Detailed procedures are presented which allow reproducible preparation of T4 gene 32 protein, a helix-stabilizing protein essential for DNA replication and genetic recombination in T4 bacteriophage-infected cells. Although 32 protein can be purified to better than 99% homogeneity by any one of several procedures, these methods have been developed to remove trace amounts of contaminating deoxyribonucleases, which are present in high levels in the original infected cells. Two alternative preparations are presented, each involving three chromatographic steps. Both 32 proteins obtained are essentially "nuclease-free," when tested at physiological salt concentrations. However, we show here that the phenyl-Sepharose chromatography step, which is necessary to remove an exonuclease activity active only at low salt concentrations, also removes a second protein present in trace amounts. In some cases, retention of this second protein is desirable, since it is essential for obtaining RNA primed, de novo DNA chain starts in an in vitro DNA replication system, when this system is contaminant-free, when tested at physiological salt concentrations. From this, and in other laboratories (6-8), it has also been used for marking regions of single-stranded DNA (and RNA) in cytological preparations viewed by electron microscopy (9-12). For most of these purposes, a concentration of 32 protein greater than 100 μg/ml is required, presumably because its cooperative binding to DNA single strands becomes energetically more favorable at such higher protein concentrations (13). At such 32 protein concentrations, trace contamination with one or more of the many deoxyribonucleases present in T4 bacteriophage-infected cells may become a major problem. In our experience, complete removal of all such nucleases has proven to be quite difficult, possibly because they associate with the gene 32 protein in some specific manner.

In this communication, we present purification methods which reproducibly yield "nuclease-free" preparations. One of these methods yields a 32 protein which also contains minor amounts of a second protein which is required for RNA primer synthesis in the T4 in vitro system. This preparation, called "32-PC," allows extensive in vitro DNA synthesis with the further addition of only five other T4 replication proteins: the products of T4 genes 43, 44, 62, 45, and 41 (6). The other preparation, called "32-PS," is preferred when 32 protein is used for any other purpose, or when a seventh T4-induced replication protein, which appears to be the T4 gene 61 protein, can be separately added to complete the replication system (6, 8).

MATERIALS AND METHODS

Chromatographic Matrices—Single-stranded DNA cellulose (0.8 mg of DNA/ml of packed cellulose) was prepared as previously described (14), using calf thymus DNA obtained from Worthington (highly polymerized grade). Norleucine-Sepharose was prepared as described elsewhere (15), and contained 13 to 17 μmol of bound norleucine/packed ml of Sepharose 2B. Phenyl-Sepharose, obtained from Pharmacia, had approximately 40 μmol of ligand bound/packed ml of Sepharose 4B. Both of these hydrophobic Sepharose columns were washed extensively with distilled water and then equilibrated with the appropriate starting buffer. Columns were reused after a final high salt wash (or low salt wash for the hydrophobic matrices), followed by re-equilibration with the starting buffer containing 0.02% NaN₃, in which they were stored at 4°C.

Enzymes—DNase I (bovine pancreatic) was obtained from Worthington (grade D). The purified products of T4 genes 41, 43, 44-62, and 45 were obtained by procedures described previously (15, 16) and in some cases were provided by Dr. C. F. Morris. Nuclease S1 was purified according to the procedure of Vogt (17). Bovine serum albumin was obtained from Sigma and, prior to its use in diluent and in enzyme assays, it was incubated at 100°C for 10 min in 62% glycerol to reduce contaminating nuclease activities to very low levels. Alternatively, a nuclease-free protein was prepared from Pen- tex bovine serum albumin (Miles) by incubating a 2 mg/ml solution in the presence of 12 mM N-ethylmaleimide for 2 h at 37°C. After the sulfhydryl reagent was inactivated by a 15-min further incubation with 140 mM β-mercaptoethanol and 10 mM Tris-HCl (pH 8.8), the albumin was extensively dialyzed for storage in 1 mM Na₂EDTA, 10 mM Tris-HCl (pH 8.1), 62% glycerol at 20°C.

Nucleic Acids—T7, fd, and PM-2 DNAs were isolated by phenol extraction, after the respective bacteriophages had been purified by CsCl density gradient centrifugation (10). The T7 DNA, as isolated, contained about one nick per every two strands. Short DNA fragments were prepared from 0.1 ml of a concentrated solution of DNA (0.1 to 0.3 mg/ml) in a buffer containing 1 mM NaCl, 0.1 mM Na₂EDTA, and 0.1 mM Tris-HCl (pH 8.1). The DNA solution was placed in 0.5-ml conical polyethylene tubes (Sarstedt) and repeatedly sonicated for 15-s intervals with the microtip of a Branson sonifier. Deoxy- and ribonucleoside triphosphates were obtained from Sigma. Methyl[H]thymidine triphosphate was obtained from New England Nuclear.

