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Purification and Characterization of the Coliphage N4-coded Single-stranded DNA Binding Protein*

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We have purified and characterized a single-stranded DNA binding protein (N4 SSB) induced after coliphage N4 infection. It has a monomeric molecular weight of 31,000 and contains 10 tyrosine and 1–2 tryptophan amino acid residues. Its fluorescence spectrum is dominated by the tyrosine residues, and their fluorescence is quenched when the protein binds single-stranded DNA. Fluorescence quenching was used as an assay to quantitate binding of the protein to single-stranded nucleotides. The N4 single-stranded DNA binding protein binds cooperatively to single-stranded nucleic acids and binds single-stranded DNA more tightly than RNA. The binding involves displacement of cations from the DNA and anions from the protein. The apparent binding affinity is very salt-dependent, decreasing as much as 1,000-fold for a 10-fold increase in NaCl concentration. The degree of cooperativity (ω) is relatively independent of salt concentration. At 37 °C in 0.22 M NaCl, the protein has an intrinsic binding constant for M13 viral DNA of 3.8×10^4 M^{-1}, a cooperativity factor ω of 300, and binding site size of 11 nucleotides per monomer. The protein lowers the melting point of poly(dA·dT)·poly(dA-dT) by >60 °C but cannot lower the melting transition or assist in the renaturation of natural DNA. N4 single-stranded DNA binding protein enhances the rate of DNA synthesis catalyzed by the N4 DNA polymerase by increasing the processivity of the N4 DNA polymerase and melting out hairpin structures that block polymerization.

Proteins that bind to single-stranded DNAs with high affinity but without sequence specificity have been purified and characterized from several sources (1). Among these are the filamentous phages M13 and fd gene V products (2, 3), the phage Ike PIKE protein (4), the bacteriophage T4 gene 32 protein (5), the bacteriophage T7 gene 2.5 protein (6), the *Escherichia coli* F sex factor-coded SSF protein (7), and SSB protein, the single-stranded DNA binding protein present in uninfected *E. coli* (8). Biochemical studies have revealed that these proteins lower the melting temperature of DNA by binding stoichiometrically and, in many cases, cooperatively to single-stranded DNA (5). With the exception of SSB protein which stimulates the T7-coded DNA polymerase (9), single-stranded DNA binding proteins only stimulate their cognate DNA polymerases (10). Genetic and biochemical studies have shown that T4 gene 32 protein and *E. coli* SSB are required for replication, recombination, and repair (1, 11). Recent evidence suggests that both proteins are also involved in transcriptional activation (12, 13). fd and M13 gene V proteins are essential for viral replication, being responsible for the synthesis of viral progeny strands by binding to them and preventing their conversion to the duplex replicative intermediate (14). Furthermore, T4 gene 32 and fd gene V proteins are translational repressors of its own synthesis (15) and of fd gene II synthesis (16), respectively, by binding to the respective mRNAs.

Our laboratory has been studying the mechanism of replication of coliphage N4. The N4 genome is a linear double-stranded DNA molecule of 72 kilobase pairs with noncomplementary 3′ single-stranded ends (17, 18). Replication of the N4 genome is independent of the host DNA replication machinery responsible for initiation and elongation of DNA synthesis (19). Functions required for the processing of Okazaki fragments are required, however (19). Analysis of *in vivo* N4 replicative intermediates indicates that the origin of N4 DNA replication lies near or at the ends of the genome (20). In order to elucidate the mechanism of initiation and termination of N4 DNA replication, we have developed an *in vitro* DNA replication system from N4-infected cells which requires N4-coded functions (21). In this paper, we present the purification of the N4-coded single-stranded DNA binding protein (N4 SSB) using an *in vitro* complementation assay for N4 DNA replication with mutant-infected cell extracts. The physical and DNA binding properties of the purified protein are presented along with studies of its interaction with the N4-coded DNA polymerase (22).

MATERIALS AND METHODS

Bacterial Strains and Phages—*E. coli* D110 (*polA1 end1 thy*) and bacteriophage N4 wild type, *dnp am25*, and *dbp am33A7* were described previously (17, 23). N4 *dnp am25* contains an amber mutation in the structural gene for the DNA polymerase (22).

Chemicals—[^{3}H]dTTP (65 Ci/mmol) was purchased from ICN Pharmaceuticals and [α-^{32}P]dCTP (3000 Ci/mmol) and [γ-^{32}P]ATP (5,000 Ci/mmol) were purchased from Amersham Corp. Single-stranded DNA cellulose was prepared by the method of Litman *et al.* (24) using salmon testes DNA type III from Sigma and CF-11 dry cellulose from Whatman. The buffer used in all experiments was 50

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mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1 mM dithiothreitol. Other chemicals were as described (22).

DNAs—Template DNAs were prepared as described (22). All homopolymers and poly(dA-dT)·(dA-dT) were purchased from Pharmacia LKB Biotechnology Inc., dissolved in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and their concentrations determined spectrophotometrically using extinction coefficients provided by Pharmacia.

Preparation of Receptor Crude Extracts for Complementation Assay from dbp Amber Mutant N4-infected E. coli—E. coli D110 grown to 6 × 10^8 cells/ml were infected with *dbp am33A7* and crude extracts were made as described previously (21).

Replication and in Vitro Complementation Assays—The replication assay was performed as described previously (21). For *in vitro* complementation, receptor crude extract containing 10–15 μg of total protein and varying amounts of crude or fractionated donor extracts were added to the standard replication mixture and replication activity determined as described previously (21).

