, inasmuch as a wide variety of amber mutants have already been isolated and characterized, and many of these seem to have important DNA-related functions (Epstein et al., 1963).

In one experiment, T4 *amA453* (gene 32) was used for infection. When the 0.60 M NaCl eluate from DNA-cellulose was examined by polyacrylamide electrophoresis, the result presented in Fig. 6B was obtained. Notice that one of the major bands of the wild type polyacrylamide gel pattern has completely disappeared. This protein is missing in another gene 32 mutant (*amE315*) and is present

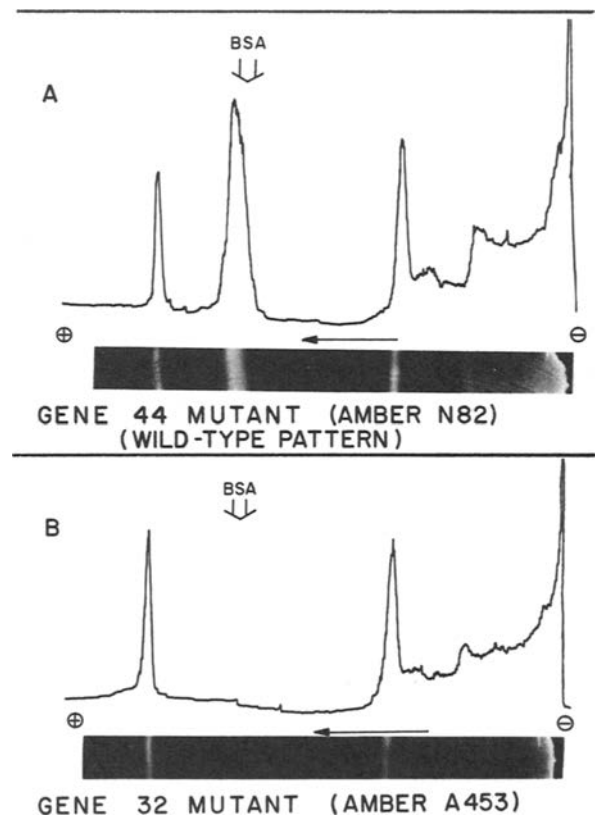


FIGURE 6. Polyacrylamide gel pattern of DNA-binding proteins from *E. coli* infected with amber mutants of bacteriophage T4. Experimental details are those described in Figs. 4 and 5. (A) 0.60 M NaCl eluate of extract from cells infected with T4 *amN82* (gene 44). (B) 0.60 M NaCl eluate of extract from cells infected with T4 *amA453* (gene 32). The gel patterns for the *amA453* crude extract and 0.15 M NaCl eluate were indistinguishable from those given in Fig. 5. The single band difference in the 0.60 M eluate was maintained in the patterns obtained when urea-denatured samples were run through polyacrylamide gels containing 8 M urea (Jovin et al., 1964).

in normal amounts in all other amber mutants tested, including mutations in genes 41, 42, 43, 44 (Fig. 6A), 45, and 30. It has therefore been tentatively identified as the gene 32 protein (henceforth abbreviated as '32 protein'). This protein binds to either T4, *E. coli*, or calf thymus native DNA-cellulose in the presence of 0.15 M NaCl, and is eluted in the 0.60 M NaCl fraction.

Because of the possibility that alteration of a secondary factor could be masking the presence of normal 32 protein in T4 *amA453* infected cells, the results just described were tested by double-label experiments using mixed mutant extracts. Identical cultures of cells were separately infected with T4 *amN82* (gene 44) and with T4 *amA453* (gene 32) bacteriophage. From 4 to 8 min after infection, one-half of each culture was labeled with ^3H -leucine while the other half was labeled with $^{35}\text{S}\text{O}_4^{2-}$. The ^3H -labeled T4 *amN82* infected cells were mixed with an equal quantity of the ^{35}S -labeled T4 *amA453* infected cells and vice versa, and the two mixed extracts were prepared and chromatographed as in Fig. 4. To further enrich for 32 protein, the 0.60 M NaCl peak from each DNA-cellulose column was fractionated with Sephadex G-200. The results of these Sephadex columns are shown in Fig. 7. The 32 protein appears at a V_e/V_0 near 2.0 and is labeled only in the T4 *amN82*-infected extract. It accounts for at least 0.5–1.0% of the total soluble protein synthesized between 4 and 8 min after infection (25°). This confirms our original interpretation: with an amber mutation in gene 32, a protein (32 protein) which is normally capable of binding to DNA can no longer be made.

The biological role of gene 32. T4 mutants deficient in gene 32 function have been placed by Epstein et al. (1963) in the 'D₀' class, meaning that they do not synthesize detectable quantities of phage DNA. More recently, the use of more sensitive techniques has confirmed the severe block in DNA-replication in the *amA453* mutant of gene 32 (Kozinski and Felgenhauer, 1967). In addition, in biparental crosses *amA453* mutants, but not other amber mutants of the D₀ class, fail to form the 'joint recombinant' DNA molecules which apparently represent the initial products of genetic recombination (Tomizawa et al., 1966; Kozinski and Felgenhauer, 1967). A purely genetic study, in which amber mutations were only partially suppressed during genetic crosses, provides independent evidence that the 32 gene product is needed for genetic recombination (H. Berger, pers. commun. 1968).