Protein Monitoring and Quantitation—Column elution profiles

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were monitored by absorbance at 280 nm with an Isco UA5 monitor equipped with a 0.35-mm path length flow cell obtained from LKB. Protein concentrations were determined by a modification of the procedure of Lowry et al. (19), using bovine serum albumin as a standard.

DNA Synthesis Assay—Protein fractions were assayed for their ability to stimulate DNA synthesis in a reaction mixture composed of assay buffer (67 mM potassium acetate, 33 mM Tris acetate (pH 7.8), 10 mM magnesium acetate, and 0.5 mM dithiothreitol), ribo- and deoxyribonucleoside triphosphates (0.5 mM each of dATP, dGTP, dUTP, and dTTP; 0.167 mM each of the four dNTPs; and 1 mM [3H]dTTP at a final specific activity of 100 to 200 cpm/pmol of dTTP) in the reaction NaCl, NaEDTA, and DNA template (0.25 mg/ml single-stranded, circular fd DNA or 12 µg/ml of double-stranded, linear T7 DNA). To reconstitute the minimum replication complex capable of catalyzing DNA synthesis on a double-stranded DNA template (6), a subset of the purified T4 bacteriophage replication proteins was used in the reaction mixture (100 µg/ml of 32 protein, 60 µg/ml of 43 protein, 20 µg/ml of 45 protein, and 20 µg/ml of a tight complex of 44 and 62 proteins). Where noted, the RNA-priming proteins, 61, or 41 protein, or both, were also added at 0.1 and 20 µg/ml, respectively. The assay mixture was incubated at 37°C and the reaction was terminated by trichloroacetic acid precipitation onto glass fiber filters. The radiolabeled, acid-insoluble DNA product was then collected and measured by standard liquid scintillation counting techniques.

Growth of Phage-infected Cells—Escherichia coli B8 was cultured at 37°C in a 100-liter New Brunswick fermentor in M9 minimal medium (20), supplemented with 0.3% casein hydrolysate (Difco). At a cell density of 7 × 10³/ml, T4 bacteriophage (amN81, gene 41) were added at a multiplicity of 5 to 10, and 40 min after infection the cells were rapidly chilled and harvested by centrifugation. The cell paste (210 g) was placed on disposable plastic weighing trays, wrapped in plastic bags, quick-frozen by immersion in liquid nitrogen, and stored at −80°C. These frozen cells have been used for protein preparations over a 2-year time period with no apparent change in protein yields or properties. Alternatively, the T4 bacteriophage triple mutant amN134, amBL392, amE219 (genes 33-, 55-, 58-61), obtained by single-step infection with a triple amber mutant (in T4 genes 33, 55, and 58), was used for infection under similar conditions. Consistent with the results reported with this mutant (21), we found about a 4-fold increase in the yield of 32 protein compared to an amN81 infection (e.g. see Table I).

Buffers used in Purification—Note that all per cent compositions are w/v and that the pH values are those measured at 20°C. L buffer: 0.1 M NaCl, 40 mM Tris-HCl (pH 8.1), 10 mM MgCl₂, 2 mM CaCl₂, 1 mM NaEDTA, 1 mM β-mercaptoethanol, and 20 µg/ml of DNase I. DC buffer: 0.1 M NaCl, 20 mM Tris-HCl (pH 8.1), 5 mM NaEDTA, 1 mM β-mercaptoethanol. DC buffer: 0.1 M NaCl, 20 mM Tris-HCl (pH 8.1), 1 mM NaEDTA, 1 mM β-mercaptoethanol, and 10% glycerol. DC buffer: same as DC, except 0.5% NaCl. DC buffer: same as DC, except 0.1 M NaCl. DC buffer: same as DC, except 0.1% NaCl. DC buffer: same as DC, except 0.01 M NaCl. DC buffer: same as DC, except 0.001 M NaCl. Buffer: 0.5 M ammonium phosphate (0.18 M K₂HPO₄, 0.013 M KH₂PO₄, pH 8.1), 0.5 M NaCl, 1 mM NaEDTA, 1 mM β-mercaptoethanol, and 10% glycerol. NS buffer: same as NS, except 0.5 M potassium phosphate. NS buffer: same as NS, except 1.0 M potassium phosphate. NS buffer: same as NS, except 2.0 M potassium phosphate. PC buffer: 0.1 M NaCl, 20 mM Tris-HCl (pH 8.1), 1 mM NaEDTA, 1 mM β-mercaptoethanol, and 10% glycerol. PC buffer: 0.1 M NaCl, 30 mM Tris-HCl (pH 8.1), 1 mM β-mercaptoethanol, and 10% glycerol. PC buffer: same as PC, plus 14% (NH₄)₂SO₄, and 10 mM MgCl₂. PC buffer: same as PS, plus 28% (NH₄)₂SO₄, and 20 mM MgCl₂. PS buffer: same as PS, plus 10 mM MgCl₂. S buffer: 0.1 M NaCl, 20 mM Tris-HCl (pH 8.1), 1 mM NaEDTA, 1.5 mM dithiothreitol, and 10% glycerol. S buffer: same as S, except 62% glycerol.