Purification of the N4-coded Single-stranded DNA Binding Protein—The starting material for purification was a crude lysate from 22 liters of N4-infected *E. coli* D110 cells and is identical to fraction 1 of the purification procedure for the N4-coded DNA polymerase described previously (22). To remove contaminating nucleotides, the lysate was dialyzed overnight against 4 liters of buffer A (50 mM Tris-HCl, pH 8, 1 mM EDTA, 2 mM 2-mercaptoethanol, 10% glycerol) plus 40 mM NaCl and incubated on ice for 90 min in the presence of 5 mM $CaCl_2$ and micrococcal nuclease at a concentration of 1 μg/ml. Those proteins that precipitated between 31.5 and 62.5% ammonium sulfate were resuspended in 30 ml of buffer A plus 40 mM NaCl and dialyzed against 4 liters of this buffer (Fraction II). Fraction II was loaded at 30 ml/h onto a 30-ml single-stranded DNA cellulose column equilibrated with buffer A plus 40 mM NaCl. The column was washed with 150 ml of buffer A plus 0.7 M NaCl, and the N4 SSB was eluted with a 300-ml linear gradient from 0.7 to 2 M NaCl in buffer A. Eluted fractions were analyzed by polyacrylamide gel electrophoresis (25), and those that contained N4 SSB in greater than 90% abundance were pooled and dialyzed into buffer B (50 mM KPO_4, pH 7.0, 1 mM EDTA, 1 mM 2-mercaptoethanol) plus 1.5 M $(NH_4)_2SO_4$ (Fraction III). N4 SSB eluted between 0.7 and 1.2 M NaCl. Fraction III was loaded onto a Pharmacia H-5/5 phenyl-Superose column equilibrated with buffer B plus 1.5 M $(NH_4)_2SO_4$, and, after washing with 10 column volumes of this buffer, the protein was eluted with a 60-column volume linear gradient of 1.5–0 M $(NH_4)_2SO_4$ in buffer B. Fractions from this column containing pure N4 SSB were dialyzed into storage buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl) and frozen at −70 °C (Fraction IV). The concentration of pure N4 SSB was determined by the method of Bradford (26), and the fractions taken at each step during the purification were analyzed on 15% polyacrylamide gels in the presence of sodium dodecyl sulfate (25).

Amino Acid Analysis—0.2 mg of N4 SSB was hydrolyzed according to the method of Inglis (27) in 1 ml of 4 M methylsulfonic acid containing 1% phenol and 0.8 mg/ml tryptamine. The protein was not reduced and alkylated prior to hydrolysis. The mixture of amino acids was adjusted to pH 2 and analyzed on a Beckman 118CL amino acid analyzer using a sodium citrate buffer and W3H column resin. The number of tyrosines and tryptophans present per monomer of N4 SSB was independently determined by the method of Goodwin and Morton (28).

Fluorescence Spectra and Titrations—Fluorescence spectra were obtained using a Perkin-Elmer MPF-4 fluorescence spectrophotometer fitted with a corrected spectra unit. The emission and excitation spectra were measured at 25 °C using a 3-ml cuvette, a scan speed of 60 nm/min, and slit widths of 2 and 3 nm for excitation and emission wavelengths, respectively.

Fluorescence titration data were collected on a Perkin-Elmer MPF-44E fluorescence spectrophotometer with slit widths of 5 nm for both excitation and emission wavelengths. Binding of polynucleotides to N4 SSB was followed by exciting at 274 nm and measuring the decrease in tyrosine fluorescence at 303 nm. A computer controlled Hamilton syringe was used to add successive amounts of titrant and data points were collected between additions as described previously (29). All fluorescence data were corrected for dilution due to addition of titrant. Forward titrations, in which protein is added to a cuvette containing nucleotide, were corrected by subtracting the linear increase in fluorescence due to addition of protein alone. This increase was determined by repeating each forward titration in the absence of polynucleotide. The binding site size (n) was determined by performing reverse titrations, in which nucleotide is added to a cuvette containing protein in standard binding buffer. We assumed the nucleotide binding sites on the protein were saturated when further additions of nucleotide no longer decreased protein fluorescence. During these titrations, changes in fluorescence due to inner filter effects were ignored because the total amount of polynucleotide added would have caused, at most, a 3% decrease in total fluorescence. The apparent affinity, $K\omega$, was determined by the "salt back-titration" method of Kowalczykowski *et al.* (29). During these titrations, inner filter effects were ignored since they do not affect the analysis. In all titrations, the equations of McGhee and Von Hippel (30) were used to quantitate the binding of N4 SSB to single-stranded DNA. Values for the intrinsic association constant, K, and cooperativity factor, ω, were determined by the method of Kowalczykowski *et al.* (31).

Denaturation and Renaturation of DNA by N4-coded Single-stranded DNA Binding Protein—Melting of poly(dA-dT)·poly(dA-dT) at 25 °C by N4 SSB was monitored by measuring the increase in absorbance at 260 nm upon addition of poly(dA-dT)·poly(dA-dT) to a solution of N4 SSB. Denaturation or renaturation of N4 DNA as a function of temperature in the presence of N4 SSB was followed by measuring the change in UV absorbance at 260 nm using a Hewlett Packard 8450A spectrophotometer with a cuvette thermostatically controlled by a Hewlett Packard 89100A Temperature Controller set to increase the temperature of the cuvette chamber 1 °C/min.

Stimulation of N4-coded DNA Polymerase—The templates and assays were as published (4) except for addition of N4 SSB as outlined in each figure legend. Reactions containing N4 SSB had [α-^{32}P]dCTP (20 Ci/mM) and were terminated by adding EDTA to 15 mM and heating for 10 min at 65 °C. Then $MgCl_2$ and NaCl were added to 30 and 60 mM, respectively. The DNA was cleaved with 5 units of *AluI* at 37 °C for 3 h, ethanol-precipitated, and analyzed on an 8% polyacrylamide sequencing gel.

RESULTS

In Vitro Complementation between dbp 33am7 Replication Extracts and Other DNA Replication-deficient Extracts—We have developed a crude *in vitro* replication system that is specific for exogenous N4 DNA and is dependent on the activity of N4-coded functions required for *in vivo* replication (21). As shown in Fig. 1, replication extracts from *dbp am33A7*-infected cells are unable to replicate DNA *in vitro*. However, addition of *dnp am25*-infected cell extracts, which are also unable to replicate N4 DNA (21), restores *in vitro*