The apparent dual function of gene 32 in both DNA replication and genetic recombination may be explained if the 32 protein catalyzes a reaction

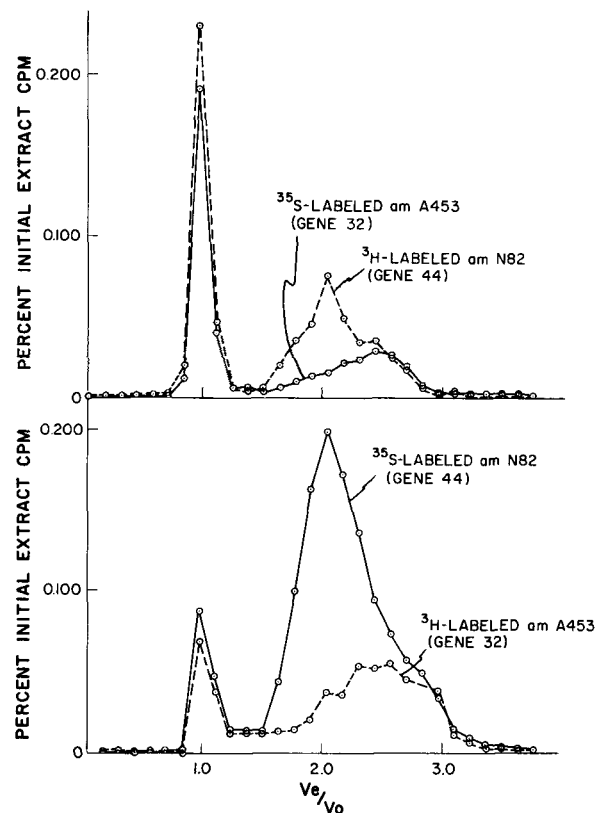


FIGURE 7. Sephadex G-200 fractionation of labeled (4 to 8 min, 25°) bacteriophage T4-induced, DNA-binding proteins. A 40 cm $\times$ 0.8 cm I.D. column of Sephadex G-200 at 4° was equilibrated with a buffer consisting of 0.10 M NaCl, 0.001 M Na₂EDTA, 0.001 M mercaptoethanol, 0.02 M Tris-HCl, pH 8.1, 10% glycerol and 500 $\mu\text{g}/\text{ml}$ BSA. Aliquots (0.3 ml) of 0.60 M NaCl DNA-cellulose eluates, obtained as described in the text, were washed through the column with this buffer at 2 ml/hr. The fractions obtained were counted in Brays-Cab-O-Sil scintillation fluid. The results shown have been corrected for channel overlap between the two radioisotopes; only 25–30% of the labeled protein applied to the column was recovered.

common to both processes. Alternatively, genetic recombination itself might be required for productive T4 DNA replication (Tomizawa et al., 1966). One simple explanation of this type is that recombination might be needed to form a DNA circle from the terminally repetitious DNA ends of an infecting linear molecule (MacHattie et al., 1967), and a circle could be necessary for productive DNA replication to begin. This model readily accommodates the fact that genetic recombination appears not to be necessary for bacteriophage lambda development (Signer et al., this volume), since with lambda the capability for circle formation is already inherent in the 'sticky-end' structure of the mature phage DNA (Hershey and Burgi, 1965; Strack and Kaiser, 1965).

The above analysis predicts that gene 32 function

will not be needed for bacteriophage T4 development once T4 DNA replication has been successfully initiated. To test this prediction, at various times after infection we have taken temperature-sensitive gene 32 mutants growing at 25° and shifted them to 42° to complete their development. In Fig. 8, the final phage yield obtained is plotted as a function of the time allowed for phage growth at the permissive temperature (25°). As predicted, no phages are produced if the temperature is shifted prior to the time that DNA synthesis begins. As the shift is made at later times, more and more phages are obtained. However, the phage yield does not show the dramatic rise that the model predicts for shifts at later times; for example, at 25 min, when 20% of the normal amount of DNA has already been made, a shift to 42° results in only a 30% phage yield. Direct measurements of DNA synthesis in temperature-sensitive gene 32 mutants before and after the temperature shift have also been

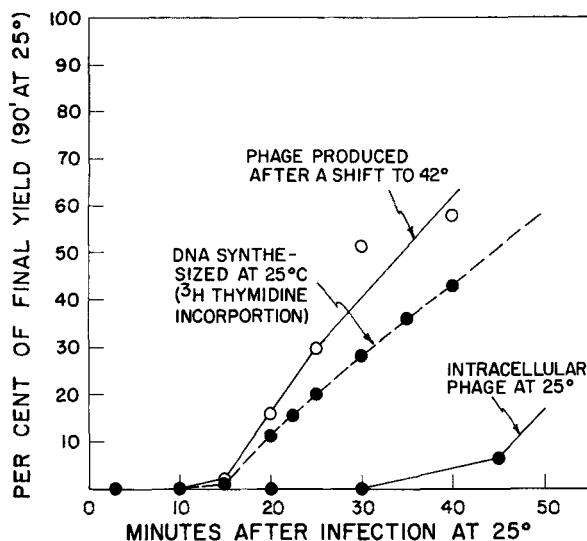


FIGURE 8. Temperature sensitivity of T4 gene 32 mutant *tsL170*. *E. coli* B3 was grown at 37° to 3×10^8 cells/ml in M9 minimal medium containing 0.3% casein hydrolysate and 5 μ g/ml thymidine. After a 15 min pre-incubation at 25°, the cells were infected by addition of 5 phage per bacterium (plus 25 μ g/ml tryptophan). Five minutes later, 0.2 μ Ci/ml of 14 C-thymidine and 20 μ g/ml of cold thymidine were added. To monitor DNA synthesis, aliquots were removed periodically into an equal volume of cold 10% trichloroacetic acid, collected and washed on membrane filters, and counted in a toluene-based scintillator. To follow phage growth, aliquots of the 25° culture were taken at the indicated times and either diluted into broth containing CHCl_3 ('intracellular phage at 25°'), or diluted 5-fold into fresh 42° media (without added thymidine) for further growth at 42° until CHCl_3 addition at 85 min after infection ('phage produced after a shift to 42°'). As a control, if wild type phage are used for this experiment, a full yield of progeny phage is obtained regardless of the time of the temperature transfer. Three other temperature-sensitive mutants of gene 32 (*tsL171*, *tsL94* and *tsL67*) gave results which were indistinguishable from those shown above for *tsL170*. All mutants were obtained from Dr. R. S. Edgar.

made, and the results are shown in Fig. 9A. The increase in temperature to 42° allows DNA synthesis to continue at the normal rate for approximately 5 min; but after this time further synthesis is retarded and a net degradation ensues. If wild-type phage are used for infection, DNA synthesis continues to completion after the same temperature shift (Fig. 9B), and a normal phage yield is obtained. It is thus clear that, in direct conflict with our simple model, gene 32 function is essential to T4 DNA-replication *throughout* the infectious cycle.