Preparation of the Cell Extract—A 60-g portion of frozen cells was added to 180 ml of a freshly prepared lysis buffer containing pancreatic DNase I (“L buffer,” see above). A homogeneous suspension of thawed cells was obtained by very brief mixing of the frozen pellet in a Waring blender at low speed. During this treatment the temperature was maintained by placing the mixture in a beaker containing an ice-water bath. The mixture was then quickly centrifuged at low speed (3 min at 5,000 rpm in a Sorval GSA rotor) to eliminate air entrapped by the blending process. The partially lysed cell suspension obtained was sonicated with three bursts from the large probe of a Branson Sonifier at the maximum power setting. Care was taken during this procedure to prevent the temperature of the lysate from rising above 15°C. Lysis was monitored by following the decrease in light scattering measured at 590 nm. Sonication was terminated when a 50-fold dilution of the suspension in lysis buffer showed that its turbidity had decreased to approximately 15% of the initial value. At this point, greater than 95% of the cells are disrupted, as determined by direct counts of intact cells with a Petroff-Hauser counter. DNase digestion was continued by incubation of the cell lysate at 15°C for 30 min. All subsequent operations were performed at 4°C. The extract was centrifuged at low speed (12,000 rpm in a Sorval GSA rotor) for 30 min following by a high speed centrifugation of the supernatant in a Beckman type 30 rotor for 2 h at 30,000 rpm. The clear, yellow light supernatant was carefully removed to constitute the cleared lysate (Fraction I).

In order to block the partial chymotryptic deavage of 32 protein, which otherwise yields trAce components of proteolytic fragments (22), a freshly dissolved DNase I solution is used (to reduce activation by contaminating chymotrypsin) and a protease inhibitor 1 mM TPCK,¹ can be added to the lysis buffer. For the same reason, the protein preparation should be carried through to the norleucine-Sepharose step (Fraction III, below) as quickly as possible.

RESULTS

Purification of Gene 32 Protein—Gene 32 protein has been purified from the cleared lysate obtained from 60 g of T4 bacteriophage-infected E. coli cell paste. Two alternative purification schemes are presented here, which diverge only at the last chromatographic step. The common steps are single-stranded DNA cellulose chromatography followed by hydrophobic chromatography on norleucine-Sepharose. These procedures are followed by either a phosphocellulose column (to yield 32-PC) or a phenyl-Sepharose column (to yield 32-PS), as the final purification step. Both of the 32 protein preparations obtained are greater than 99% homogeneous and are essentially indistinguishable as judged by SDS-polyacrylamide gel electrophoresis. However, the two preparations differ greatly in their ability to stimulate DNA replication in a reconstituted T4 in vitro replication system, when a circular single-stranded DNA is used as template (for explanations, see below).

The results from the two different purification schemes are summarized in Table I. In Preparation A, yielding 32-PC, a total of 41 mg of 32 protein was obtained after three chromatographic steps. In Preparation B, yielding 32-PS, a total of 106 mg of 32 protein was obtained. The increased yield of 32 protein isolated in Preparation B is due to the use of cells infected with a triple amber mutant (in T4 genes 33, 55, and 61) which overproduces 32 protein as the starting material, rather than to any particular aspect of the Preparation B procedure (data not shown).

The details of these two procedures follow, along with characterizations of the final proteins obtained. Since the 32 protein constitutes such a large portion of the total protein in the T4-infected cell, the initial DNA affinity column step yields such a pure protein that subsequent purification can be monitored by simply following the total protein profile. Assays are then performed across the major peak of protein obtained, both to test for the removal of unwanted impurities (such as nucleases), and to ascertain that the full activity of the 32 protein in the T4 in vitro DNA replication system has been retained.

In this laboratory, we have also used, on occasion, a variety of other chromatography steps for the preparation of 32 protein not described below or listed in Table I. For completeness, the elution behavior of 32 protein when chromatographed on several of these additional columns is briefly summarized in Table II.

DNA-Cellulose Chromatography—Fraction I, prepared as described under “Materials and Methods,” was dialyzed at 4°C against three 2-liter changes of DC buffer with rapid mixing and at least 6 h between buffer changes. (It is essential

¹ The abbreviations used are: TPCK, toluene-3-sulfonyl l-phenylalanine chloromethyl ketone; SDS, sodium dodecyl sulfate; ss DNA, single-stranded DNA; ds DNA, double-stranded DNA.
that this dialysis reduce divalent cation concentrations sufficiently to prevent DNase I digestion of the DNA-cellulose column matrix). The dialysate was centrifuged in a Sorval GSA rotor for 5 min at 10,000 rpm to remove a light precipitate which formed. Prechilled glycerol was then added to the supernatant at a ratio of 1 g of glycerol/10 ml of extract, to yield Fraction Ia.