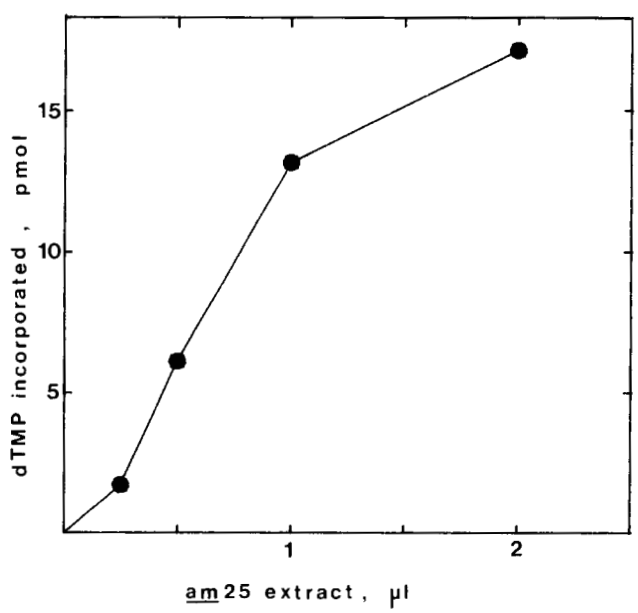

FIG. 1. **Complementation of *dbp am33A7*-infected cell extracts for *in vitro* N4 DNA synthesis by *dnp am25*-infected cell extracts.** 50 μl *in vitro* N4 DNA replication reactions containing 2 μg of N4 DNA, 0.5 μl of *dbp am33A7*-infected cell extract, and the indicated amount of *dnp am25*-infected cell extracts were performed as described under "Materials and Methods."

DNA replication activity. This assay allowed us to purify the protein missing in *dbp am*33A7-infected cell extracts and subsequently identify it as a N4-induced, single-stranded DNA binding protein.

Purification of the N4 Single-stranded DNA Binding Protein—Initially, the N4 SSB purification required four columns (32). The purified protein showed a very high affinity for single-stranded DNA and had no detectable RNA polymerase, DNA polymerase, ATPase, or primase activities (32). A nitrocellulose filter retention assay demonstrated that the protein had a much higher binding affinity for single-stranded than double-stranded DNA (32). Therefore, a faster purification strategy, using single-stranded DNA affinity chromatography as the first step, was developed (Table I). Fractions I and II of the purification do not restore *in vitro* DNA replication activity to *dbp am*33A7-infected cell extracts but in the remaining purification steps *dbp am*33A7 complementing activity copurifies with single-stranded DNA binding activity. Because of our inability to assay for N4 SSB in Fractions I and II, the 35% yield in Fraction III is estimated from the relative amounts of N4 SSB present in single-stranded DNA cellulose column fractions as judged by band intensities on silver-stained polyacrylamide gels. The increase in total activity after phenyl-Superose chromatography is unexpected as the total amount of protein in Fractions III and IV is almost identical. The difference in activity may be due to the extremely low protein concentration of Fraction III or the removal of a potent inhibitor of replication activity. Fig. 2 shows that Fraction IV consists of a single polypeptide with a monomeric molecular weight of 31,000. This suggests that the N4 single-stranded DNA binding protein is the only protein missing from *dbp am*33A7-infected cells and therefore the N4 *dbp* gene codes directly for it or controls its induction. The native protein aggregates in solutions of 0.1 or 0.4 M KCl (32).

Amino Acid Analysis of the N4-coded Single-stranded DNA Binding Protein—The bacteriophage T4 gene 32 protein and fd phage gene 5 protein are single-stranded DNA binding proteins containing phenylalanine and tyrosine residues which are thought to interact with DNA bases when they bind to single-stranded DNA (33–36). Therefore, the number of aromatic amino acid residues present in N4 SSB was determined by two methods. Table II lists the amino acid composition after hydrolyzing the protein in 4 M methylsulfonic acid to quantitate tryptophan. The predicted molecular weight from the amino acid composition is 30,092 and is in good agreement with the molecular weight determined by polyacrylamide gel electrophoresis. The analysis shows that each molecule of N4 SSB contains 17 phenylalanines, 10 tyrosines, and 2 tryptophans.

TABLE I

Purification of N4 single-stranded DNA binding protein

Fraction	Volume	Protein	Activity	Purification	Yield
	ml	*mg*	*units*[a]	*-fold*	*%*
I. S100 lysate	296	947.2			
II. 30–60% ammonium sulfate precipitate	16	461			
III. Single-stranded DNA cellulose	108.5	4.4	6.32×10^4	75[b]	35
IV. Phenyl-Superose	6	4.5	2.14×10^5	244	35

[a] One unit is the amount of protein which catalyzes the incorporation of 1 pmol of nucleotide into acid-precipitable material in 20 min at 30 °C.

[b] Calculated from amount of N4 SSB and total *E. coli* protein in S100 lysate.

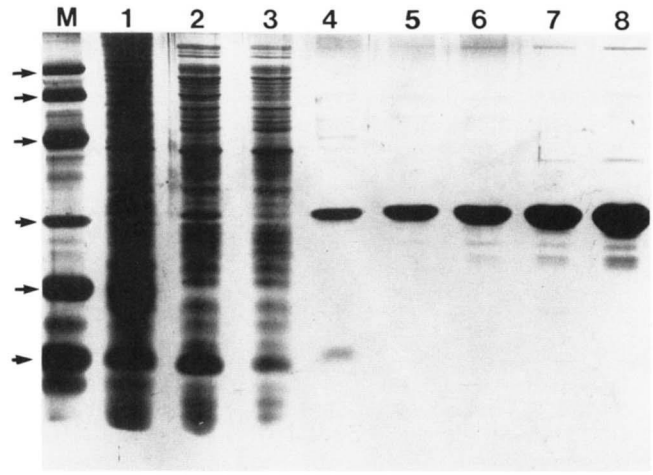

FIG. 2. **Polyacrylamide gel electrophoresis of isolated protein fractions during N4 SSB purification.** Protein fractions at various stages of the SSB purification were subjected to 15% polyacrylamide sodium dodecyl sulfate gel electrophoresis (23) and the proteins visualized by silver staining (55). The lane marked *M* contains a mixture of the following proteins from *top* to *bottom*: phosphorylase B (M_r 97,400), bovine serum albumin (M_r 66,200), ovalbumin (M_r 42,699), carbonic anhydrase (M_r 31,000), soybean trypsin inhibitor (M_r 21,500), and lysozyme (M_r 14,400) as molecular weight markers. *Lane 1* contains 16 μg of Fraction 1 in Table I. *Lanes 2* and *3* contain 8 μg of the single-stranded DNA column load and flow-through fractions respectively. *Lane 4* contains 2.25 μg of the pooled single-stranded DNA cellulose column fractions. *Lane 5–8* contain 2.25, 4.5, 7.5 and 15 μg of the purified N4 SSB protein.