Of special interest is the fact that DNA synthesis does not stop immediately after the temperature shift in Fig. 9A, but rather seems to continue at an approximately normal rate for about 5 min at 42°. Identical behavior was observed for the two temperature-sensitive gene 32 mutants tested. Possibly 32 protein is needed for initiation or for termination of a round of DNA replication, but not for the polymerization part of the reaction. On the basis of the observed structure of replicating phage lambda DNA molecules (Ogawa et al., 1968), one can derive a detailed model of DNA replication which does in fact require a recombination-like event for periodic reinitiation of DNA synthesis (B. Alberts, unpubl. observations). An understanding of the precise function of 32 protein would enable such a model to be suitably tested.

Cooperative binding of gene 32 protein to single-stranded DNA. As the first step in an attempted analysis of the function of the gene 32 protein in *in vitro* studies, we set out to separate it from other DNA-binding proteins in preparative quantities. In the process of scaling up our procedures, an interesting and presumably biologically significant cooperative binding of this protein to single-stranded DNA was discovered. At the low protein concentration used in the experiment shown in Fig. 4, 32 protein remains bound to *either native or denatured* DNA-cellulose in the presence of 0.15 M NaCl, but is released with 0.60 M NaCl. Chromatography using higher concentrations of protein does not alter this pattern of binding when *native* DNA-cellulose is used. However, with denatured DNA-cellulose high protein concentrations cause the majority of the gene 32 protein to remain bound in 0.60 M NaCl and to elute instead in the 2.0 M NaCl fraction. This change in DNA affinity might be due to the increased number of 32 protein molecules present in concentrated extracts, but it could be equally well attributed to an increase in some other substance. In order to distinguish between these two possibilities, a dilute radioisotopically labeled extract was divided in half and mixed with a 100-fold excess of a concentrated, unlabeled extract,

