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Purification and Characterization of the Bacteriophage T7 Gene 2.5 Protein

A SINGLE-STRANDED DNA-BINDING PROTEIN*

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Bacteriophage T7 gene 2.5 protein has been purified to homogeneity from cells overexpressing its gene. Native gene 2.5 protein consists of a dimer of two identical subunits of molecular weight 25,562. Gene 2.5 protein binds specifically to single-stranded DNA with a stoichiometry of ~7 nucleotides bound per monomer of gene 2.5 protein; binding appears to be noncooperative. Electron microscopic analysis shows that gene 2.5 protein is able to disrupt the secondary structure of single-stranded DNA. The single-stranded DNA is extended into a chain of gene 2.5 protein dimers bound along the DNA. In fluorescence quenching and nitrocellulose filter binding assays, the binding constants of gene 2.5 protein to single-stranded DNA are 1.2×10^6 M^{-1} and 3.8×10^6 M^{-1}, respectively. *Escherichia coli* single-stranded DNA-binding protein and phage T4 gene 32 protein bind to single-stranded DNA more tightly by a factor of 25. Fluorescence spectroscopy suggests that tyrosine residue(s), but not tryptophan residues, on gene 2.5 protein interacts with single-stranded DNA.

Bacteriophage T7 encodes most of its own replication proteins including an RNA polymerase (gene 1), DNA polymerase (gene 5), helicase and primase (gene 4), DNA ligase (gene 1.3), endonuclease (gene 3), and exonuclease (gene 6) (for review, see Richardson, 1983). The product of gene 2.5 has been implicated in T7 DNA replication, recombination, and repair (Reuben and Gefter, 1973; Scherzinger *et al.*, 1973; Araki and Ogawa, 1981a, 1981b; Nakai and Richardson, 1988). It was originally purified based upon its strong, specific affinity for single-stranded DNA and its ability to stimulate DNA synthesis by T7 DNA polymerase[1] (Scherzinger *et al.*, 1973; Reuben and Gefter, 1974). Gene 2.5 protein has been thought to be analogous in function to the *Escherichia coli* single-stranded DNA-binding (SSB)[2] protein and the phage T4 gene 32 protein; both of these proteins have a strong, specific affinity for single-stranded DNA and can stimulate DNA synthesis. In addition, like T7 gene 2.5 protein (Reuben and Gefter, 1973; Scherzinger *et al.*, 1973; Araki and Ogawa, 1981a, 1981b; Nakai and Richardson, 1988), both *E. coli* SSB protein and the T4 gene 32 protein have been shown by genetic and biochemical studies to be required for DNA replication, recombination, and repair (see Chase and Williams, 1986). *E. coli* SSB protein can substitute for gene 2.5 protein functionally; T7 phage have been isolated which contain mutations in gene 2.5 based upon their inability to grow on *E. coli* mutants that have a defective SSB protein (Araki and Ogawa, 1981a).

Single-stranded DNA-binding proteins that stimulate DNA polymerases are thought to act nonenzymatically, coating the DNA and removing secondary structures. Other examples of such proteins, in addition to T7 gene 2.5 protein, T4 gene 32 protein, and *E. coli* SSB protein, are the phage N4 SSB protein (Lindberg *et al.*, 1989), phage ϕ29 gene 5 protein (Gutierrez *et al.*, 1991), yeast stimulatory protein (Brown *et al.*, 1990), herpes simplex virus DNA-binding protein ICP8 (Ruyechan and Weir, 1984), adenovirus DNA-binding protein (Lindenbaum *et al.*, 1986), and human SSB protein (Kenny *et al.*, 1989). T4 gene 32 protein has been shown to interact specifically with the T4 DNA polymerase based on sedimentation analysis (Huberman *et al.*, 1971) and affinity chromatography (Formosa *et al.*, 1983). A specific interaction has been inferred between T7 gene 2.5 protein and the T7 DNA polymerase (Reuben and Gefter, 1973) and the T7 helicase/primase (Nakai and Richardson, 1988), based upon the ability of gene 2.5 protein to stimulate these enzymes. In the accompanying paper, we demonstrate directly a physical interaction between gene 2.5 protein and both T7 DNA polymerase and helicase/primase (Kim *et al.*, 1992). In addition, we show that gene 2.5 protein, *E. coli* SSB protein, and T4 gene 32 protein all increase the processivity of T7 DNA polymerase on single-stranded DNA. In this paper we describe the purification of gene 2.5 protein from cells overexpressing the gene, characterization of its physical properties, and its interaction with DNA.

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[1] We refer to the T7 gene 5 protein-thioredoxin complex as "T7 DNA polymerase" acknowledging the fact that T7 gene 5 protein in the absence of thioredoxin has all the catalytic activities present in T7 DNA polymerase (Tabor *et al.*, 1987). Thioredoxin binds to gene 5 protein in a one-to-one complex, increasing the affinity of gene 5 protein for a primer-template complex, thus stimulating the processivity of DNA synthesis (Tabor *et al.*, 1987; Huber *et al.*, 1987).

[2] The abbreviations used are: SSB, single-stranded DNA binding; BSA, bovine serum albumin; FPLC, fast protein liquid chromatography; DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EM, electron microscopy; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate.

EXPERIMENTAL PROCEDURES

Materials

Bacterial Strains and Plasmids—pAR511-2.5, the plasmid used to overexpress T7 gene 2.5, was kindly provided by Dr. F. W. Studier (Brookhaven National Laboratory). This plasmid was transformed into *E. coli* BL21(DE3) (Studier and Moffatt, 1986). The procedure for the growth and induction of the cells for the overproduction of gene 2.5 protein was based on a bacteriophage T7 RNA polymerase expression system (Studier and Moffatt, 1986).

Proteins—T7 gene 2.5 protein was purified as described under "Results." *E. coli* SSB protein and T4 gene 32 protein were obtained from U. S. Biochemical Corp. Bovine serum albumin (BSA) and lysozyme were from Miles Laboratory. *E. coli* SSB protein was purified according to Chase *et al.* (1980) or was purchased from U. S. Biochemical Corp. Both preparations were greater than 99% homogeneous as judged by electrophoresis in the presence of sodium dodecyl sulfate.

DNA and Nucleotides—T7 [^{3}H]DNA (10 cpm/pmol) was prepared as described by Hinkle and Chamberlin (1972). Unlabeled T7 DNA was prepared as described by Richardson (1966). Single-stranded M13mp7 [^{3}H]DNA (45 cpm/pmol) was prepared as described by Matson and Richardson (1983). Unlabeled single-stranded M13mp18 DNA was prepared as described by Tabor *et al.* (1987). Wild-type M13 single-stranded DNA for electron microscopy was isolated as described (Register and Griffith, 1986). Salmon sperm DNA was from Sigma. Single-stranded DNA cellulose was prepared using heat-denatured salmon sperm DNA by the procedure of Alberts and Herrick (1971). Unlabeled nucleotides were from Pharmacia LKB Biotechnology Inc. ^{3}H-Labeled nucleoside triphosphates were from Du Pont-New England Nuclear and were further purified by fast protein liquid chromatography (FPLC) using a Mono Q column (Pharmacia).

Other Materials—DEAE-cellulose (DE52) and DE81 filter discs were from Whatman. Sephacryl S-200, DEAE-Sephacel, and Superose 12 were from Pharmacia. Protein standards (high molecular weight kit), benzamidine, and phenylmethylsulfonyl fluoride were from Sigma. Bio-Gel A-5m and isoelectric focusing protein standards (pI 4.6–9.6) were from Bio-Rad.

Methods

Molecular Weight Determination by Gel Filtration—The native molecular weight of gene 2.5 protein was determined by gel filtration FPLC using a Superose 12 column. The buffer for all experiments was 50 mM KPO_4, pH 7.0, 150 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, and 10% glycerol. Chromatography was carried out at 4 °C with a flow rate of 0.5 ml/min, and the A_{280} was measured. 18 μg of gene 2.5 protein was applied to the column. A standard curve of K_{av} *versus* $\log_{10} M_r$ was determined by chromatographing independently the following: protein standards (Sigma, high molecular weight kit), blue dextran (for the void volume, v_o), and deoxyadenosine (for the total volume, v_t). K_{av}, the fractional retention, was calculated according to the formula $K_{av} = (v_e - v_o)/(v_t - v_o)$, where v_e is the peak elution volume for each protein.