Fraction Ia was applied to a single-stranded DNA cellulose column (packed volume 63 ml) which had been equilibrated with DC buffer. The column was eluted batchwise in three 90-ml steps comprised of DC, DC, then DC buffers, which contain NaCl at final concentrations of 0.1 M, 0.6 M, and 2.0 M, respectively. All flow rates were 40 ml/h. The particular protein elution profile obtained in Preparation A for the two highest salt steps is shown in Fig. 1A. Fractions 30 to 33 (18 ml), eluting at 2.0 M NaCl with an absorbance at 280 nm greater than 1, were pooled to form Fraction II.

Hydrophobic Chromatography—A norleucine-Sepharose column (packed volume of 10 ml) was equilibrated with Buffer NS, which contains 1 M potassium phosphate. Fraction II was diluted with an equal volume of NS buffer and loaded at 5 ml/h onto the norleucine-Sepharose column. The column was washed with 15 ml of NS buffer and then step-eluted at 5 ml/h by reducing the phosphate concentration to 0.5 M with 20 ml of NS buffer. As a second elution step, 20 ml of NS buffer was passed through the column. The protein elution profile obtained from this column in Preparation A is shown in Fig. 1B. Essentially no UV-absorbing material is detected in the final lowest salt elution (NS buffer). The protein-containing fractions eluting with 0.5 M potassium phosphate (NS buffer) were pooled to comprise Fraction III (16 ml).

Advantages and Disadvantages of 32-PC and 32-PS Preparations—As noted above, after the norleucine-Sepharose step, our purification scheme for 32 protein branches, with Fraction III being further purified by one of two separate procedures. Preparation A utilizes phosphocellulose chromatography to yield a 32 protein preparation designated as 32-PC. This protein fraction is >99% pure as judged by SDS-polyacrylamide gel electrophoresis, but it still contains an exonucleolytic activity which is detectable only at very low salt concentrations. As this nuclease is inactive at the salt concentrations routinely used for DNA polymerization reactions (see below), 32-PC has been used as the source of 32 protein in much of the previously published work from this laboratory (7, 23, 24). The second purification scheme utilizes phenyl-Sepharose chromatography to yield a protein fraction designated as 32-PS. 32-PS has no detectable exonucleolytic activities (see below). However, in addition to the removal of exonuclease activity, a seventh T4-coded protein which is required in trace amounts for RNA primer synthesis is removed from 32 protein by the phenyl-Sepharose chromatography step (but not by the phosphocellulose chromatography). This protein recently has been isolated separately and partially purified; and it has been tentatively identified as the T4 gene 61 product (6, 8). When this “gene 61 protein” is not available separately, the 32-PC preparation must be used for complete reconstitution of the T4 in vitro DNA replication system (see below).

Preparation of 32-PC—Fraction III (16 ml) was dialyzed against PC buffer to yield Fraction IIIa. A column of Whatman P-11 phosphocellulose (5 ml packed volume) was thoroughly equilibrated by rinsing it overnight with PC buffer. Fraction IIIa was applied to the column at a flow rate of 10 ml/h, and 4-ml fractions were collected. The column was then washed with 20 ml of additional PC buffer. The 32 protein does not bind to the phosphocellulose matrix under these conditions, and the breakthrough fractions with an absorbance at 280 nm greater than 1 were pooled (18 ml). These combined fractions were quickly dialyzed against Buffer S, followed by dialysis against the same buffer containing 62% glycerol (Buffer S). The concentrated protein solution obtained (Fraction IV PC)
**T4 Gene 32 Protein**

**Nuclease Assays**—The presence of both exo- and endodeoxyribonuclease contaminants in the 32 protein fractions has been monitored carefully throughout these purification schemes. The results of exonuclease assays are presented in Table III, and the results of sensitive endonuclease assays are presented in Table IV. Note that, when such assays are designed to detect nuclease activities on single-stranded DNA, it is important to test 32 protein at saturating, rather than only at saturating, protein to DNA ratios. Otherwise, an activity which hydrolyzes only free (non-32 protein-complexed) single-stranded DNA will escape detection.

### Table III

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Nuclease Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>Low activity</td>
</tr>
<tr>
<td>III</td>
<td>High activity</td>
</tr>
</tbody>
</table>

**Fig. 1.** Protein elution profile from the single-stranded DNA cellulose column used to purify 32 protein. The 2.0 M NaCl eluate constitutes Fraction II. Chromatography was carried out as described in the text, with a flow rate of 40 ml/h. Four-milliliter fractions were collected. B, Norleucine-Sepharose chromatography of Fraction II. Chromatography was carried out as described in the text, with a 5 ml/h flow rate. Each fraction contains 1.5 ml.