TABLE II

Amino acid composition of N4 single-stranded DNA binding protein

Amino acid	Molar ratio	Amino acid	Molar ratio	Amino acid	Molar ratio
Ala	38	His	4	Pro	9
Arg	5	Ile	11	Ser	18
Asx	28	Leu	20	Thr	21
Cys	1	Lys	20	Trp	2
Gly	40	Met	1	Tyr	10
Glx	33	Phe	11	Val	18

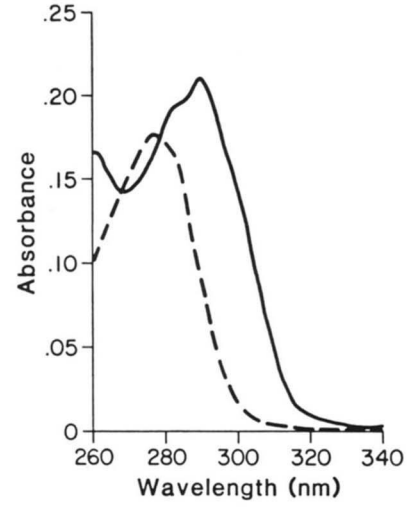

FIG. 3. **Ultraviolet light absorbance spectrum of N4 SSB at pH 2 (– – –) and 13 (——).** N4 SSB was diluted 2-fold into solutions of 0.01 M HCl or 0.1 M NaOH and the absorbance measured as a function of wavelength in a Perkin Elmer λ 5 UV/VIS spectrophotometer with a scan speed of 30 nm/min, a slit width of 2 mm, and a 0.5-s response time.

Fig. 3 shows the UV absorbance spectra for N4 SSB at pH 2 and 13. Analysis of the spectra by the method of Goodwin and Morton (28) predicts 11.1 tyrosines and 0.88 tryptophans per monomer of N4 SSB which is in close agreement with the amino acid analysis for those two amino acids. At this point we do not know the number of surface and buried tyrosine residues in N4 SSB.

Fluorescence Spectrum of SSB—The intrinsic fluorescence of aromatic amino acid residues in the binding sites of fd phage and T4 phage single-stranded DNA binding proteins is quenched appreciably when these proteins bind single-stranded DNA, and fluorescence quenching has been used as an assay to quantitate the binding of these proteins to single-stranded DNA (29, 37). To that end, we investigated the fluorescence properties of the N4 SSB. The emission spectrum closely matches that of *N*-acetyltyrosinamide with a maximum emission centered around 303 nm (Fig. 4*a*). If the protein is excited at 295 nm, where only tryptophan residues are expected to absorb energy, there is only a slight emission centered around 345 nm and almost total loss of fluorescence at 303 nm (data not shown). This suggests that fluorescence in the native protein is due to tyrosine emission and not to tryptophan fluorescence that has been shifted to lower wave lengths. Fig. 4*b* shows the fluorescence spectrum of N4 SSB in 6 M guanidine HCl when excited at 277 nm. The emission spectrum more closely matches the spectrum of tryptophan fluorescence free in solution as expected for a denatured protein containing both tryptophan and tyrosine. The two spectra indicate that in the native protein tryptophan fluorescence is quenched and that there is no energy transfer from the fluorescent tyrosine side chains to the tryptophan residues.

Fig. 4*a* also shows that the fluorescence emission of the native protein centered at 303 nm is quenched 50%, after correcting for inner filter effects due to poly(dA_{200}), in the presence of a saturating concentration of poly(dA_{200}). In contrast, the decrease in protein fluorescence in 6 M guanidine HCl (see Fig. 4*b*) is entirely due to inner filter effects. With subsaturating amounts of poly(dA_{200}), the percent of maximum quenching for the native protein is directly proportional to the concentration of poly(dA_{200}). Since this relationship was independent of protein concentration, we used fluorescence quenching to quantitate binding of N4 SSB to single-stranded DNAs.

Binding Site Size of N4 SSB Protein—As a first step in quantitating the binding of N4 SSB to single-stranded DNA, the binding site size, *n*, was determined for a variety of polynucleotides. These values were obtained through a series of reverse titrations in standard binding buffer. Fig. 5*a* shows examples with poly(dA_{200}) at three different protein concentrations. In the standard buffer, nucleotide binding is expected to be stoichiometric and *n* is obtained from the ratio of the nucleotide to protein concentration at saturation as defined under "Materials and Methods." The results are presented in Table III along with the percent maximal quenching for each polynucleotide. With the exception of poly(dU) and poly(rA), each value of *n* is the average of three reverse titrations performed at different protein concentrations. The binding site size remains constant for all polynucleotides tested and is equal to 11 ± 2. N4 SSB has a high affinity to poly(dT_{200}) and will bind stoichiometrically in buffers containing 0.4 M NaCl. A series of reverse titrations with poly(dT_{200}) in 0.4 M NaCl gives a binding site size of 11 as well (data not shown), indicating that the binding site size of N4 SSB does not change with salt concentration. The maximal percent of protein fluorescence quenching varies from polynucleotide to polynucleotide (Table II). However, there is no direct correlation between the percent of maximum quenching and the polynucleotide composition (*i.e.* purines *versus* pyrimidines),

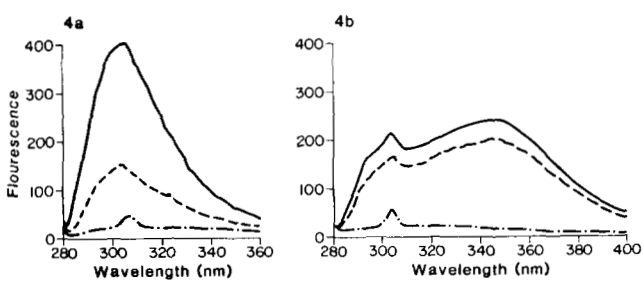

FIG. 4. **Fluorescence emission spectra of N4 SSB.** *a*, the fluorescence emission spectra of a 2 μM solution of N4 SSB either alone (——) or in the presence of 30 μM poly(dA) (– – –) was measured as described under "Materials and Methods" using an excitation frequency of 277 nm. Fluorescence emission spectrum of the standard binding buffer is also shown (– · –). *b*, fluorescence emission spectra of 6 M guanidine hydrochloride (– · –) and 1.3 μM N4 SSB in 6 M guanidine hydrochloride alone (——) or in the presence of 30 μM poly(dA) (– – –) using an excitation frequency of 277 nm.