Molecular Weight Determination by Velocity Sedimentation—The native molecular weight of gene 2.5 protein was also examined by velocity sedimentation. This was done to correct for the contribution of molecular shape to the apparent molecular weight determined by gel filtration, thus permitting the calculation of the true molecular weight (Siegel and Monty, 1966). Six identical linear gradients of 5–20% sucrose were prepared in 150 mM KPO_4, pH 7.0, 1 mM β-mercaptoethanol, and 5 mM $MgSO_4$, in 14 × 89 mm Beckman Ultraclear centrifuge tubes. Samples were sedimented for 22 h at 35,000 rpm at 4 °C in a Beckman SW 41 rotor. The sedimentation velocities of protein standards (thyroglobin, apoferritin, β-amylase, alcohol dehydrogenase, BSA, and carbonic anhydrase) were determined independently as markers. 15 μg of gene 2.5 protein was dialyzed against 150 mM KPO_4, pH 7.0, 1 mM β-mercaptoethanol, and 5 mM $MgSO_4$ and sedimented together with thyroglobin. 400-μl fractions were collected from the bottom of the tubes with a needle puncture device (Hoefer Scientific Co.) connected to a peristaltic pump. Proteins were monitored by measurement of the A_{214}.

Nitrocellulose Filter Binding Assay—Gene 2.5 protein was monitored during its purification using the filter binding assay described by Whitter and Chase (1980). Reaction mixtures (50 μl) contained 40 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 10 μM single-stranded M13mp7 [^{3}H]DNA (45 cpm/pmol), 50 μg/ml BSA, and aliquots of the fractions to be assayed. After incubation for 30 min at 25 °C, the reaction mixtures were diluted to 3 ml with 40 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, and 50 μg/ml BSA, and then filtered through a 0.45-μm HAWP nitrocellulose filter (Millipore) at a flow rate of 5 ml/min. The filters were then washed with 5 ml of 40 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, and 50 μg/ml BSA, dried, and the radioactivity retained on each filter was determined in the presence of a toluene-based scintillation fluor. One unit of activity is defined as the amount of gene 2.5 protein capable of retaining 1 nmol of nucleotides of M13 DNA on the filter.

The relative binding affinities of T7 gene 2.5 protein, T4 gene 32 protein, and *E. coli* SSB protein for double-stranded T7 [^{3}H]DNA and single-stranded M13mp7 [^{3}H]DNA were compared using the filter binding assay described by Matson and Richardson (1985). The standard binding reaction mixture (25 μl) contained 40 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 50 μg/ml BSA, either 10 μM single-stranded M13mp7 [^{3}H]DNA (45 cpm/pmol) or 30 μM double-stranded T7 [^{3}H]DNA, and varying concentrations of the DNA-binding protein. Where indicated (see Fig. 4), the reaction mixtures contained either a 1 mM concentration each of dGTP, dATP, dTTP, and dCTP, or a 1 mM concentration each of rGTP, rATP, rUTP, and rCTP. After incubation for 30 min at 25 °C, the reaction mixtures were diluted to 3 ml with 40 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, and 50 μg/ml BSA. The diluted samples were immediately filtered through nitrocellulose. The filters were washed and dried, and the radioactivity retained on each filter was determined in the presence of a toluene-based scintillation fluor. Background levels (less than 5%) of ^{3}H-labeled DNA binding to filters in the absence of single-stranded DNA-binding proteins have been subtracted from each point. A maximum efficiency of retention of ^{3}H-labeled DNA by single-stranded DNA-binding proteins was more than 60% of the DNA on a nitrocellulose filter. Values of minimum estimates of apparent binding constants were calculated from the free ligand concentration at the midpoint of the titration curves determined with varying concentrations of the DNA-binding proteins (McGhee and von Hippel, 1974).

Fluorescence Spectroscopy—Details of the spectroscopic methods used here are described in the accompanying paper (Kim *et al.*, 1992). In fluorescence quenching experiments, gene 2.5 protein was titrated with the indicated nucleic acid by addition of 5–20-μl aliquots from a concentrated stock solution to the protein solution. Fluorescence was measured on a precision spectrofluorometer equipped with two Bausch and Lomb monochrometers using an excitation wavelength of 277 nm and an emission wavelength of 348 nm. The changes in gene 2.5 protein fluorescence upon addition of poly(dT) (fluorescence quenching, percent Q) were corrected for background, dilution of the protein, photobleaching, and inner filter effects. The correction for inner filter effect was made based on the consideration that exciting light at 277 nm is attenuated by 10 $A_{277/2}$ or $F_{corr} = F_{obs} \cdot$ antilog $(A_{277/2})$ (Lakowicz, 1983). All experiments were carried out in 50 mM $NaPO_4$, pH 7.7, 50 mM NaCl, and 0.1 mM EDTA at 25 °C. Poly(dT) concentration was determined from the specific absorbance at 264 nm and expressed as mol of PO_4 assuming an extinction coefficient of 8.1×10^3 M^{-1} cm^{-1}. The concentration of gene 2.5 protein was determined from the specific absorbance at 280 nm using an extinction coefficient of 2.58×10^4 M^{-1} cm^{-1}.

Values of site size and minimum estimates of apparent binding constant were derived from the stoichiometric points of the titrations of gene 2.5 protein with poly(dT) according to $K_a = \phi/(1 - \phi)^2$[gene 2.5 protein], where ϕ represents the fractional saturation of the protein at the stoichiometric point (Kelly *et al.*, 1976). The data are shown as the percent quenching of protein fluorescence (percent Q) *versus* the ratio of the concentration of poly(dT) in terms of phosphate to the concentration of monomer gene 2.5 protein.

Binding of Gene 2.5 Protein to Single-stranded DNA for Electron Microscopy—To examine the binding of gene 2.5 protein to single-stranded DNA, gene 2.5 protein (2.2 mg/ml) was diluted in 20 mM Hepes/NaOH, pH 7.5, 0.1 mM EDTA, or 50 mM $NaPO_4$, pH 7.5, 50 mM NaCl, mixed with single-stranded wild-type M13 DNA at 1 μg/ml, and incubated for 10 min at 37 °C. Ratios of gene 2.5 protein to DNA ranged from 10:1 (μg of gene 2.5 protein per μg of single-stranded DNA) to 60:1, or a molar ratio equal to 0.13–0.76 monomers of gene 2.5 protein per nucleotide of single-stranded DNA.

Electron Microscopy—Following the binding of gene 2.5 protein to single-stranded DNA, the samples were lightly fixed with 0.6% glutaraldehyde for 5 min at 22 °C and then passed through a 2-ml column of Bio-Gel A-5m equilibrated in 10 mM Tris·HCl, pH 7.5, 1 mM EDTA. The fixed sample was mixed with spermidine hydrochloride (to 0.5 mM) and then applied for 5 s to the EM support consisting

of a thin glow-charged carbon film supported by a mesh copper grid. The sample was then rapidly frozen by plunging into liquid ethane chilled in liquid nitrogen. The frozen sample was transfered to a Wiltek-modified Balzers 300 Freeze-etch system and the water sublimed for 2 h at −85 °C and 1 h at −50 °C. The sample was then rotary shadowcast with tungsten at −170 °C and 10^{-7} torr (1 torr = 133 pascals). Samples to be examined without fixation were diluted directly from the incubation mixture, mixed with spermidine, applied to the EM support, frozen, and processed as described above. Micrographs were taken on a Phillips EM 400 TLG. Length measurements were made by projecting the micrographs onto a Summagraphics digitizing table coupled to an IBM-PC computer programmed with software developed in this laboratory.