**Fig. 2.** Analysis by SDS-polyacrylamide gel electrophoresis of the 32 protein-containing fractions. A, Lanes A through C contain 10 μg each of Fraction II, III, IV-PC, respectively (all from Preparation A). Lanes D through F display the same fractions, but contain 10 pg each of Fraction II, III, IV-PC, respectively (all from Preparation A). Lanes G through I display the same fractions, but contain 10 pg each of Fraction II, III, IV-PC, respectively (all from Preparation A). Lanes J through L display the same fractions, but contain 10 pg each of Fraction II, III, IV-PC, respectively (all from Preparation A).

32 protein peak, it is usually best not to pool too many fractions together.

**Protein Purity**—All of our 32 protein preparations have been analyzed at each step in the purification by polycrylamide gel electrophoresis in the presence of SDS. Typical results are shown in Fig. 2, where both 10 μg and 100 μg of total protein from each fraction have been analyzed, in order to reveal even very minor contaminants. These results show that the initial DNA cellulose chromatography step provides the major purification, with the additional steps mainly serving to remove relatively minor protein bands. Nevertheless, these additional steps are essential for our purposes, since they remove nuclease which can interfere with the utilization of 32 protein in many cases (see below).

A detailed SDS-polyacrylamide gel analysis of a side fraction obtained from the norleucine-Sepharose column is shown in Fig. 2A, lane G. In this low salt (0.02 M potassium phosphate) wash, there is a complex mixture of contaminants, which together comprise less than 1% of the total protein applied to the column. The majority of these protein bands are initially present at such low concentrations relative to 32 protein that they can be detected on gels only after they have been purified away from the bulk of the gene 32 protein. Clearly, a myriad of minor protein species are separated from the 32 protein fraction by this hydrophobic chromatography step.

**Fig. 2A** Slow DNA migration. DNA is stored at -20°C. If 32-PC is maintained in this way at -20°C at a high protein concentration (>2 mg/ml), its activity (assayed as the stimulation of extensive in vitro DNA synthesis without the separate addition of gene 61 protein) is stable for months. This implies that both the 32 protein, and the relatively labile “contaminant” of active 61 protein in the 32-PC preparation, are stabilized under these conditions.

**Preparation of 32-PS**—A 10-ml column of phenyl-Sepharose was equilibrated with PS, buffer, which contains 14% (NH₄)₂SO₄. Fraction III was dialyzed against two changes of PS, buffer. The dialyze was then diluted with an equal volume of PS, buffer, which contains twice the concentration of MgCl₂ and (NH₄)₂SO₄ as PS, so that the final sample was essentially in PS, buffer. The sample was loaded onto the column at a flow rate of 14 ml/h. The column was washed with 15 ml of PS, buffer and then eluted with a 100-ml reverse linear gradient spanning from 14 to 0% (NH₄)₂SO₄ (PS₃ to PS₅) buffer. Finally, the column was washed with 15 ml of PS₃ buffer to complete the 32 protein elution. Fractions 66 to 69 (3 ml each), which had an absorbance at 280 nm >1.0 and contained about 1% (NH₄)₂SO₄, were concentrated by dialysis versus solid sucrose. These fractions were then dialyzed against S, buffer containing 10% glycerol, followed by S₂ buffer containing 62% glycerol for storage as above. As a trace of nuclease can sometimes be detected in the trailing edge of the
**Table III**

**Assay for single strand and double strand specific endodeoxyribonuclease activities in the 32 protein fractions**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Step</th>
<th>ss DNA solubilized</th>
<th>ds DNA solubilized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/ml/10 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>ss DNA cellulose</td>
<td>16.9</td>
<td>2.2</td>
</tr>
<tr>
<td>III</td>
<td>Norleucine-Sepharose</td>
<td>2.9</td>
<td>0.5</td>
</tr>
<tr>
<td>IV-PC</td>
<td>Phosphocellulose</td>
<td>&lt;1</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td>No protein</td>
<td></td>
<td>&lt;1</td>
<td>&lt;0.4</td>
</tr>
</tbody>
</table>

**Table IV**

**Assay for single strand and double strand specific DNA endodeoxyribonuclease activity in the 32 protein fractions**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Concentration µg PM-2 DNA nicked/ml</th>
<th>Concentration µg fd DNA nicked/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction II</td>
<td>20</td>
<td>153</td>
</tr>
<tr>
<td>Fraction III</td>
<td>20</td>
<td>142</td>
</tr>
<tr>
<td>Fraction IV-PC</td>
<td>20</td>
<td>&lt;40</td>
</tr>
<tr>
<td>Fraction IV-PS</td>
<td>200</td>
<td>42</td>
</tr>
<tr>
<td>Fraction IV-PS</td>
<td>200</td>
<td>&lt;28</td>
</tr>
</tbody>
</table>