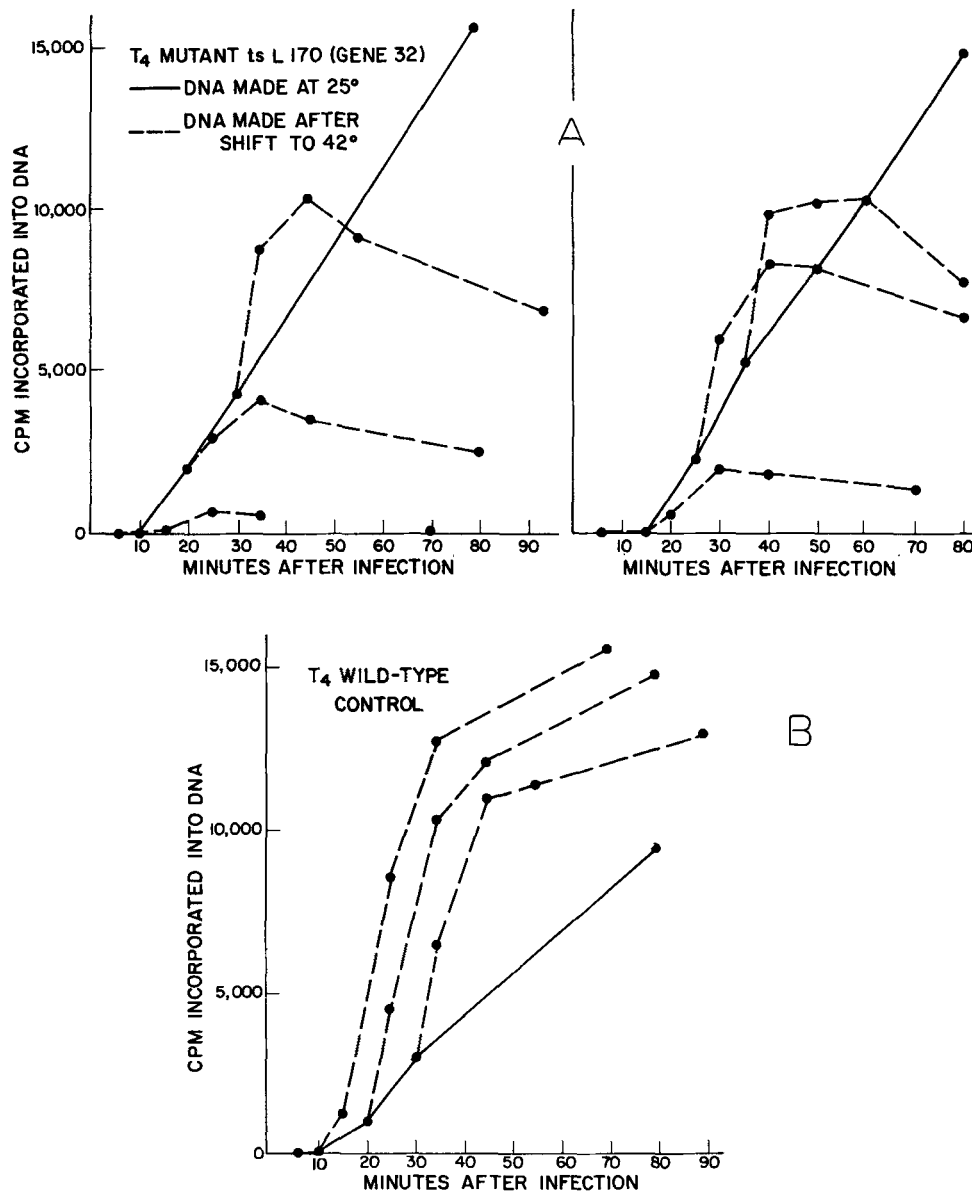


FIGURE 9. Dependence of T4 DNA synthesis on continued gene 32 function. (A) *E. coli* B₃ cells were infected with T4 *tsL170* phage (m.o.i. = 5) and labeled with ¹⁴C-thymidine as in Fig. 8, except that aliquots were taken into TCA both before and after the temperature shift. Two separate experiments are shown. Identical results were obtained when *tsL171* was used for the infection. (B) The experimental details were as above, except that wild-type phage were used.

made in one case (Expt. B) from cells infected with a T4 amber mutant deficient in gene 32 and in the other case (Expt. A) from cells infected with T4 *amN82*, a DNA-negative mutant which makes normal amounts of 32 protein. The concentrated mixed extracts were then chromatographed on two identical denatured DNA-cellulose columns. The 0.60 M NaCl and 2.0 M NaCl elution profiles obtained from each column are compared in Fig. 10. The radioisotopically labeled extract used had been prepared by mixing separate ³⁵S-labeled T4 *amN82* and ³H-labeled T4 *amA453* cultures, so that the

32 protein is identified by ³⁵S enrichment. From Fig. 10 it is clear that this enrichment shifts from the 0.60 M to the 2.0 M NaCl fraction (tubes 13–15) only when the concentrated, unlabeled extract contains 32 protein (Expt. A). We therefore conclude that it is a high concentration of this protein itself that enables binding to single-stranded DNA in 0.60 M NaCl. This means that individual molecules of 32 protein must interact when bound to single-stranded DNA and suggests a possible model of the type illustrated in Fig. 11, in which protein monomers line up along exposed strands

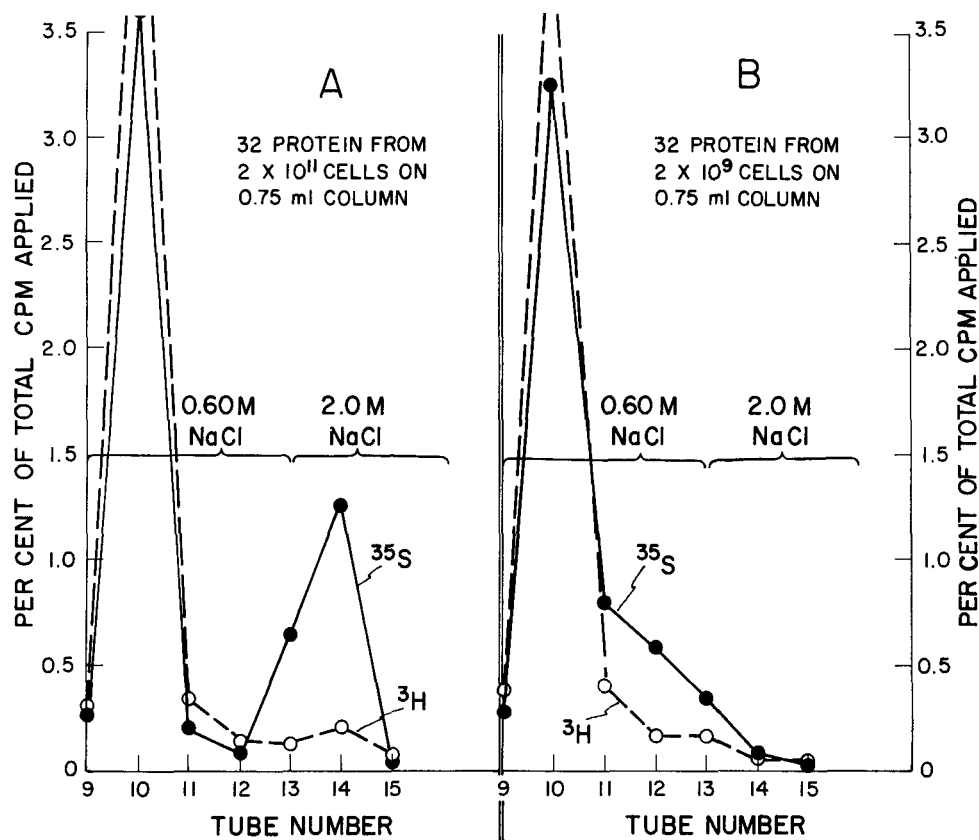


FIGURE 10. Cooperative binding of 32 protein to denatured DNA-cellulose. Unlabeled extracts were prepared by phase system extraction (Fig. 3) from *E. coli* B infected for 45 min at 31° with either T4 amN82 or T4 amA453. Just before loading onto denatured calf thymus DNA columns (0.75 ml packed volume containing 1050 µg DNA), unlabeled extract from 2 × 10¹¹ cells was mixed with a small amount of the special radioisotopically labeled extract described in the text. Chromatography was performed by the standard procedures. (A) Unlabeled extract from T4 amN82-infected cells. (B) Unlabeled extract from T4 amA453-infected cells. A similar result was obtained when this experiment was repeated using denatured T4 DNA-cellulose columns.