Other Methods—During the purification of gene 2.5 protein, protein concentrations were determined by the method of Bradford (1976) using BSA as a standard. The hydrolysis of nucleoside triphosphates was assayed by measuring the formation of ^{3}H-labeled nucleoside diphosphates from ^{3}H-labeled nucleoside triphosphates (Matson and Richardson, 1983). DNA concentrations were determined from the specific absorbance at 264 nm (for poly(dT)) and 260 nm (all other DNA) and expressed as mol of PO_4. Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS-PAGE) and staining with Coomassie Brilliant Blue were carried out according to Laemmli (1970).

RESULTS

Purification of the T7 Gene 2.5 Protein

Several procedures have been described for the purification of single-stranded DNA-binding proteins (Alberts and Frey, 1970; Reuben and Gefter, 1974; Herrick and Alberts, 1976; Chase *et al.*, 1980). The purification scheme described here is a modification of these procedures. The purification of gene 2.5 protein from 20 g of cells is summarized in Table I. Gene 2.5 protein was monitored both by its binding to single-stranded M13 [^{3}H]DNA using a nitrocellulose filter binding assay and by SDS-PAGE analysis (Fig. 1*A*). All purification steps were carried out at 0–4 °C unless otherwise indicated.

Overproduction of Gene 2.5 Protein—The plasmid pAR511-2.5 contains gene 2.5 under the control of T7 RNA polymerase. Gene 2.5 was overexpressed by induction of T7 RNA polymerase in the strain *E. coli* BL21(DE3) (Studier and Moffatt, 1986). *E. coli* BL21(DE3) containing pAR511-2.5 was grown overnight in 500 ml of 1% tryptone, 0.5% yeast extract, 1% NaCl, 0.1% casamino acids, 20 mM KPO_4, pH 7.4, and 50 μg/ml ampicillin. This culture was used to inoculate 10 liters of 2% tryptone, 1% yeast extract, 0.5% NaCl, 0.2% casamino acids, 40 mM KPO_4, pH 7.4, and 50 μg/ml ampicillin in a New Brunswick fermenter. The cells were incubated with aeration at 37 °C. At a cell density corresponding to A_{590} = 4.5, isopropyl β-D-thiogalactopyranoside was added at a final concentration of 0.4 mM to induce the expression of T7 RNA polymerase and thus gene 2.5 protein (Studier and Moffatt, 1986). After induction, the cells were incubated for 3 additional h and then harvested by centrifugation at 6,000 × *g* for 10 min in a Sorvall GS-3 rotor. The cell paste was resuspended in 2.5 liters of 50 mM Tris·HCl, pH 7.5, 25 mM EDTA, and 10% sucrose, and again harvested by centrifugation. The cell paste (88 g) was resuspended in 400 ml of 50 mM Tris·HCl, pH 7.5, 1 mM EDTA, and 10% sucrose, and 90-ml aliquots (20 g of cells) were frozen in liquid N_2 and stored at −80 °C.

Preparation of Cell Extract—Frozen cells (20 g in 90 ml) were thawed overnight on ice. Two ml of lysozyme (10 mg/ml) and 11 ml of 50 mM Tris·HCl, pH 7.5, 1 mM EDTA, 10% sucrose, 1 M NaCl, 100 mM bezamidine chloride, and 5 mM phenylmethylsulfonyl fluoride were added. After incubation of the mixture for 45 min on ice with intermittent stirring, 24 ml of 50 mM Tris·HCl, pH 7.5, 1 mM EDTA, 5 M NaCl was added to bring the final concentration of NaCl to 1 M. The cells were heated in a 37 °C water bath with constant stirring until the temperature reached 20 °C and then cooled in an ice-water bath until the temperature was reduced to 4 °C. The lysate was centrifuged for 45 min at 40,000 rpm in a Beckman Ti-45 rotor. The supernatant (120 ml) was fraction I (Fig. 1*A*, *lane 1*).

DEAE-Cellulose Chromatography—A column of Whatman DE52 DEAE-cellulose (5.8 cm^2 × 30 cm) was prepared and equilibrated with 20 mM Tris·HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM DTT, and 10% glycerol (buffer A) containing 0.4 M NaCl. Fraction I was diluted with buffer A to give a conductivity equivalent to buffer A containing 0.4 M NaCl. The diluted fraction I (~300 ml) was applied to the column. Gene 2.5 protein is not retained under these conditions. The flow-through fractions (~300 ml) were pooled to give fraction II (Fig. 1*A*, *lane 2*).

Ammonium Sulfate Precipitation—To 300 ml of fraction II, ammonium sulfate was added to 80% saturation (155 g) over a period of 60 min and was stirred slowly for an additional 60 min. The precipitate was collected by centrifugation at 10,000 × *g* for 45 min in a Sorvall GSA rotor and dissolved in 50 ml of buffer A containing 50 mM NaCl (fraction III).

Sephacryl S-200 Chromatography—A column of Sephacryl S-200 (3.8 cm^2 × 60 cm) was prepared and equilibrated with buffer A containing 50 mM NaCl. Fraction III was applied to the column and eluted with buffer A containing 50 mM NaCl. From this point on gene 2.5 protein was monitored by absorbance at 280 nm, SDS-PAGE, and its ability to bind single-stranded DNA using the nitrocellulose filter binding assay as described under "Experimental Procedures." Fractions (132 ml) containing single-stranded DNA binding activity were pooled (fraction IV; Fig. 1*A*, *lane 3*).

Single-stranded DNA-Cellulose Chromatography—A column of single-stranded DNA-cellulose (2.5 cm^2 × 12 cm) containing approximately 5 g of single-stranded DNA-cellu-

TABLE I

Purification of T7 gene 2.5 protein from 20 g of induced E. coli BL21(DE3) containing the plasmid pAR511-2.5

Fraction	Step	Protein	Total units[a]	Specific activity
		mg		*units/mg protein*
I	Extract	1,770	ND[b]	
II	DEAE-cellulose	1,426	ND[b]	
III	Ammonium sulfate	986	ND[b]	
IV	Sephacryl S-200	760	24,500	32
V	Single-stranded DNA-cellulose	48	6,672	139
VI	DEAE-Sephacel	36	5,724	159
VII	Mono Q	34	5,472	160

[a] One unit is equal to 1 nmol of nucleotide of M13 DNA bound as determined by the nitrocellulose filter binding assay described under "Experimental Procedures."

[b] Not determined. The number of units of gene 2.5 protein could not be determined in these fractions because of the presence of contaminating single-stranded DNA-binding proteins.

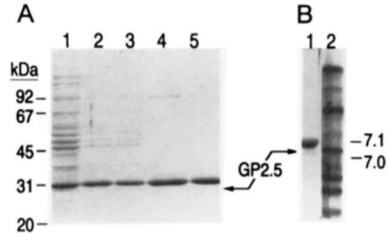

FIG. 1. **Purification of the T7 gene 2.5 protein.** *A*, analysis of the purification of gene 2.5 protein by 12% polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate. Purification of gene 2.5 protein is described in the text and is summarized in Table I. *Lane 1*, cell extract (fraction I); *lane 2*, DEAE-cellulose pool (fraction II); *lane 3*, Sephacryl S-200 pool (fraction IV); *lane 4*, single-stranded DNA cellulose pool (fraction V); *lane 5*, DEAE-Sephacel pool (fraction VI). The positions of molecular mass standards are indicated at the left. *B*, isoelectric focusing of gene 2.5 protein by polyacrylamide tube gel electrophoresis was basically performed as described (Williams *et al.*, 1983). *Lane 1*, gene 2.5 protein (fraction VI); *lane 2*, isoelectric focusing protein standards. After electrophoresis, the gels were stained with Coomassie Brilliant Blue.

lose (5.4 mg of denatured salmon sperm DNA/g of cellulose) was prepared by the procedure of Alberts and Herrick (1971). The column was equilibrated in buffer A containing 50 mM NaCl. Fraction IV was applied to the column at a rate of 48 ml/h. Gene 2.5 protein was eluted by a step gradient containing increasing NaCl concentrations, with each step (100 ml) containing buffer A plus either 0.1, 0.5, 1.0, or 2.0 M NaCl. One-ml fractions were collected. Most of the gene 2.5 protein eluted in 30 ml of buffer A plus 1.0 M NaCl (fraction V; Fig. 1*A*, *lane 4*).