As indicated by the results shown in Tables III and IV, the major portion of contaminating single and double strand specific endodeoxyribonuclease activities are removed from the 32 protein preparation by the norleucine-Sepharose chromatography step, which yields Fraction III (Table IIIA). Phosphocellulose chromatography (to yield Fraction IV-PC) removes all of the residual activity which can be detected in the buffer used for our DNA synthesis assays, which has an ionic strength in the physiological range. However, a single-stranded exonuclease activity remains associated with 32-PC, which is detected when the concentration of potassium acetate in the nuclease assay mixture is reduced to 25 mM. This exonuclease activity is seen to be removed from Fraction III by the phenyl-Sepharose step, which yields 32-PS (Table IIIB). Endodeoxyribonuclease contaminants in each fraction of 32 protein are assayed by incubating 32 protein either with covalently closed, circular PM-2 DNA, or with circular, single-stranded fd DNA. The scission of a single phosphodiester bond in either one of these molecules produces a major conformational change, allowing nicked and closed circular species to be readily separable by agarose gel electrophoresis (for example, see Fig. 4 of Ref. 15). In reconstruction experiments, this technique is able to detect a pancreatic DNase I contamination of only $10^{-11}$ g/ml (3.2 x $10^{-5}$ Kunitz units/ml). The results obtained when the various 32 protein fractions are tested in this way are presented in Table IV. The bulk of contaminating endodeoxyribonuclease activities active on both single- and double-stranded DNAs are seen to be removed from 32 protein by either the phosphocellulose chromatography or phenyl-Sepharose chromatography steps (to yield Fractions IV-PC and IV-PS in Table IV). As a result, even when tested at a concentration of 200 µg/ml, and incubated with DNA for 30 min at 37°C under DNA synthesis conditions, both 32-PC and 32-PS cause less than one nick per 60,000 DNA nucleotides (see also Ref. 15).

Quantitation of the 32 Protein Activity Recovered—Although in vivo studies demonstrate that the protein encoded by gene 32 is essential for DNA replication (3, 25), a simple in vitro assay of 32 protein-dependent DNA synthesis has been technically difficult to construct. Complementation assays of protein-dependent DNA synthesis, such as the mutant-infected cell lysate system devised by Barry and Alberts (26), show no reproducible gene 32 protein-dependent DNA synthesis.

In contrast, the products of T4 genes 41, 43, 44, 62, and 45 can be readily isolated on the basis of their ability to complement a defective mutant-infected cell lysate (7, 15, 16, 27). It is possible to use these highly purified proteins (with added DNA template and ribo- and deoxyribonucleoside triphosphate substrates) to construct a DNA synthesis assay which is completely dependent on the addition of 32 protein. However, stimulation or inhibition of the DNA synthesis reaction by contaminating nucleases, DNA ligases, or other proteins which modify DNA substrates or products can in principle distort the measurement of the activity of 32 protein in this assay. Nevertheless, when each fraction of 32 protein obtained during the purification was tested for activity in this way, the

No protein

<table>
<thead>
<tr>
<th>Protein</th>
<th>Concentration µg PM-2 DNA nicked/ml</th>
<th>Concentration µg fd DNA nicked/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction II</td>
<td>20</td>
<td>153</td>
</tr>
<tr>
<td>Fraction III</td>
<td>20</td>
<td>142</td>
</tr>
<tr>
<td>Fraction IV-PC</td>
<td>20</td>
<td>&lt;40</td>
</tr>
<tr>
<td>Fraction IV-PS</td>
<td>200</td>
<td>42</td>
</tr>
<tr>
<td>Fraction IV-PS</td>
<td>200</td>
<td>&lt;28</td>
</tr>
<tr>
<td>DNase I</td>
<td>$1 \times 10^{-4}$</td>
<td>&gt;4,000</td>
</tr>
<tr>
<td>No protein</td>
<td>&lt;28</td>
<td>&lt;56</td>
</tr>
</tbody>
</table>
which have been displaced by new DNA synthesis (see Ref. 6). As expected for a reaction requiring de novo chain starts, such as that in Panel B, its addition to the reaction in Panel A stimulates total synthesis only 1.5- to 2-fold (6).

Specific activity of the 32 protein appeared to be roughly unchanged from Fraction II through Fraction IV (28). We therefore believe that both 32-PS and 32-PC proteins have been isolated in a highly active form.