FIGURE 11. A possible model for the cooperative binding of 32 protein to single-stranded DNA. If binding actually occurs in the suggested manner, 32 protein would unravel the short, imperfectly paired double-helical 'hairpins' which are thought to predominate in single-stranded nucleic acids (Fresco et al., 1960). Under physiological conditions of salt and temperature, these regions of weak secondary-structure act as kinetic barriers and prevent denatured DNA molecules containing complementary nucleotide sequences from renaturing in vitro. With purified DNA, renaturation is facilitated by any condition, such as high temperature (Marmur and Doty, 1961), organic solvents (Subirana, 1966), or mild alkali (Doerfler and Hogness, 1968), which preferentially melts out the imperfect helices in single-stranded DNA while leaving intact the more stable, perfect double-helix formed between matching complementary strands. Providing that the binding of 32 protein to single-stranded DNA leaves the bases suitably exposed, this binding could serve to speed up renaturation in vivo in a manner analogous to the action of these mild denaturing conditions in vitro. The existence of some substance in cells which acts in this way to facilitate renaturation is implicitly assumed in most models of genetic recombination.

A POSSIBLE MODEL FOR THE COOPERATIVE BINDING OF 32 PROTEIN TO DNA

CONDITION	STABLE IN 0.6 M NaCl
LOW CONCENTRATION OF 32 PROTEIN ON DENATURED DNA 	NO
HIGH CONCENTRATION OF 32 PROTEIN ON DENATURED DNA 	YES
HIGH CONCENTRATION OF 32 PROTEIN ON NATIVE DNA 	NO

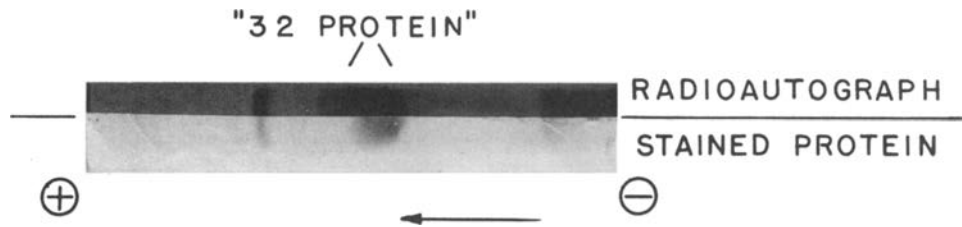


FIGURE 12. Polyacrylamide gel electrophoresis of purified gene 32 protein. The one-step purification was carried out on a denatured DNA-cellulose column by methods similar to those described in Fig. 10. Polyacrylamide gels were processed according to Fig. 5.

of DNA and bind in cooperative units. The failure to observe the same effect of 32 protein concentration on the strength of its binding to *native* DNA tends to discourage the alternate interpretation that a new, tightly binding oligomer of this protein is formed when its concentration is sufficiently high.

Physical properties of 32 protein. Because so few proteins remain bound to DNA in 0.60 M NaCl, with the type of chromatographic procedure outlined in Fig. 10 we can obtain an approximate 300-fold purification of 32 protein in a single step. At this point, stained polyacrylamide gels show 32 protein to be the major protein component present, with two or three minor protein bands also visible. A photograph of such a gel is displayed in Fig. 12.

Physical characterization of purified 32 protein has been attempted by a combination of two different techniques: gel filtration and sucrose

gradient velocity sedimentation (Siegel and Monty, 1966). The behavior of 32 protein on Sephadex G-200 in pH 8.1 buffer containing 0.10 M NaCl is shown in Fig. 13. From its elution position, this protein should have a frictional coefficient which is about 0.85 times as great as the frictional coefficient of the *E. coli* alkaline phosphatase marker (Laurent and Killander, 1964). In a sucrose gradient, the sedimentation rate of 32 protein is 0.5 times the rate of alkaline phosphatase in the same Sephadex buffer. Assuming constant $\bar{v}$, the Svedberg equation gives:

$$\frac{M_1}{M_2} = (S_1/S_2)(f_1/f_2).$$

If the molecular weight of alkaline phosphatase is assumed to be 80,000 (Garen and Levinthal, 1960), we obtain a molecular weight for 32 protein of about 35,000 daltons. Compared to the roughly