DEAE-Sephacel Chromatography—A column of DEAE-Sephacel (2.5 $cm^2 \times 28$ cm) was prepared and equilibrated with buffer A containing 0.1 M NaCl. Fraction V was dialyzed against buffer A to reduce the conductivity to that of buffer A containing 0.1 M NaCl. The dialyzed fraction V was applied to the column at a flow rate of 23 ml/h, and the resin was washed with 150 ml of buffer A containing 0.1 M NaCl. Gene 2.5 protein was eluted with a 300-ml linear gradient from 0.1 to 0.5 M NaCl in buffer A at a flow rate of 23 ml/h. One-ml fractions were collected. Gene 2.5 protein eluted at approximately 0.25 M NaCl. The fractions (15 ml) containing gene 2.5 protein were pooled and analyzed by SDS-PAGE (fraction VI; Fig. 1*A*, *lane 5*). Fraction VI appears to be homogeneous as a single band judged by electrophoresis under the denaturing conditions (Fig. 1*A*, *lane 5*), but it contains a low level of single-stranded DNA-dependent nucleoside 5′-triphosphatase activity.

Mono Q FPLC—To remove a contaminating single-stranded DNA-dependent nucleoside 5′-triphosphatase in fraction VI, several portions of fraction VI were dialyzed against buffer A and were chromatographed separately on the Mono Q column (HR 5/5, Pharmacia) equilibrated in buffer A. In each run, the column was washed with 10 ml of buffer A and eluted with a 45-ml gradient of buffer A containing 0–600 mM NaCl. Single-stranded DNA dependent ATPase was assayed across the column. The DNA-dependent ATPase activity eluted from the column slightly before the bulk of the gene 2.5 protein. Fractions of gene 2.5 protein showing no ATPase were pooled, dialyzed against 20 mM KPO_4, pH 7.4, 0.1 mM DTT, 0.1 mM EDTA, and 50% glycerol at 4 °C, and stored at −20 °C (fraction VII).

Purity of Gene 2.5 Protein—36 mg of gene 2.5 protein were recovered from 20 g of induced cells. For comparison, 0.25 mg of gene 2.5 protein had been previously purified from 10 g of T7 phage-infected cells (Scherzinger *et al.*, 1973). After electrophoresis of the purified gene 2.5 protein under denaturing conditions, staining with Coomassie Blue produced a single band corresponding to a molecular weight of approximately 27,000 (Fig. 1*A*, *lane 5*). Although the gene 2.5 protein present in fraction VI appears homogeneous, we found a low level of single-stranded DNA-dependent nucleoside 5′-triphosphatase activity (five molecules of ATP hydrolyzed to ADP and P_i per min per monomer of gene 2.5 protein) in the fraction. FPLC chromatography of the gene 2.5 protein fraction VI on a Mono Q column as described above separated the ATPase from the gene 2.5 protein. The DNA-dependent ATPase activity eluted just prior to the appearance of the gene 2.5 protein. Fraction VII of gene 2.5 protein has no detected nucleoside 5′-triphosphatase activity (less than 0.1 molecule of ATP hydrolyzed per min per monomer of gene 2.5 protein). The contaminating ATPase present in fraction VI represents less than 1% of the protein in this fraction.

Physical Properties of the T7 Gene 2.5 Protein

SDS-PAGE Analysis and Isoelectric Focusing—The physical properties of gene 2.5 protein determined in this study are summarized in Table II. The molecular weight of gene 2.5 protein deduced from the DNA sequence of its gene is 25,562 (Dunn and Studier, 1983). By SDS-PAGE analysis, it migrates as a single band of approximately 27,000 Da (Fig. 1A, *lane 5*). Previous reports of the molecular weight of gene 2.5 protein by SDS-PAGE analysis range between 25,000 and 31,000 (Scherzinger *et al.*, 1973; Reuben and Gefter, 1973). Gene 2.5 protein has an isoelectric point of 7.0 ± 0.1 (Fig. 1*B*, *lane 1*). This is in spite of the fact that 15 of the carboxyl-terminal 21 residues are acidic (Dunn and Studier, 1983).

Native Molecular Weight Analysis—The molecular weight of native gene 2.5 protein was determined by gel filtration and sedimentation velocity analysis. Analysis by gel filtration chromatography was carried out by FPLC using a Superose 12 column (Fig. 2*A*). A plot of K_{av} versus $\log_{10} M_r$ was derived from the elution profiles of a number of protein standards and was used to estimate the native molecular weight of gene 2.5 protein. The calculated M_r of 53,700 is approximately twice that of a gene 2.5 protein monomer.

To confirm that native gene 2.5 protein is a dimer, we determined its molecular weight using a second hydrodynamic method, sedimentation velocity centrifugation (Siegel and Monty, 1966). The sedimentation of native gene 2.5 protein was compared with the sedimentation of six different protein standards, one of which (thyroglobin) was sedimented together with gene 2.5 protein (Fig. 2*B*). Gene 2.5 protein sedimented with a coefficient of 3.7 S, to give a M_r of 53,000. We conclude from these two independent measurements that the native form of gene 2.5 protein consists of a dimer of two identical subunits of molecular weight 25,562.

TABLE II

Summary of the physical properties of the T7 gene 2.5 protein

Subunit molecular weight	
From DNA sequence analysis[a]	25,562
From SDS-PAGE analysis	27,000
Native molecular weight	
From gel filtration analysis	54,000
From sedimentation velocity analysis	53,000
Stokes radius	33.1 Å
Frictional coefficient	1.18
Sedimentation coefficient ($s_{20,w}$)	3.7 S
Isoelectric (pI)	7.0 ± 0.1
Thermostability (T_m)[b]	53 °C

[a] Dunn and Studier (1983).

[b] Determined with differential scanning calorimeter as described (Keating *et al.*, 1988).

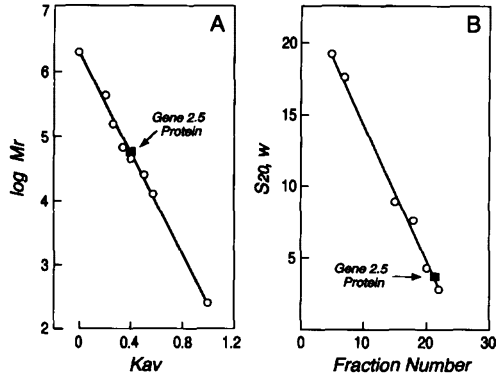

FIG. 2. **Molecular weight determination of the T7 gene 2.5 protein.** *A*, gel filtration chromatography. Chromatography was carried out using a Superose 12 column as described under "Experimental Procedures." The K_{av} *versus* the $\log_{10} M_r$ were plotted for the standards (○), and the best-fit line was determined by a least squares analysis. The K_{av} of 0.386 for gene 2.5 protein (■) intersected the best-fit line to give a predicted molecular weight of 53,700. The standards were: blue dextran (2,000 kDa), ferritin (440 kDa), human IgG (160 kDa), BSA (68 kDa), ovalbumin (45 kDa), chymotrypsinogen (25 kDa), cytochrome *c* (12.5 kDa), and cytidine (246 Da). *B*, sedimentation velocity centrifugation. The conditions for sedimentation are described under "Experimental Procedures." The sedimentation distance of each protein standard (○) was plotted as a function of its $s_{20,w}$ value. The sedimentation distance of gene 2.5 protein (■) intersected the best-fit line to give a value of 3.7 S. Fraction 1 was the first fraction collected from the bottom of the tube. The standards and their $s_{20,w}$ values were: thyroglobin (19.2 S), apoferritin (17.6 S), β-amylase (8.9 S), alcohol dehydrogenase (7.6 S), BSA (4.3 S), and carbonic anhydrase (2.8 S).