Behavior of 32 Protein Preparations in DNA Synthesis Reactions: T7 DNA Template—To obtain any substantial in vitro DNA synthesis at physiological salt concentrations on a nicked, double-stranded DNA template such as T4 or T7 DNA, five purified T4 proteins are essential (the gene 32, 44/62, 45, and 43 proteins; see Ref. 6). As expected for a reaction which starts at a nick, the synthesis observed is enhanced by prior DNase I treatment, and is inhibited by prior ligase treatment of the template DNA. Moreover, all of the newly replicated DNA is template linked, as shown by co-migration of incorporated label with template DNA on alkaline agarose gel electrophoresis. As shown in Figs. 3A and 4A, high concentrations of 32 protein are required in this reaction. Both of the 32 protein preparations described here, 32-PC and 32-PS, are equally effective in stimulating DNA synthesis on such templates. The major products, when viewed in the electron microscope, are linear double-stranded molecules with several long single-stranded branches, the latter presumably representing strands of the original double helical template DNA which have been displaced by new DNA synthesis (see Ref. 6).

Fdg DNA Template—In contrast to their similar behavior in the reaction on a nicked, duplex template (Fig. 3A), the two 32 protein preparations are very different in their ability to stimulate DNA synthesis on a single-stranded, circular template such as fd DNA. Here, unlike the case just discussed, an RNA-primed, de novo initiation of replication is essential to obtain synthesis. As shown in Fig. 3B, 32-PC efficiently stimulates synthesis on fd DNA. But upon the substitution of 32-PS for 32-PC, essentially no synthesis is obtained. Subsequent studies, detailed elsewhere, have been carried out to determine the reason for this striking difference in the 32-PC and 32-PS preparations. These studies have shown that, in addition to the removal of residual exonuclease activity from the 32 protein preparation, the phenyl-Sepharose chromatography step removed an additional required protein from the 32 protein fraction. This protein, tentatively identified as the product of the DNA-delay gene 61, has an essential role in RNA primer synthesis (6). The 32-PS protein regains the capacity to stimulate replication in the fd assay when partially purified 61 protein is added to the reaction, or when suboptimal concentrations of 32-PC are present to provide this protein (6). Therefore, we feel that both 32-PC and 32-PS preparations are fully active in supplying the actual 32 protein requirements.

Optimal 32 Protein Concentration for in Vitro DNA Replication—High concentrations of 32 protein (100 to 200 µg/ml) are required to support extensive in vitro DNA replication on either double-stranded or single-stranded DNA templates. The amount of DNA synthesis observed on a nicked duplex T7 DNA template, as a function of the 32 protein concentration, is shown in Fig. 4A. Below a concentration of 25 to 50 µg/ml of 32 protein, very little DNA synthesis is observed. Near-maximal stimulation of synthesis is supported by a broad range of intermediate 32 protein concentrations from 100 to 400 µg/ml, while very high 32 protein concentrations (up to 800 µg/ml) only slightly depress the maximum incorporation observed.

A corresponding titration to determine the optimal 32 protein concentration for extensive rolling circle DNA replication in a reaction on a single-stranded, circular fd DNA template (6, 7) is shown in Fig. 4B. Once again, a threshold requirement for 25 to 50 µg/ml of 32 protein is observed. But in this case, the optimum 32 protein concentration increases with the time...


**Table V**

<table>
<thead>
<tr>
<th>Time of DNA Synthesis</th>
<th>pmol dTMP incorporated/5 μl</th>
<th>Per cent double-stranded DNA (by S1 resistance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.4</td>
<td>96</td>
</tr>
<tr>
<td>5</td>
<td>24.6</td>
<td>83</td>
</tr>
<tr>
<td>10</td>
<td>281.5</td>
<td>82</td>
</tr>
<tr>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ds T7 DNA control</td>
<td>104</td>
<td></td>
</tr>
<tr>
<td>as T7 DNA control</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

The threshold concentration of 32 protein which is required for significant DNA synthesis in Fig. 4 appears to indicate a minimum concentration of free 32 protein which is needed for helix destabilization. Consistent with such a view, we find that the threshold 32 protein level is relatively insensitive to changes in the initial DNA template concentration (data not shown).

As shown in Table V, the primary product of the complete in vitro reaction is double-stranded DNA, as determined by its resistance to S1 nuclease treatment. However, even at 20% of the total concentration of 32 protein whose concentration is 0.1 μg/ml, the total concentration of single-stranded DNA could have had reached 30 μg/ml after long reaction times in the experiment shown in Fig. 4B. As single-stranded DNA can bind at least 12 times its own weight of 32 protein (1), a large amount of the 32 protein present could have become sequestered in a tight DNA protein complex. Consequently, the increase of the optimum 32 protein concentration seen at extended reaction times in Fig. 4B is most likely due to the generation of so much DNA product that it markedly decreases the concentration of free (non-DNA-bound) 32 protein.

**DISCUSSION**

The purification procedures presented in this communication are unusual in that their primary purpose is the removal of trace contaminants from an already "nearly pure" protein. These protocols were developed after the utilization of numerous alternative chromatographic steps were unsuccessful for complete nuclease removal from 32 protein. Many of these other purification steps nevertheless destroyed the ability of the 32 protein to stimulate extensive DNA synthesis in the T4 in vitro system containing five other DNA replication proteins (those corresponding to T4 genes 43, 44/62, 45, and 41). In retrospect, it is now clear that these "inactivations" were actually due to the removal of 32 protein of a seventh required T4 replication protein, recently identified and purified in this laboratory, which probably corresponds to the product of T4 gene 61 (6, 8).