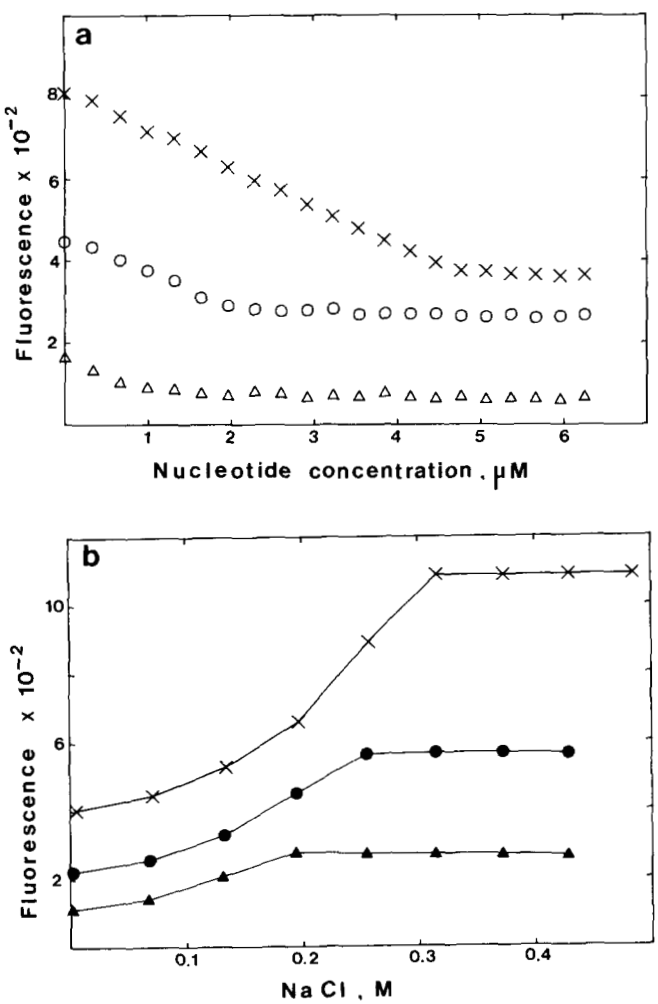

FIG. 5. **Stoichiometric binding (*a*) and salt back-titration (*b*) of N4 SSB to poly(dA).** *a*, fluorescence of N4 SSB at concentrations of 0.086 μM (△), 0.172 μM (○), and 0.344 μM (×) was measured as a function of poly(dA) concentration as described under "Materials and Methods." *b*, fluorescence of N4 SSB at concentrations of 0.29 μM (△), 0.58 μM (○), and 1.164 μM (×) and containing just enough poly(dA_{200}) to saturate the protein, assuming a binding site size of 11 nucleotides, was measured as a function of NaCl concentration as described under "Materials and Methods."

TABLE III

Binding parameters of N4 single-stranded DNA binding protein to different polynucleotides

Polynucleotide	n	Q_{max}	log $K\omega$/log[NaCl]	[NaCl][a]	$K\omega$[b]
		%		M	$M^{-1} \times 10^{-6}$
Poly(rU)	9.5 ± 0.5	71 ± 13	−1.8 ± 0.2	0.059	0.38
Poly(rA)	10.7[c]	63[c]	−2.1 ± 0.2	0.072	0.38
Poly(dA)	12.2 ± 1.1	50 ± 7	−2.2 ± 0.1	0.15	1.8
Poly(dU)	11.4[c]	84[c]	−2.6 ± 0.3	0.70	81.0
Poly(dI)	11.7 ± 1.1	70 ± 2	−2.5 ± 0.7	0.68	60.0
Poly(rI)	11.2 ± 2	82 ± 10	−2.6 ± 0.8	0.34	13.0
M13 25 °C	10.9 ± 0.03	86 ± 7	−5.2 ± 3	0.41	105.0
M13 37 °C		86 ± 4	−2.2 ± 0.5	0.28	7.0
poly(dU)-NaBr	10.5 ± 1.32		−3.4 ± 0.3	0.39	27.0

[a] At $K\omega = 1 \times 10^7$.
[b] At 0.22 M NaCl.
[c] Only two data points were collected so standard deviations could not be calculated.

the degree of stacking between bases and/or the strength of binding to N4 SSB.

Binding Affinities of N4 SSB to Different Polynucleotides—The binding affinity, $K\omega$, of the N4 SSB to different polynucleotides as a function of salt concentration was determined using the salt back-titration method of Kowalczykowski *et al.* (29). In this method, NaCl is added to a solution of N4 SSB that is saturated with polynucleotide. As the salt concentration increases, protein-polynucleotide complexes dissociate resulting in an increase in protein fluorescence. Total release of nucleotide is assumed when further increases in salt concentration cause no further increase in protein fluorescence. The final value of fluorescence was always 90–100% of the initial uncomplexed protein fluorescence. Fig. 5*b* shows protein fluorescence as a function of NaCl concentration from three salt back-titrations performed with poly(dA_{200}). The midpoint of each salt back-titration, defined by the salt concentration where one-half of the protein fluorescence initially quenched by the polynucleotide has been recovered, is assumed to represent the point at which half of the polynucleotide is still bound by protein. The binding isotherms of McGhee and Von Hippel (30) predict that, for cooperatively binding proteins ($\omega > 10\ n$), $K\omega$ is equal to the inverse of free protein concentration at half-saturation. The N4 SSB shows moderate cooperativity when binding to polynucleotides (see below) and values of $K\omega$ for poly(dA_{200}) were determined from the midpoints of each of the salt back-titrations in Fig. 5*b*. The results, along with those obtained at two other initial protein concentrations and with other homopolymers, are plotted in Fig. 6 according to the method of Record *et al.* (38). The data points for each homopolymer show a linear dependence of log($K\omega$) on log[NaCl], and Table III lists the slope of each line that best fits the data points for that homopolymer. Poly(dT_{200}) was not included in this set of experiments since it binds too tightly to N4 SSB and is not released until the salt concentration exceeds 1 M (data not shown). Poly(rC_{200}) was not included since it did not bind N4 SSB in the standard buffer (data not shown).