Differential Scanning Calorimeter Analysis—Differential scanning calorimeter analysis is a measure of the thermostability of proteins. We analyzed the gene 2.5 protein to determine the temperature at which it denatures. Gene 2.5 protein had a transition temperature (T_m) of 53 °C (data not shown). For comparison, wild type T4 gene 32 protein has a T_m of 56.3 °C (Williams and Konigsberg, 1983).

DNA Binding Properties of the T7 Gene 2.5 Protein

Nitrocellulose Filter Binding Analysis—We used a nitrocellulose filter binding assay to compare the binding of three single-stranded DNA-binding proteins (T7 gene 2.5 protein, *E. coli* SSB protein, and T4 gene 32 protein) with single-stranded M13 [^{3}H]DNA and double-stranded T7 [^{3}H]DNA (Fig. 3). For each experiment, the association constant was calculated by comparing the amount of DNA retained on the filter in the presence of varying amounts of protein with the maximum amount of DNA retained in the presence of saturating levels of the protein. Both *E. coli* SSB protein and T4 gene 32 protein have a binding constant of about 10^8 M^{-1} for single-stranded DNA, comparable to the values reported by Williams and Chase (1990). In contrast, the binding constant of gene 2.5 protein for single-stranded DNA is 3.8×10^6 M^{-1}, lower than that of *E. coli* SSB protein or T4 gene 32 protein by a factor of 25. All three proteins show a specific affinity for single-stranded DNA, binding only very weakly to double-stranded DNA (Fig. 3), consistent with previous reports (Scherzinger *et al.*, 1973; Sigal *et al.*, 1972; Williams and Konigsberg, 1983).

Since gene 2.5 protein contains an amino acid sequence that shares homology with the ATP binding site of other proteins (see "Discussion"), we examined whether the presence of rNTPs or dNTPs had an effect on the binding of gene 2.5 protein to single- or double-stranded DNA (Fig. 4). The presence of rNTPs or dNTPs in the binding reaction had no significant effect on the binding constant of gene 2.5 protein for single- or double-stranded DNA.

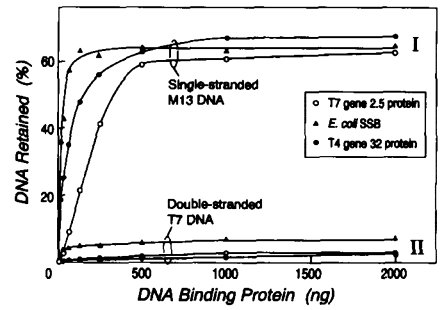

FIG. 3. **Analysis of protein binding to single- and double-stranded DNA by nitrocellulose filter binding assay.** The single-stranded DNA-binding proteins analyzed were T7 gene 2.5 protein (○), T4 gene 32 protein (●), and *E. coli* SSB protein (▲). Each reaction mixture (25 μl) contained the indicated amount of single-stranded DNA-binding protein and either 80 ng of M13mp7 [^{3}H] DNA (*I*) or 240 ng of T7 [^{3}H]DNA (*II*). After incubation for 10 min at 30 °C, each mixture was filtered through nitrocellulose, and the amount of radioactivity retained was determined as described under "Experimental Procedures."

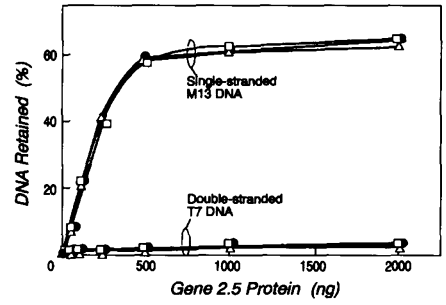

FIG. 4. **The effect of nucleoside triphosphates on the binding of T7 gene 2.5 protein to single- and double-stranded DNA.** Nitrocellulose filter binding assays were carried out as described under "Experimental Procedures." Each reaction mixture (25 μl) contained the indicated amount of gene 2.5 protein and either 80 ng of single-stranded M13mp7 [^{3}H]DNA or 240 ng of double-stranded T7, [^{3}H]DNA in the absence of nucleoside triphosphates (△) or in the presence of a 1 mM concentration each of dGTP, dATP, dTTP, and dCTP (□) or a 1 mM concentration each of rGTP, rATP, rUTP, and rCTP (○).

Fluorescence Spectroscopy—When proteins bind to single-stranded DNA, the intrinsic fluorescence of their aromatic amino acid residues is quenched. This fluorescence quenching has been used as an assay to quantitate the binding of proteins to single-stranded DNA (Williams *et al.* 1983; Kowalczykowski *et al.*, 1981). We have used this procedure to analyze the binding properties of gene 2.5 protein. The absorption of energy at 277 nm by proteins is caused by tyrosine and tryptophan residues. Gene 2.5 protein contains 11 tyrosine and 3 tryptophan residues (Dunn and Studier, 1983). In the absence of DNA, when a solution containing gene 2.5 protein is excited at 277 nm, the emission maximum is 355 nm. Since the emission maximum of free tryptophan yields at 350 nm, the tryptophyl residues must be fully exposed. In the presence of increasing amounts of single-stranded M13 DNA, this emission maximum is shifted to 340 nm, and fluorescence is quenched in proportion to the amount of single-stranded DNA present (Fig. 5*A*). In a control experiment, single-stranded DNA did not quench the fluorescence of BSA, a protein that does not bind to DNA (data not shown). These data suggest that either tyrosine or tryptophan residues in gene 2.5 protein are important in binding to single-stranded DNA.

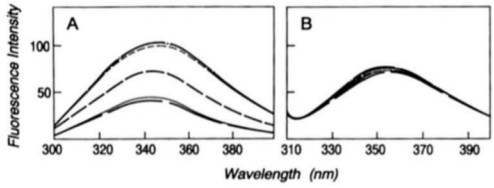

FIG. 5. **Fluorescence emission spectra of T7 gene 2.5 protein.** An excitation wavelength of 277 nm (*A*) or 295 nm (*B*) was used to determine the emission spectra of gene 2.5 protein (3.9 μM) either in the absence of single-stranded DNA (—·—) or in the presence of 1 μM (- - - -), 9 μM (– –), 15 μM (——), or 25 μM (— —) M13mp18. Fluorescence measurements were carried out as described under "Experimental Procedures." The binding buffer was 50 mM $NaPO_4$, pH 7.7, 50 mM NaCl, and 0.1 mM EDTA.

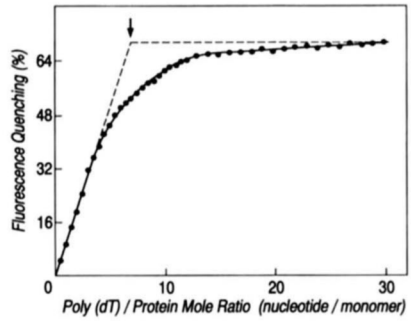

FIG. 6. **Fluorescence quenching of T7 gene 2.5 protein by single-stranded DNA.** Fluorescence measurements were carried out as described under "Experimental Procedures" using an excitation wavelength of 277 nm and measuring the emission at 348 nm. Samples contained 50 mM $NaPO_4$, pH 7.7, 50 mM NaCl, 0.1 mM EDTA, 3.9 μM gene 2.5 protein, and varying amounts of poly(dT) DNA. The stoichiometry of gene 2.5 protein binding was determined by the intersection of the initial and final slopes (indicated by the *arrow*).

When a protein is excited at 295 nm, only tryptophan residues (and not tyrosine residues) absorb energy (Lakowicz, 1983). When a solution of gene 2.5 protein was excited at 295 nm, the emission maximum was 355 nm. The emission spectrum did not change with the presence of a saturating amount of single-stranded DNA (Fig. 5*B*), suggesting that tryptophan residues in the native gene 2.5 protein are not involved in DNA binding. Thus, the fluorescence quenching observed at 277 nm is a result of an interaction of tyrosine residues in gene 2.5 protein with the single-stranded DNA.