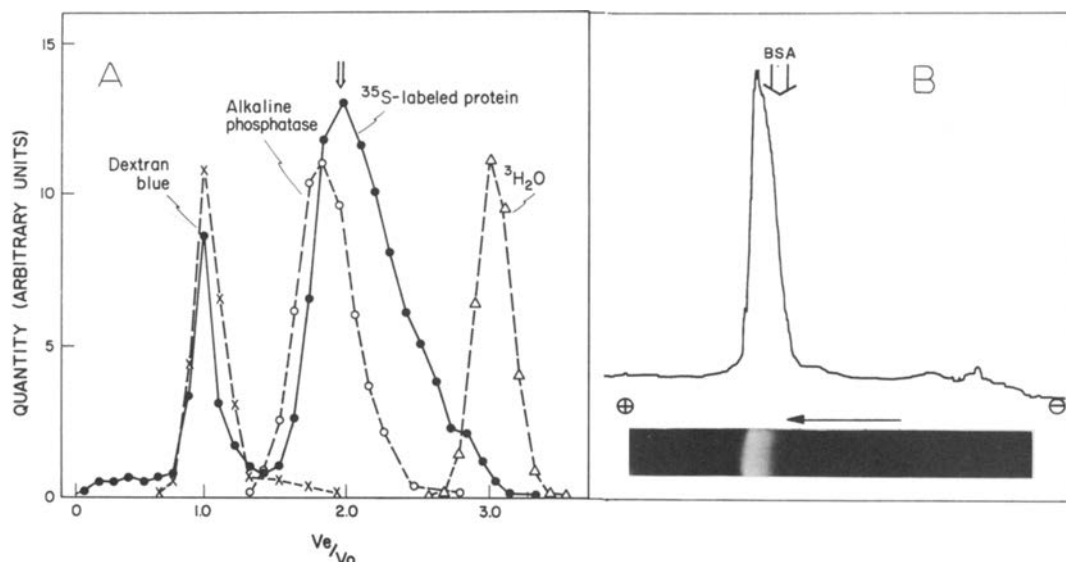


FIGURE 13. Characterization of 32 protein on Sephadex G-200. (A) Details were as described in Fig. 7, except that the 2.0 M NaCl peak from a denatured DNA-cellulose fractionation (Fig. 10A) was applied to the Sephadex column. About 70% of the ^{35}S -labeled protein was recovered. Alkaline phosphatase was assayed by the standard colorimetric assay (Garen and Levinthal, 1960) with 0.1 mM ZnCl_2 added. (B) This polyacrylamide gel autoradiograph of the peak Sephadex fraction (arrow in (A) above) was obtained by the procedures outlined in Fig. 5. It proves that the protein in these fractions is indeed 32 protein, and thus substantiates the results in Fig. 7.

spherical alkaline phosphatase molecule, 32 protein, because of its relatively large frictional coefficient, must either have considerably more water of hydration or, more likely, have a non-spherical shape (axial ratio of a prolate ellipsoid of about 4:1). Each 32 protein molecule could therefore be as much as 120 Å long, which is sufficient to complex with perhaps 20 contiguous DNA nucleotides.

Purified 32 protein binds both to native and to thermally denatured T4 or *E. coli* DNAs, as judged by cosedimentation of the radioisotopically labeled protein with these DNAs to the bottom of a sucrose gradient (in 0.10 M NaCl, 0.001 M Na₃-EDTA, 0.02 M Tris-HCl, pH 8.1). It is likely that more detailed studies of purified 32 protein and its complexes with DNA will enable its precise biological function to be ascertained.

THE ISOLATION OF PROTEINS WITH A SPECIFIC AFFINITY FOR ONE SPECIES OF DNA

Among the most interesting of all DNA-binding proteins are the regulatory proteins which control DNA expression. These proteins require a special nucleotide sequence for complex formation with DNA *in vivo* and might be expected to recognize only DNA containing this sequence *in vitro*. Selective DNA-binding has in fact been observed with both the lactose and phage λ repressors (Gilbert and Muller-Hill, 1967; Ptashne, 1967b).

A variation of DNA-cellulose chromatography has been developed whereby proteins exhibiting selective DNA affinity *in vitro* can be isolated and identified. Since free DNA in solution is not retained by DNA-cellulose, proteins can be partitioned between one type of DNA in solution (mobile phase) and another type of DNA immobilized on the cellulose (stationary phase). With an appropriate choice of conditions, the proteins remaining bound to the DNA-cellulose will be those preferring the type of DNA on the column.

In developing the methodology, we have attempted to isolate T4-induced proteins with a specific affinity for UV-irradiated T4 DNA by using such a DNA vs. DNA-cellulose partition system. In order to prevent possible slowly exchanging, nonspecific proteins from complexing with the DNA-cellulose, an excess of unirradiated T4 DNA was added and incubated with the extract. The extract was then pumped slowly over a UV-irradiated T4 DNA-cellulose column, followed by an extensive unirradiated T4 DNA wash. After the wash, proteins left bound to the column were released by the usual salt elutions. The results of some preliminary experiments are summarized in Fig. 14. In total, about 0.5% of the T4-induced

soluble protein appears to bind preferentially to the UV-irradiated T4 DNA. However, as also shown in Fig. 14, 0.2% binding was observed to an *unirradiated* T4 DNA-cellulose column in an otherwise identical control experiment run simultaneously. The difference, 0.3% of the total soluble phage-induced protein, is reproducible and represents preferential binding to UV-irradiated DNA. In extracts made from cells infected with T4 *v*₁, a UV-sensitive bacteriophage mutant (Harm, 1963), this preferential binding appears to be eliminated (Fig. 14). We suspect therefore that proteins exhibiting preferential binding to the irradiated DNA are part of the bacteriophage repair system. Further studies are in progress which should clarify this point.

With respect to methodology, the experiment in Fig. 14 reveals two main factors limiting the resolution currently attainable with DNA vs. DNA-cellulose partition chromatography. The first of these is the low but finite binding (0.05 to 0.1%) of proteins to the cellulose matrix itself. This background can be reduced if the extract is first passed through a removable column of plain cellulose overlaying the DNA-cellulose column bed. In addition, further purification or pretreatment of the cellulose might be helpful. However, a second important factor is that the exchange of nonspecific DNA-binding proteins into the mobile phase was incomplete in Fig. 14, as is clear from the binding observed to the unirradiated T4 DNA-cellulose control. This could reflect selective binding of proteins to some unique structural feature of the DNA complexed to cellulose. Inasmuch as single-stranded T4 DNA was included in the DNA wash, the hypothetical structure which is recognized would have to be more complicated than exposed single-strands. It seems, therefore, more likely that the efficiency of exchange with the mobile phase can be carried to any extent desired, depending only on the ratio of DNA concentrations in the mobile and stationary phases and on the volume and flow rate of the DNA wash employed.