The relative affinity of N4 SSB for each polynucleotide can be determined from the data presented in Fig. 6 by comparing values of $K\omega$ at a given salt concentration or values of salt concentration needed to give a common value of $K\omega$. Table III lists the expected affinity of each polynucleotide for N4 SSB at 0.22 M NaCl, and the salt concentration for each polynucleotide needed to achieve a $K\omega$ value of 4×10^6. The

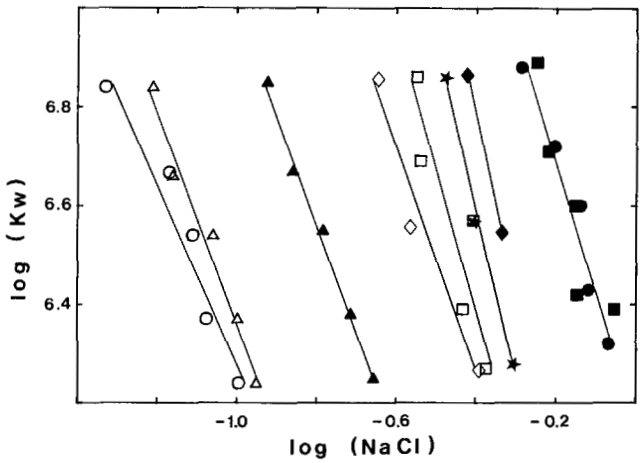

FIG. 6. **Binding affinity of N4 SSB to different polynucleotides.** The salt back-titrations performed for each polynucleotide were as described in Fig. 5. ○, poly(rU); △, poly(rA); ▲, poly(dA); □, poly(rI); ■, poly(dI); ●, poly(dU); ◆, M13 viral DNA at 25 °C; ◇, M13 viral DNA at 37 °C; ★, poly(dU) in NaBr instead of NaCl.

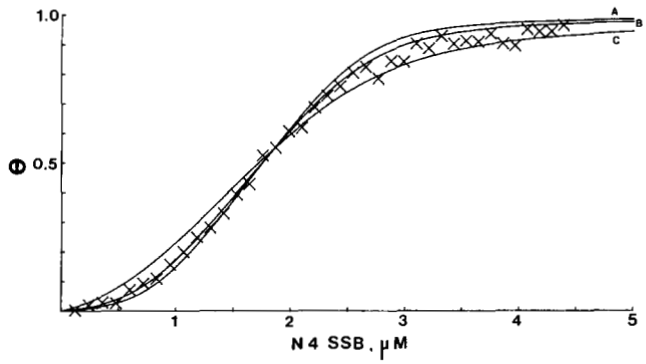

FIG. 7. **Forward titration of N4 SSB with M13 (+) single-stranded DNA.** The forward titration was performed in 0.22 M NaCl at 37 °C and a M13 (+) single-stranded DNA concentration of 1.57 μM as described under "Materials and Methods." Θ represents fractional lattice saturation. *Solid lines* represent theoretical curves in which $n = 11$ and $K = 1.1 \times 10^5$, $\omega = 100$ for *A*; $K = 3.8 \times 10^4$, $\omega = 300$ for *B*; $K = 2.3 \times 10^4$; $\omega = 500$ for *C*.

data show that N4 SSB has different affinities for different homopolymers but maintains the same binding site size. In addition, N4 SSB always shows a higher binding affinity for a particular deoxyribopolynucleotide *versus* the same ribopolynucleotide. Two series of salt back-titrations with M13 DNA at 25 and 37 °C (Fig. 6) show that the binding affinity of N4 SSB for single-stranded DNA is lower at higher temperatures.

Forward Titrations of N4 SSB Protein and Nucleic Acids—The binding specificity demonstrated by N4 SSB in Fig. 6 could be due to differences in the value of K or ω for each polynucleotide. The salt back-titration method cannot easily determine independent values for K and ω. Therefore a series of forward titrations were performed with natural and synthetic DNAs at salt concentrations high enough so that the binding was nonstoichiometric. Fig. 7 shows such a titration with M13 DNA at 37 °C in standard binding buffer containing 0.22 M NaCl. Using the McGhee and Von Hippel theoretical binding isotherms (29, 30), the data are best fit with an intrinsic association constant of $3.8 \times 10^4\ M^{-1}$ and a cooperativity factor of 300. The values of K and ω from Fig. 8 are listed in Table IV along with values obtained from additional forward titrations. In general, the product of $K\omega$ for each titration listed in Table IV is in excellent agreement with the value extrapolated from Fig. 7. Over a range of salt concen-

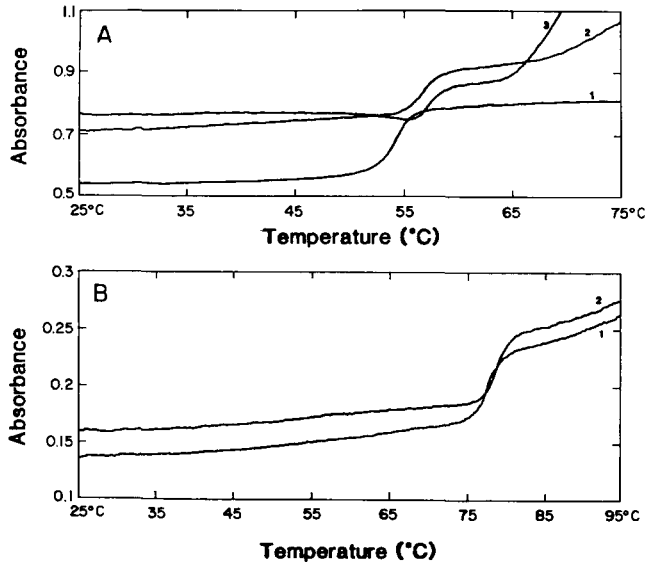

FIG. 8. **Melting of poly(dA-dT)·(dA-dT) (*A*) and N4 DNA (*B*) by N4 SSB.** *A*, *curves 1*, *2*, and *3* are the absorbance of a 63 μM solution of poly(dA-dT)·(dA-dT) in the presence of 0 μM SSB, 0 mM NaCl; 3.1 μM SSB, 12 mM NaCl; 4.7 μM SSB, 18 mM NaCl, respectively. *B*, *curves 1* and *2* are the absorbance of a 20 μM solution of N4 DNA in standard binding buffer alone or in the presence of 2.0 μM N4 SSB and 7.7 mM NaCl (2), respectively.