We also used fluorescence quenching to quantitate the binding of gene 2.5 protein to single-stranded DNA (poly(dT)). When poly(dT) was added in increasing amounts to a solution containing gene 2.5 protein, the extent of fluorescence quenching was directly proportional to the concentration of the DNA (Fig. 6). At a saturating concentration of poly(dT) (a ratio of approximately 25 mol of thymidylate residues to 1 mol of gene 2.5 protein), essentially all the gene 2.5 protein was complexed to the DNA. From the data presented in Fig. 6, a binding constant of 1.2×10^6 M^{-1} is calculated for the interaction of gene 2.5 protein and poly(dT), based on the method of Kelly *et al.* (1976). The average number of nucleotides bound by gene 2.5 protein was determined by the intersection of the initial and final slopes (*arrow* in Fig. 6); this corresponds to a stoichiometry of ~seven nucleotides bound per gene 2.5 protein monomer.

Electron Microscopic Analysis of the T7 Gene 2.5 Protein and DNA Interactions

Visualization of Gene 2.5 Protein Bound to Single-stranded DNA in the Absence of Magnesium—Electron microscopic visualization of complexes formed between gene 2.5 protein and single-stranded M13 DNA provides a means of accessing the degree of cooperativity of protein binding to single-stranded DNA and the nature of the structures produced. Complexes were formed in a buffer containing 20 mM Hepes/NaOH, pH 7.5, 1 mM EDTA, at ratios of protein to single-stranded DNA ranging from 10:1 to 60:1 (μg of protein/μg of single-stranded DNA), or 0.13–0.76 monomers of gene 2.5/nucleotide. Following complex formation, the samples were lightly fixed and passed over Bio-Gel A-5m prior to preparation for EM. The EM preparative method used here (see "Experimental Procedures") involves rapid freezing, sublimination of the ice, and rotary shadowcasting, the latter steps carried out in an ultra high vacuum system to eliminate artifacts of air drying and contamination of the sample by pumping oils.

As shown in Fig. 7, as increasing amounts of gene 2.5 protein were added to the single-stranded M13 DNA in the absence of magnesium, an increasing fraction of the single-stranded M13 DNA molecules changed from compact bush-like structures typical of protein-free single-stranded DNA to relatively open circular structures. With 0.13 monomers/nucleotide (10:1) (*panel A*), the majority of the single-stranded DNA molecules appeared protein-free, but a few were relatively open and clearly complexed with protein, typical of the structures commonly seen at higher protein to DNA ratios. With increasing protein, the fraction of opened structures increased until at a ratio of 40:1 (0.5 monomers/nucleotide) (*panel C*) all of the single-stranded DNA molecules were in the form of open circular structures.

The open circular structures frequently contained regions

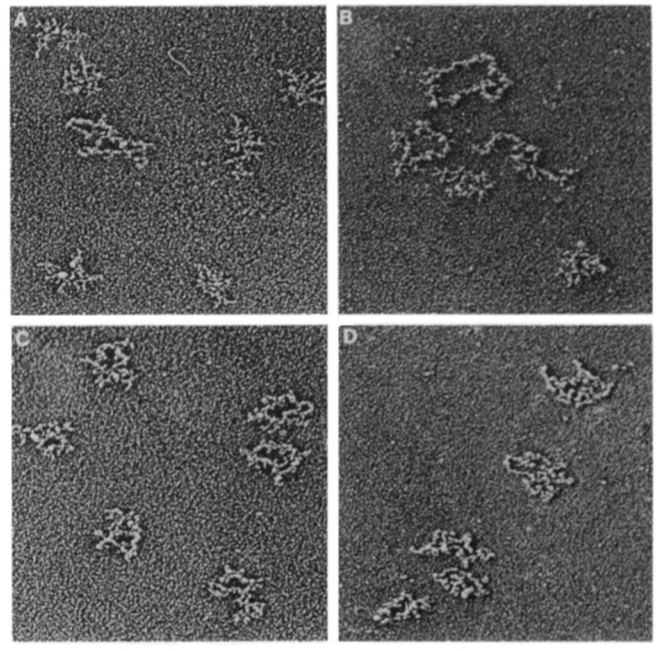

FIG. 7. **Visualization of the binding of gene 2.5 protein to single-stranded DNA.** Gene 2.5 protein was incubated with single-stranded M13 DNA at weight ratios of 10:1 (*A*), 20:1 (*B*), 40:1 (*C*), and 60:1 (*D*), which correspond to molar ratios of gene 2.5 protein monomer to nucleotide of 0.13 (*A*), 0.26 (*B*), 0.50 (*C*), and 0.76 (*D*). The samples were fixed with glutaraldehyde, purified by gel filtration through Bio-Gel A-5m, and prepared for EM by rapid freezing, freeze-drying in a cryopumped evaporator at 10^{-7} torr and rotary shadowcasting with tungsten, as described under "Experimental Procedures." The bush-like structures that predominate in *A* are identical to what is seen when protein-free single-stranded M13 DNA is prepared for EM using this procedure (not shown). The images are shown in *reverse contrast*. The *bar* at the *bottom* equals 0.1 μm.

that were compact or not fully extended. Measurements of the length of the gene 2.5 protein-single-stranded M13 DNA circles revealed a variation in length ranging from 0.6 to 1.6 Å/nucleotide (n = 21) (based on 3.4 Å/base pair for duplex DNA). In contrast, in the same experiment, when single-stranded M13 DNA was bound by a saturating amount of *E. coli* SSB protein, the lengths of these complexes varied by only 10% (1.1–1.2 Å/nucleotide; n = 5). The compact regions may result from incomplete removal of the secondary structure of the single-stranded DNA or possibly an intertwining of the gene 2.5 protein-single-stranded DNA strand about itself. The diameter of the gene 2.5 protein-single-stranded DNA filament measured approximately 6 nm, significantly less than the 8–10-nm diameter of SSB protein-single-stranded DNA complexes.

When gene 2.5 protein-single-stranded M13 DNA complexes were taken directly from the incubation mixture, frozen without fixation, and examined at higher magnification (Fig. 8), loose chains of protein particles bound along the single-stranded DNA were seen. Occasionally the particles bound to the DNA or free on the background appeared as two smaller particles nearly touching (*arrow*, Fig. 8). This appearance and their size (single proteins of 25 kDa are too small to be seen easily by this EM method) argue that these particles represent dimers of gene 2.5 protein, in agreement with the solution studies.

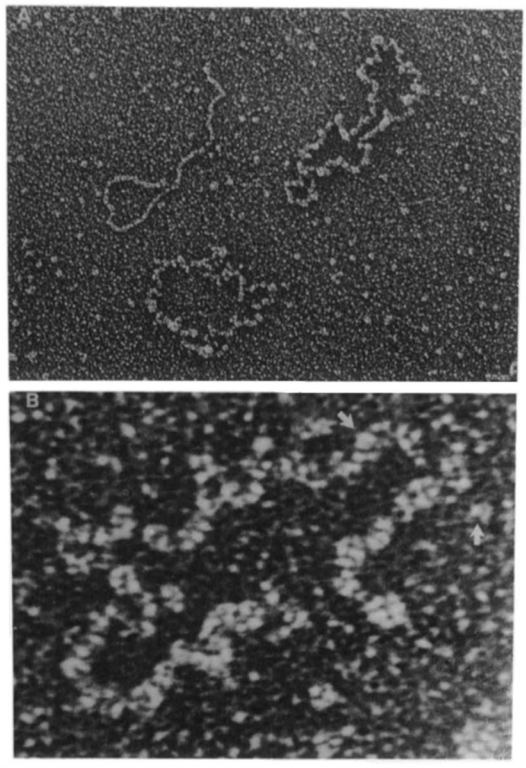

FIG. 8. **High magnification image of unfixed complexes of gene 2.5 protein and single-stranded DNA.** Gene 2.5 protein was complexed with single-stranded M13 DNA at a ratio of 40:1 (as in Fig. 7*C*) and applied directly to the EM support without fixation. The sample was then prepared for EM as described for the fixed samples in Fig. 7. *A*, a double-stranded DNA fragment is shown together with individual molecules of gene 2.5 protein. The size of the protein molecules is consistent with that of a dimer of gene 2.5 protein. *B*, a high magnification view of single-stranded DNA coated with gene 2.5 protein. Apparent dimers of the gene 2.5 protein can be seen spaced along the single-stranded DNA (for example, see *arrow*). The images are shown in *reverse contrast*. The *bar* at the *bottom* is equal to either 0.1 μm (*A*) or 0.04 μm (*B*).