Preparation A, producing 32-PC, has the advantage of removing from 32 protein all detectable nucleases active in the physiological salt range where the T4 in vitro system operates, while at the same time conserving sufficient amounts of the 32 protein to allow efficient RNA-primed DNA replication without its separate addition. Preparation B, yielding 32-PS, is preferred whenever the presence of this second activity in the 32 protein preparation is not required. In 32 PS, we are unable to detect any deoxyribonuclease activity (using concentrations of 32 protein up to 200 μg/ml), even at low salt concentrations (Tables III and IV). Similar sensitive assays have failed to detect either ATPase, ribonuclease H, or ribonuclease activities in the 32 PS preparation (data not shown). It might be noted that, although most of the alternative column steps in Table II were tried, phenyl-Sepharose was the only one capable of separating the residual exounucleases in 32-PC from the 32 protein.

There are several points to be made concerning the results obtained during the course of these attempted purifications. The first is that there appear to be numerous activities and minor protein bands which adhere rather specifically to the 32 protein, thereby making them quite difficult to remove from it. Many of these minor protein bands may, in fact, complex with 32 protein in vivo. Single-stranded DNA probably does not exist as such inside of cells, being present instead as a tight complex with helix-stabilizing protein(s). Any enzyme which operates on single-stranded DNA in the T4-infected cell, presumably recognizes such DNA as a 32 protein-DNA complex. The extensive genetic studies carried out by Mosis et al. (29) have suggested that specific regions of the 32 protein interact in vivo with T4 DNA polymerase, DNA ligase, ribonuclease H, and recombination nucleases. If many of the interactions between 32 protein and other proteins involve hydrophobic interactions, we can rationalize the fact that the most useful columns for our purifications have been the hydrophobic matrices, norleucine-Sepharose and phenyl-Sepharose. As a separate study, it would be of interest to analyze those proteins which associate with 32 protein in detail. Preliminary experiments, utilizing affinity columns to which 32 protein is linked, have been promising in this regard.

Analogously, Molineux and Getter (30, 31) have described a variety of interactions of the E. coli helix-stabilizing protein with E. coli exonucleases and DNA polymerases (see Ref. 32).

The second major point to be made in connection with the above discussion is that it is very difficult to know when one has removed all of the trace contaminants in 32 protein which could have an important biochemical function in the particular assay being used. While 32 protein is required normally at levels of 100 to 400 μg/ml (see Fig. 4), minor contaminants might be active in an enzymatic role at concentrations of 0.1 μg/ml or less. Even given sensitive analytical techniques such as SDS-polyacrylamide gel electrophoresis (see Fig. 2), one can rarely assure the purity of any protein to greater than 99.9%, as would be necessary to rule out contaminants at this level.

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B. Alberts, unpublished experiments.
ment of Biochemistry, University of Washington Medical School, St.
Louis, some of the experiments by M. B. were carried out.

REFERENCES

3. Epstein, R. H., Bolle, A., Steinberg, C. M., Kellenberger, E., Boy
de la Tour, E., Chevalley, R., Edgar, R. S., Susman, M., Den-
Symp. Quant. Biol. 28, 375-384
247-253
175
Acad. Sci. U. S. A. 72, 4800-4804
Quant. Biol. 43, 489-494
341-350
693-702
72, 4506-4510
166, 141-145
13. Von Hippel, P. H., Jensen, D. E., Kelly, R. C., and McGhee, J. D.
(1977) in Nucleic Acid-Protein Recognition (Vogel, H. J., ed)
217
15. Morris, C. F., Hama-Inaba, H., Mace, D., Sinha, N. K., and
254, 6797-6802
18. Yamauro, K. R., Alberts, B. M., Denhardt, R., Lawlimore, L.,
(1951) J. Biol. Chem. 193, 265-275
York
251, 7251-7262
23. Alberts, B., Morris, C. F., Mace, D., Sinha, N. K., Bittner, M., and
Moran, L. (1975) in DNA Synthesis and Its Regulation, (Gou-
lain, M., Hanawalt, P., and Fox, C. F., eds) Vol. 3, pp. 241-269,
W. H. Benjamin, Menlo Park
Liu, C.-C., Mace, D., Moran, L., Morris, C. F., Piperno, J., and
Sinha, N. K. (1977) in Nucleic Acid-Protein Recognition (Vo-
A. 69, 2717-2721
N. J.
S. A. 71, 3858-3869
Purification of the T4 gene 32 protein free from detectable deoxyribonuclease activities.

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