TABLE IV

Summary of binding parameters from "forward" titrations of N4 single-stranded DNA binding protein to different polynucleotides

Polynucleotide	NaCl	n	$K \times 10^{-4}$ [a]	ω [a]	$K\omega$	$K\omega$ [b]
	M		M^{-1}		$M^{-1} \times 10^{-6}$	
Poly(dU)	0.6	11	5.4 ± 0.6	200 ± 25	9.2	5.4
Poly(dA)	0.12	11	2.3 ± 0.3	300 ± 50	6.9	7.0
Poly(rA)	0.05	11	6.5 ± 0.3	150 ± 50	9.8	10.0
M13 DNA 25 °C	0.30	11	3.9 ± 1	250 ± 50	9.8	16.0
M13 DNA 37 °C	0.22	11	3.8 ± 2	300 ± 50	11.4	6.6

[a] Error values listed for K and ω represent the maximum deviations of K and ω that would still generate a theoretical curve fitting the data with $K\omega$ fixed at the experimentally observed value.

[b] From Fig. 6.

tration from 0.05 to 0.6 M, the value of ω is relatively constant and changes, at most, by a factor of 2. This implies that the value of ω is independent of salt concentration and that the changes in $K\omega$ observed in Fig. 6 are due to changes in K. The last two forward titrations listed in Table IV show that ω is also temperature-independent, and therefore the decrease in binding affinity with increasing temperature shown in Fig. 6 is caused by a decrease in K as well.

Ionic Interactions between N4 SSB Protein and Nucleic Acids—Record *et al.* (38) have shown that the slopes of log K (or log $K\omega$, since ω is independent of salt concentration) *versus* log [NaCl] plots can represent $m'\psi$ or the number of ion pairs formed on protein binding multiplied by the thermodynamic extent of counterion "binding" to the polynucleotide free in solution (38). This assumes that the major force in promoting complex formation is the release of cations from polynucleotides upon protein binding and that anion release from protein upon complex formation is negligible (38). To see if anions were involved in binding, salt back-titrations were performed on poly(dU) with buffers and salt solutions containing bromide instead of chloride. As can be seen in Fig. 6 and Table III, the slope of the log $K\omega$ *versus* log [NaCl] plot increases and N4 SSB·poly(dU) complexes are less stable in bromide-containing buffers. We were unable to quantitate binding in the presence of phosphate, glutamate, or acetate, since these anions strongly quench the tyrosine fluorescence of N4 SSB. Thus, there is a net displacement of, at most, three sodium or chloride ions upon formation of the N4 SSB·SS DNA complex.

Effect of N4 SSB on Denaturation of Duplex DNA—Single-stranded DNA binding proteins can substantially lower the melting temperature of duplex DNA by complexing single-stranded DNA and shifting the helix-coil equilibrium to favor the single-stranded form (5). Fig. 8*A*, *curve 1*, shows the A_{260} of a solution of poly(dA-dT)·poly(dA-dT) as a function of temperature in the absence of N4 SSB. A single melting transition at 54 °C is observed. *Curves 2* and *3* represent the melting profile of an identical amount poly(dA-dT)·poly(dA-dT) in the presence of enough N4 SSB to saturate 50 and 75% of the polynucleotide, respectively, assuming a binding site size of 11. Again, a single melting transition is observed but the total change in absorbance is only 50 and 25% of the transition observed in the absence of N4 SSB. Since N4 SSB binds cooperatively to DNA and the transitions correspond to the amount of poly(dA-dT)·poly(dA-dT) uncomplexed with protein, we assume the melting transition of complexed DNA is below 25 °C. The dissociation of a small amount of N4 SSB·DNA complex due to the presence of salt in N4 SSB storage buffer produced the slight decrease in absorbance before the transition in *curve 3*. The higher salt concentration also shifted the poly(dA-dT)·poly(dA-dT) melting transition to 57 °C. The large increases in absorbance above 65 °C are due to light scattering from denaturation of N4 SSB.

The discrete changes in the melting profile of poly(dA-dT)·poly(dA-dT) by increasing amounts of N4 SSB suggest that it denatures DNA in a stoichiometric and cooperative fashion. Fig. 9 shows the increase in A_{260} of a solution containing N4 SSB as a function of poly(dA-dT)·poly(dA-dT) concentration. At lower polynucleotide concentrations, the increase in A_{260} is above what would be expected from the addition of double-stranded DNA alone. The additional change in A_{260} is due to the hyperchromic effect associated with melting poly(dA·dT)·poly(dA-dT). At nucleotide concentrations above 0.42 μM, further increases in A_{260} correspond to those expected for increases in DNA concentration alone. Extrap-

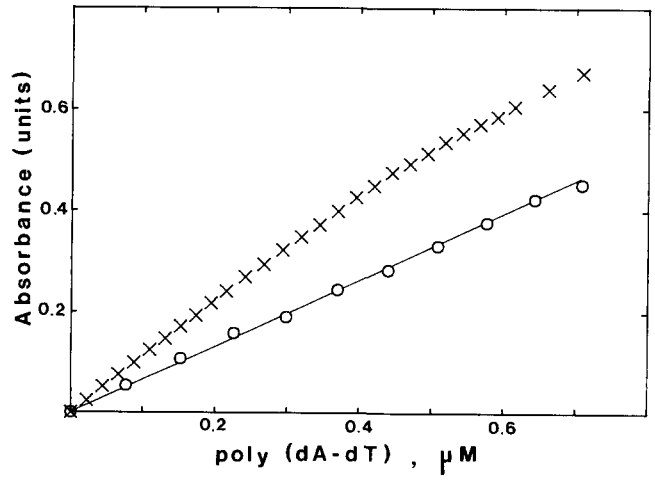

FIG. 9. **Titration of poly(dA)·poly(dT) by the N4 SSB.** The absorbance of a 3.3 μM solution of N4 SSB in standard binding buffer (×) as a function of poly(dA-dT)·poly(dA-dT) concentration was measured as described under "Materials and Methods." The absorbance of poly(dA-dT)·(dA-dT) as a function of concentration (O) in standard binding buffer is also plotted.

olation from the two linear portions of the curve presented in Fig. 9 predicts a binding site size of 12.7 nucleotides. This is slightly higher than the value obtained from the fluorescence titration data, but confirms that N4 SSB denatures poly(dA-dT)·poly(dA-dT) stoichiometrically.