Formation of the gene 2.5 protein-single-stranded M13 DNA complexes in 50 mM $NaPO_4$, pH 7.5, and 50 mM NaCl at weight ratios of protein to DNA of 10:1–60:1 (0.13–0.76 monomers/nucleotide) produced results very similar to what was seen when they were prepared in 20 mM Hepes/NaOH, pH 7.5, and 1 mM EDTA as described above. When the samples were buffered in Hepes, concentrations of NaCl above 50 mM appeared to result in the aggregation of the gene 2.5 protein-single-stranded M13 DNA complexes, as few individual complexes (or protein-free single-stranded M13 DNA circles) were observed by EM.

Visualization of Gene 2.5 Protein-Single-stranded DNA Complexes formed in the Presence of Magnesium—Because the reactions employing gene 2.5 protein generally include divalent cations, complexes of gene 2.5 protein and single-stranded M13 DNA were formed using a ratio of gene 2.5 protein to single-stranded M13 DNA of 40:1 (0.76 monomers/nucleotide) and in the presence of 10 mM magnesium and/or 3 mM ATP or a combination of 4 mM magnesium and 1 mM ATP. When these complexes were prepared as in Fig. 7 for EM and examined, they had a highly compact appearance (Fig. 9). No effect of ATP was observed on their morphology, and the complexes formed with 4 mM magnesium and 1 mM ATP appeared very similar to those formed in 10 mM magnesium with or without ATP. Although the complexes did not appear as extended loops under these conditions, it appeared that the DNA was not fully saturated with protein until input ratios of 40:1 (0.5 monomers/nucleotide) or greater were employed.

DISCUSSION

The class of proteins referred to as "single-stranded DNA-binding proteins" are distinguished by their strong, specific affinity for single-stranded DNA (see Chase and Williams, 1986). Two of the first examples of proteins that were identified and purified based on this property were the phage T4 gene 32 protein (Alberts and Frey, 1970) and *E. coli* SSB protein (Sigal *et al.*, 1972). A protein with a high affinity for single-stranded DNA was purified from phage T7-infected *E. coli* (Scherzinger *et al.*, 1973; Reuben and Gefter, 1974) and later identified as the product of gene 2.5 (Dunn and Studier, 1983). In this paper we have characterized in more detail the physical properties of T7 gene 2.5 protein and compared its properties with those of *E. coli* SSB protein and T4 gene 32 protein.

A principal role of single-stranded DNA-binding proteins

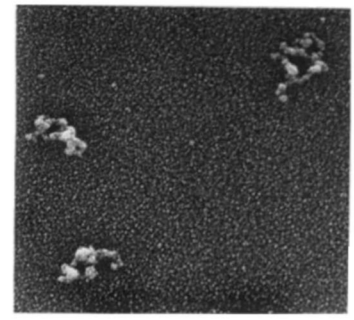

FIG. 9. **EM analysis of gene 2.5 protein-single-stranded DNA complexes formed in the presence of magnesium.** Gene 2.5 protein was assembled onto single-stranded M13 DNA using a 40:1 ratio of protein to single-stranded DNA in a buffer containing 10 mM magnesium. The sample was then fixed and prepared for EM as described in Fig. 7. The complexes formed in the presence of magnesium appear more *compacted* than the open filamentous complexes formed in the absence of magnesium (Fig. 7*C*). The image is shown in *reverse contrast*. The *bar* at the *bottom* is equal to 0.1 μm.

is to remove secondary structure in the DNA, thus stimulating DNA replication and recombination (see Chase and Williams, 1986; Meyer and Laine, 1990; Lohman and Bujalowski, 1990). These proteins act structurally and are required in stoichiometric amounts. They do not hydrolyze nucleoside triphosphates and have no known enzymatic activities. A major difference between these DNA-binding proteins is their affinity for single-stranded DNA: the affinity of *E. coli* SSB protein or the T4 gene 32 protein for single-stranded DNA (K_{assoc} = 10^8 M^{-1}) (Williams and Chase, 1990; this study) is 25–100 times higher than that of gene 2.5 protein (K_{assoc} = 1–4 × 10^6 M^{-1}). Such a major difference in affinity for single-stranded DNA questions the assignment of T7 gene 2.5 protein to this class of single-stranded DNA-binding proteins (helix-destabilizing proteins) defined by T4 gene 32 protein and *E. coli* SSB protein. For example, the affinity of the gene 2.5 protein for single-stranded DNA is virtually identical to that of the *E. coli* recA, 2.6 × 10^6 M^{-1} (Menetski and Kowalczykowski, 1985).

Nevertheless, T7 gene 2.5 protein also has properties characteristic of classic single-stranded DNA-binding proteins such as *E. coli* SSB protein and T4 gene 32 protein. It stimulates DNA synthesis on single-stranded DNA catalyzed by T7 DNA polymerase (Scherzinger *et al.*, 1973; Reuben and Gefter, 1974) and the synthesis of primers on single-stranded DNA by the T7 primase (Nakai and Richardson, 1988). In the accompanying paper (Kim *et al.*, 1992) we show that gene 2.5 protein, *E. coli* SSB protein, and T4 gene 32 protein all stimulate the processivity of T7 DNA polymerase, presumably by removing the secondary structures in the single-stranded DNA.

In considering the binding of the single-stranded DNA-binding proteins to DNA it is important to note that these measurements have been carried out in the absence of other replication proteins. *In vivo*, it has either been directly shown or strongly suggested that the proteins we have been discussing interact with other recombination and replication proteins. For example, it has been shown directly that T4 gene 32 protein interacts specifically with the T4 DNA polymerase and the recombination proteins uvsX and uvsY (Formosa *et al.*, 1983). Evidence suggests that *E. coli* SSB protein interacts with *E. coli* DNA polymerase II (Molineux and Gefter, 1974) and the *E. coli* recA protein-single-stranded DNA complex (Morrical and Cox, 1990). In the accompanying paper we show by direct physical measurements a specific interaction between T7 gene 2.5 protein and T7 DNA polymerase (Kim *et al.*, 1992). T7 DNA polymerase binds nonspecifically to single-stranded DNA with an affinity of 10^6 M^{-1} and binds specifically to a primer-template complex with an affinity of 10^9 M^{-1} (Huber *et al.*, 1987). Although the effect of the interaction of gene 2.5 protein and T7 DNA polymerase on the affinity of each protein for single-stranded DNA is not known, it is likely that physiologically it is the affinity of the complex that is important for T7 DNA replication.

T7 gene 2.5 protein shares some general structural similarities with T4 gene 32 protein, *E. coli* SSB protein, and *E. coli* recA. For example, like *E. coli* SSB protein and T4 gene 32 protein (see Chase and Williams, 1986) and *E. coli* recA protein (Benedict and Kowalczykowski, 1988), limited proteolysis of gene 2.5 protein produces several stable peptides, suggesting that all four proteins have separate domains separated by proteolytically sensitive regions.[3] In addition, the carboxyl termini of all four proteins are highly acidic; of the carboxyl-terminal 21 residues, 15 in T7 gene 2.5 protein, 6 in T4 gene 32 protein, 5 in *E. coli* SSB protein (Williams *et al.*, 1983), and 8 in *E. coli* recA protein (Sancar *et al.*, 1980) are acidic. These acidic regions are thought to play a role in the interaction of these proteins with other replication proteins. Inspection of the amino acid sequence of the gene 2.5 protein, as derived from the nucleotide sequence (Dunn and Studier, 1983), reveals that amino acid residues 24–52 and 115–143 share some homology with ATP binding sites of known ATPases (Fig. 10). We could not detect any regions of homology to this site in the other two single-stranded DNA-binding proteins used in this study, *E. coli* SSB protein and T4 gene 32 protein.