As an alternative method for isolating DNA-binding proteins having selective DNA affinities, two separate DNA-cellulose columns can be run in series. In this case, the initial column is designed to screen out DNA-binding proteins lacking specificity for the type of DNA used for the second column. This simple approach suffers from the fact that, unlike the partition method, it requires nearly absolute DNA-binding specificity on the part of the proteins to be isolated. The known difficulty of restricting RNA polymerase binding to biologically significant initiating sites on DNA (Pettijohn and Kamiya, 1967) suggests that it may be unreasonable to expect even regulatory

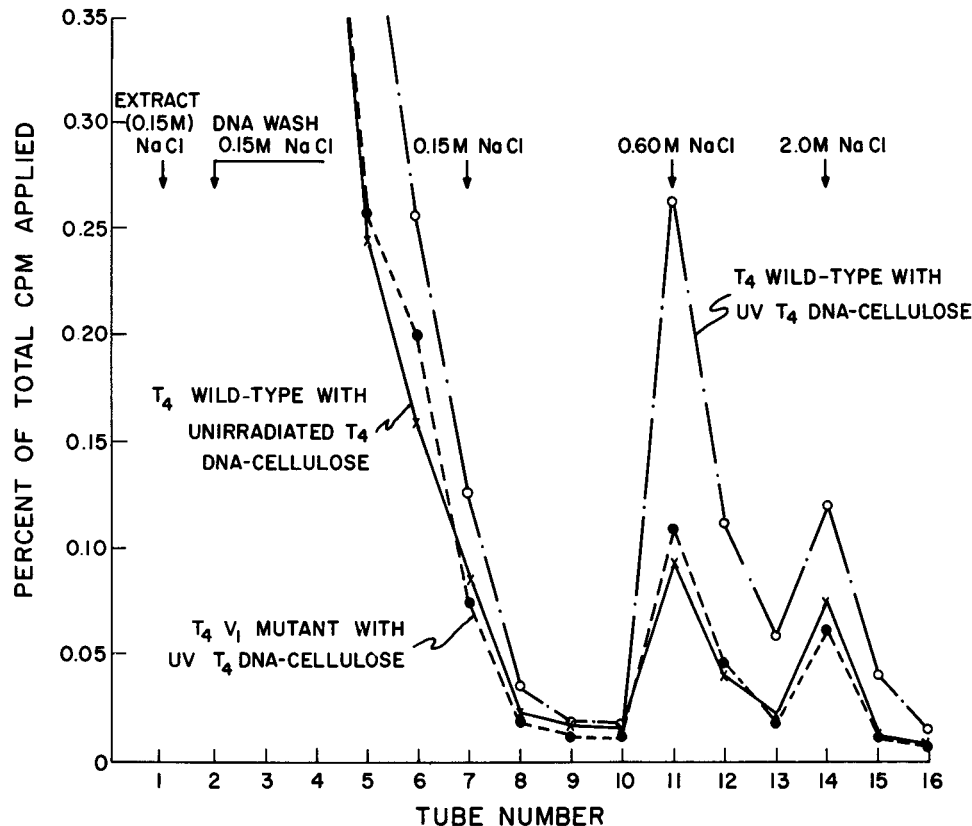


FIGURE 14. Specific binding of T4-induced proteins to UV-irradiated T4 DNA-cellulose. Native T4 DNA (500 ml of 50 $\mu\text{g}/\text{ml}$) was irradiated with constant mixing in a quartz cylinder of 8 cm diameter, surrounded by 3 germicidal lamps, type G15T8. These were placed a distance of 7 cm from the cylinder axis. The apparatus was calibrated by uridine bleaching (McLaren and Shugar, 1964); 2.5 quanta were absorbed per DNA nucleotide in the 1 min of irradiation used. Reference to the data of Wulff (1963) indicates that 5% of the thymine bases in this DNA were dimerized. After concentration by alcohol precipitation and dialysis, both the irradiated T4 DNA and an aliquot of the initial sample were adsorbed to cellulose under identical conditions. The three DNA-cellulose columns shown were run simultaneously, with the only variables being those indicated. Extracts from 5×10^{10} infected cells were made as previously described in Fig. 4, except that the DNase treatment was omitted and the dialyzed extract was clarified by centrifugation for 30 min at 38,000 rpm. One hr before loading the columns, the extract was diluted with 2 volumes of 'rinse buffer' containing 250 $\mu\text{g}/\text{ml}$ sonicated, native T4 DNA plus 250 $\mu\text{g}/\text{ml}$ of sonicated, heat-denatured T4 DNA. This same DNA-containing 'rinse buffer' was used for the DNA wash. Fractions of 0.5 ml were collected at 15 min intervals. All DNA-cellulose columns were 0.75 packed ml and contained 0.4 mg of DNA.

proteins to display such an *absolute* specificity of DNA-binding in vitro.

DISCUSSION

POTENTIAL OF THE METHOD

The DNA-cellulose procedure which has been outlined for purification of DNA and RNA polymerases from *E. coli* (Fig. 2 and 3) should serve as a simple and effective first step toward the complete purification of these, as well as other, DNA-binding enzymes. In contrast to more conventional purification schemes, this procedure should be directly applicable to isolation of the polymerases from *any* organism. Moreover, the comparative speed and mildness of the method make it less likely that sensitive enzymes will be altered from their original intracellular forms. A 1 ml DNA-

cellulose column is sufficient to adsorb the RNA polymerase from 2 g of *E. coli* cells, with no evidence of saturation (Fig. 3). Since these columns can be made in any size, the procedure can be scaled up to handle even very large quantities of protein, if desired. A different, although related, DNA-cellulose method has in fact been independently derived and used successfully for isolation of DNA polymerase on a preparative scale by Dr. R. Litman (pers. commun., 1968).

DNA-cellulose chromatography, when combined with polyacrylamide gel electrophoresis as in Fig. 5, provides a reproducible analytical 'fingerprint' of the DNA-binding proteins of an organism. This in turn enables identification of new proteins with DNA-associated functions, provided that altered fingerprints are obtained from appropriate defective mutants (Fig. 6).

LIMITATIONS OF THE METHOD

It is important to point out that, for the following reasons, fingerprints of DNA-binding proteins obtained by DNA-cellulose chromatography do not reveal *all* of the proteins which function on DNA within the cell:

(1) Evidence from several systems suggests that actively replicating DNA is intimately associated with bacterial membranes (Ganesan and Lederberg, 1965; Smith and Hanawalt, 1967; Knippers and Sinsheimer, 1968). It is therefore likely that some of the enzymes needed for DNA replication remain fixed to membrane components in a cell sonicate and are removed during centrifugation. A large fraction of the labeled protein in T4 bacteriophage-infected as well as uninfected *E. coli* is in fact particulate.