In the absence of DNA, T4 gene 32 protein is a dimer (Carroll *et al.*, 1975) and *E. coli* SSB protein is a tetramer (Williams *et al.*, 1984). When bound to single-stranded DNA, *E. coli* SSB protein remains a tetramer (Williams *et al.*, 1984), whereas T4 gene 32 protein becomes a monomer (see Chase and Williams, 1986). We have analyzed the stoichiometry of gene 2.5 protein complexed to single-stranded DNA by electron microscopy. EM visualization of the binding of gene 2.5 protein to single-stranded DNA revealed that in low salt buffers and in the absence of magnesium, gene 2.5 protein is able to disrupt the secondary structure of single-stranded DNA. In doing so, the single-stranded DNA was extended into what appeared to be a chain of gene 2.5 protein dimers bound along the DNA. Fluorescence studies described here show that gene 2.5 protein binds to single-stranded DNA at a molar ratio of approximately one gene 2.5 protein monomer/6–8 nucleotides. This is similar to the ratios of one monomer of T4 gene 32 protein bound per 5–11 nucleotides, and one monomer of *E. coli* SSB protein bound per 6–8 nucleotides (see Chase and Williams, 1986). However, from the EM observations, it appears that full saturation of single-stranded M13 DNA requires severalfold higher levels of gene 2.5 protein than that estimated from the solution measurements presented here, a ratio of one monomer of gene 2.5 protein/two nucleotides. Possible explanations are: 1) that there may be several levels of complexing the single-stranded DNA and that saturation of the fluorescence occurs before full protein binding as seen by EM, or 2) that the gene 2.5 protein may bind to poly(dT) or to ethio-derivatized single-stranded DNA more efficiently than to a large natural DNA such as M13. Subjectively, the density of gene 2.5 protein bound per unit length along the single-stranded DNA appeared to be less than what is seen when *E. coli* SSB protein, T4 gene 32 protein, or *E. coli* recA protein binds to single-stranded DNA.[4] For those proteins, the single-stranded DNA-protein filaments formed are more regular. Binding of gene 2.5 protein to single-stranded M13 DNA in the presence of magnesium required similar amounts of protein to fully complex the DNA as in the absence of magnesium; however, the structures produced were highly compact in nature. In competition with *E. coli* SSB protein, the *E. coli* SSB protein appeared to displace gene 2.5 protein or at least dominate the morphology of the resulting complexes.[4] The fluorescence quenching experiments described here show that tyrosine residues are used by gene 2.5 protein in binding to single-stranded DNA. Physical and genetic studies have also implicated an important role for tyrosine residues in the binding of the T4 gene 32 protein to single-stranded DNA (Shamoo *et al.*, 1989). In contrast, tryptophan residues, but not tyrosine residues, are involved in the binding of *E. coli* SSB protein to single-stranded DNA (see Meyer and Laine, 1990). Such a role of either tyrosine or tryptophan residues in the binding of these proteins to single-stranded DNA is consistent with a model

[3] Y. T. Kim and C. C. Richardson, unpublished results.

[4] Y. T. Kim, J. D. Griffith, and C. C. Richardson, unpublished results.

FIG. 10. **The T7 gene 2.5 protein has sequence similarity to the consensus ATP binding sites of other proteins.** The alignment of homologous sequences in the ATP binding sites of the α- and β-subunits ATPase, ATP/ADP translocase, adenylate kinase, and phosphofructokinase are from Walker *et al.* (1982). The alignment of homologous sequences in *E. coli* recA protein and phage T4 uvsX protein are from Fujisawa *et al.* (1985). The *boxes* correspond to conserved residues. The encircled aspartic acid (*D*) found in close proximity to the *boxed region* in every case may be important in binding magnesium (Walker *et al.*, 1982).

in which aromatic amino acids participate in predominantly hydrophobic interactions with the nucleic acid bases.

T4 gene 32 protein (Alberts and Frey, 1970), and under some conditions *E. coli* SSB protein (see Meyer and Laine, 1990), bind to single-stranded DNA cooperatively. *E. coli* recA protein binds cooperatively to single-stranded DNA, both in the presence and absence of nucleoside triphosphates (Menetski and Kowalczykowski, 1985). By electron microscopy, a sensitive method for observing the binding of protein to DNA, the binding of gene 2.5 protein to single-stranded DNA appeared to be much less cooperative than that of T4 gene 32 protein. When subsaturating amounts of gene 32 protein are added to M13 single-stranded DNA, two populations of molecules are seen by electron microscopy. One species appears typical of protein free single-stranded DNA circles, and the second species appears as fully extended protein-covered loops. Variation of the protein to DNA ratio only varies the fraction of molecules in the two populations. Here when subsaturating amounts of gene 2.5 protein were added to M13 single-stranded DNA three populations were observed: protein-free DNA, molecules that appeared fully complexed by gene 2.5 protein, and molecules that appeared to be partially complexed by gene 2.5 protein. At subsaturating concentrations of gene 2.5 protein, the largest number of molecules was in the latter fraction, arguing for a relatively noncooperative binding to single-stranded DNA. The binding curve of single-stranded DNA to nitrocellulose obtained with increasing concentrations of a DNA-binding protein can provide information on the cooperativity of binding although the cooperative character of the binding assay may not be apparent if the cooperativity is very low or if the efficiency is very high (Woodbury and von Hippel, 1983). In the case of gene 2.5 protein (Figs. 3 and 4), either in the presence or absence of nucleotide 5′-triphosphates, the binding curve does not appear to be a perfect rectangular hyperbola that would be indicative of fully noncooperative binding. The slight but apparent sigmoidal nature observed may be indicative of limited cooperativity.

Upon infection of *E. coli* with phage T7 a single-stranded DNA renaturation activity is induced (Sadowski *et al.*, 1980). Sadowski *et al.* (1980) suggested that T7 gene 2.5 protein is responsible for this activity. We have recently demonstrated that gene 2.5 protein facilitates the renaturation of single-stranded DNA much more efficiently than does *E. coli* recA protein, *E. coli* SSB protein, or T4 gene 32 protein.[5] This reaction does not require Mg^{2+}; however, in the absence of Mg^{2+}, a higher NaCl concentration is required. Interestingly, in the electron micrographs of the complexes formed in the absence of Mg^{2+} and NaCl, conditions under which gene 2.5 protein does not stimulate renaturation, the complexes were spread out with little evidence of protein-protein interactions beyond the nearest neighbors (Fig. 7). On the other hand, in the presence of Mg^{2+} ions, conditions under which gene 2.5 protein does catalyze renaturation of single-stranded DNA, the single-stranded DNA appeared highly compact, with some indication of intrastrand interactions (Fig. 9). It is possible that these interactions are the results of gene 2.5 protein-gene 2.5 protein interactions beyond the nearest neighbors. Such interactions could be important for the catalysis of homologous pairing. On the other hand, it is possible that the compact structures represent the inherent tendency of single-stranded DNA to base pair with itself and form cross-bridges in the presence of Mg^{2+} ions; in this regard, gene 2.5 protein is much less effective at disrupting secondary structure than *E. coli* SSB protein or T4 gene 32 protein. We are currently investigating the mechanism by which the binding of gene 2.5 protein to single-stranded DNA facilitates its ability to renature to a homologous single-stranded DNA fragment. This activity is likely to be fundamental in the essential role of T7 gene 2.5 protein *in vivo* in recombination (Araki and Ogawa, 1981a, 1981b).

[5] S. Tabor and C. C. Richardson, unpublished results.

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