(2) Some DNA-associated proteins may be lost during DNA removal from crude extracts. In the case of the polyethylene glycol-dextran two-phase extraction (Fig. 3), proteins which either remain associated with DNA or are insoluble in the presence of 5 M NaCl will be discarded. When pancreatic DNase treatment is used (Fig. 2), DNA-associated proteins which require DNA for their solubilization will be lost. It has been reported that DNase treatment of a deoxyribonucleoprotein complex isolated from *E. coli* causes the bulk of the protein in this complex to precipitate (Kadoya et al., 1964).

(3) A very substantial DNA affinity is required in order for a DNA-associated protein to be recognized as such by our procedures. Any protein with an affinity constant for DNA (K) greater than some maximum value will be poorly retarded by the DNA-cellulose and be lost in the 0.05 M NaCl rinse (Fig. 4). The classical theory of partition chromatography (Martin and Synge, 1941) enables a simple relationship to be derived between R_F , the relative movement of protein and solvent through a DNA-cellulose column, and

$$K = \frac{(\text{free protein})(\text{free DNA-sites})}{(\text{DNA-bound protein})} \\ = K_{\text{eff}} (\text{free DNA sites}).$$

Note that an 'effective' equilibrium constant, K_{eff} , has been introduced, K_{eff} is equivalent to the partition coefficient for the protein in a 'theoretical plate' consisting of an imaginary DNA-containing stationary phase immobilized on the column and a

mobile solvent phase of equal volume ($K_{\text{eff}} = \alpha \frac{A_S}{A_L}$ in the notation of Martin and Synge). Following the published derivation, we obtain:

$$\frac{1}{R_F} = \frac{A_L}{A} (K_{\text{eff}} + 1)$$

where

$$\frac{A_L}{A} = \frac{\text{actual fraction of total column volume}}{\text{occupied by the aqueous phase}}$$

$$R_F = \frac{\text{movement in the column of the position of maximum protein concentration}}{\text{simultaneous movement of surface of developing fluid in empty part of tube above column.}}$$

From this result we calculate that the position of maximum protein concentration will just leave the bottom of the column when the number of column volumes which has followed the protein onto the top of the column is equal to $1 + K_{\text{eff}}$ [alternatively

written as $1 + \frac{K}{(\text{free DNA sites})}$]. Application of

this equation to the chromatogram in Fig. 4, assuming one DNA binding site per 10 base pairs, reveals that those proteins remaining on the column after the 0.05 M NaCl rinse must have a $K < 10^{-5}$ moles/l (binding energy > 7 Kcal/mole). Many proteins have an affinity only for selected regions of the DNA, and for them the estimate of one binding site per 10 base pairs is much too high. These proteins must of course bind even more tightly in order to be detected.

(4) Some important DNA-binding proteins may represent too small a fraction of the total cell protein to produce a band in the autoradiographs of Fig. 5. This is certainly the case with regulatory proteins such as the lactose repressor (Gilbert and Muller-Hill, 1966). Detection of these proteins in DNA-cellulose eluates demands either specific assays for them or special purification methods such as selective DNA-binding and/or elution from the DNA with the appropriate inducer.

In conclusion, despite the dramatic progress already made in understanding DNA function, it is clear that vast areas of ignorance remain. It is hoped that DNA-cellulose chromatography, by opening up a new approach to the biochemical analysis of genetic mechanisms, may help to shed some light on these fascinating and vital processes.

ACKNOWLEDGMENTS

This work was supported by United States Public Health Service research grant GM-14927 and in part by USPHS biomedical sciences support grant FR-07057. We gratefully acknowledge encouragement and advice received from Drs. A. Tissieres and R. H. Epstein in Geneva, which aided this investigation in its initial stages, as well as direct contributions to development of the methodology from Mr. Glenn Herrick and Mr. Larry

Owens at Princeton. Thanks are also due to Drs. R. S. Edgar and W. Harm for providing bacteriophage mutants, and to Drs. J. R. Fresco and K. Hoogsteen for suggestions regarding the manuscript.

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DISCUSSION

A. KOZINSKI: It was assumed that the activity of the gene 32 product was involved in DNA replication and recombination. We have shown that *am453* (defective in gene 32) does replicate, but only once. Thus the gene product in question cannot be responsible for the initiation of replication. We have also demonstrated that in the nonpermissive host the DNA from *am453* undergoes

extensive breakage. In contrast to other phages, these fragments most likely do not have exposed ends—they do not stick to nitrocellulose membranes, methylated albumin columns, and they do not recombine. It is therefore quite likely that the gene 32 product is an exonuclease.

B. ALBERTS: We have looked with a variety of DNA substrates for nuclease activity associated with the 32 protein, thus far without success. It appears that 32 protein would have to exhibit a much lower nucleolytic activity per molecule than either pancreatic DNase or lambda exonuclease, for example. Of course, it may be that its nuclease activity requires special cofactors or substrates not yet tried, or that it is unusually unstable. Your interesting observations, which demonstrate that a functioning 32 gene product leads to exposure of single-stranded regions in DNA fragments in vivo, might mean that these single-stranded regions are the final products of the normal recombination process. In this case, your results may be only indirectly related to the mode of action of the 32 protein itself.



Cold Spring Harbor Symposia on Quantitative Biology

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Cold Spring Harb Symp Quant Biol 1968 33: 289-305

Access the most recent version at doi:[10.1101/SQB.1968.033.01.033](https://doi.org/10.1101/SQB.1968.033.01.033)

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