In contrast to the results with poly(dA-dT)·(dA-dT), N4 SSB, even in saturating amounts, does not lower the melting transition of N4 DNA (compare *curves 1* and *2* in Fig. 8*B*). Furthermore, N4 SSB cannot enhance the rate of renaturation of denatured N4 DNA in standard binding buffer alone or in the presence of either 5 mM $MgCl_2$, 1 mM spermidine, increased concentrations of NaCl or in buffers of lower pH (data not shown).

Stimulation of N4 DNA Polymerase by N4 SSB—Single-stranded DNA binding proteins are known to play a direct role in DNA replication, recombination, and repair (1, 11). *In vitro* studies have shown that a single-stranded DNA binding protein isolated from a particular organism will stimulate the rate of DNA synthesis of its cognate DNA polymerase. Fig. 10 shows that the N4-coded DNA polymerase is stimulated up to 30-fold by N4 SSB. Other single-stranded DNA binding proteins tested provide little stimulation of DNA synthesis and, at higher concentrations, some inhibition is observed. Under similar reaction conditions, N4 SSB is unable to stimulate the rate of DNA synthesis by the *E. coli* DNA polymerase I, T4 phage DNA polymerase, or yeast DNA polymerase I (data not shown).

The increased level of DNA synthesis seen in Fig. 10 could be due to an increase in the rate of initiation or in the processivity of the N4 DNA polymerase. Support for the latter mechanism comes from the observation that the amount of stimulation of DNA synthesis by N4 SSB is higher with DNA templates that contain longer stretches of single-stranded DNA. If DNase I "activated" calf thymus DNA is used as a template, the rate of DNA synthesis is stimulated only 5-fold by N4 SSB. In contrast, there is an 800-fold stimulation when denatured N4 DNA is used as a template (data not shown).

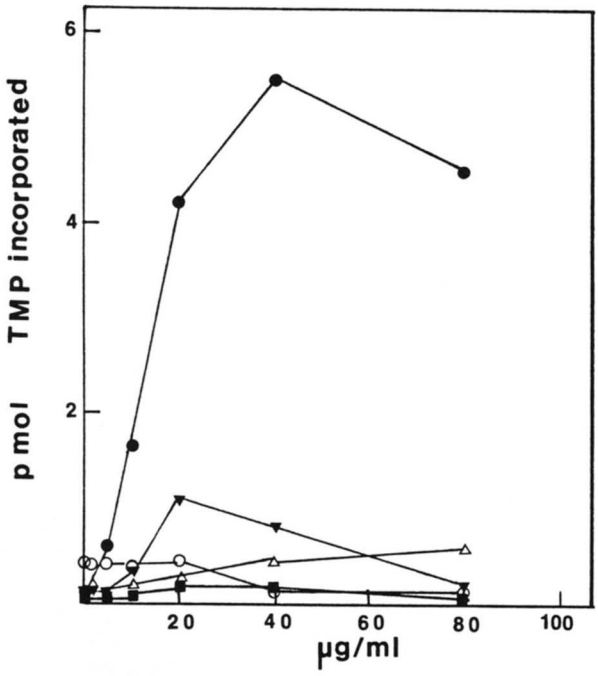

FIG. 10. **N4 DNA polymerase is stimulated specifically by N4 SSB.** DNA polymerase reactions (0.05 ml) contained 600 pmol of the hybrid of ϕX174 viral (+) single-stranded DNA and RF *Hae*III fragment 7, 0.04 unit of N4 DNA polymerase, and increasing concentrations of the following single-stranded DNA binding proteins: ●, N4 SSB; ▼, T4 phage gene 32 protein; ○, *E. coli* SSB; △, T7 gene product 2.5; and ■, fd phage gene V.

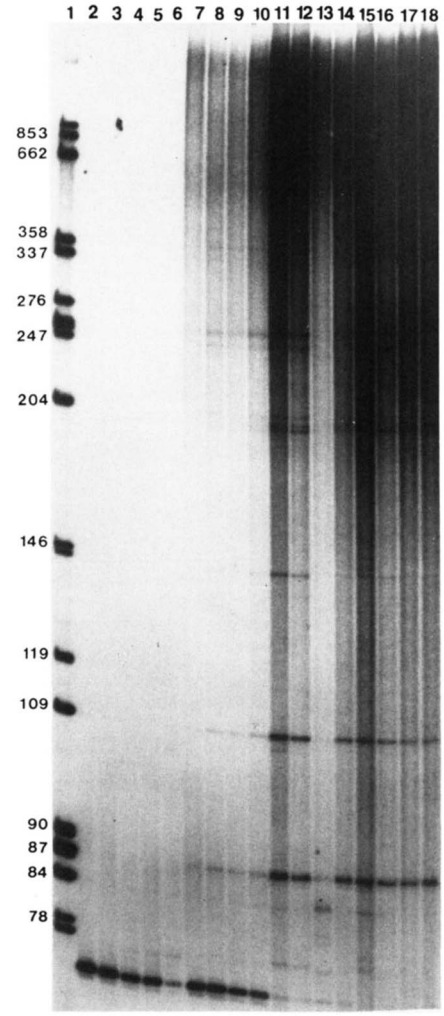

FIG. 11. **N4 SSB enhances the processivity of the N4 DNA polymerase.** *Lane 1* contains *Alu*I-restricted ϕX174 RF DNA fragments ^{32}P-3′-end-labeled as described previously. Reactions contained 200 microunits of N4 DNA polymerase and 600 pmol of template. Incubation was at 30 °C for the indicated lengths of time. *Lanes 2*, 0 s; *3*, 20 s; *4*, 40 s; *5*, 1 min; *6*, 3 min. *Lanes 7–10* are identical to *lanes 3–6* except that reactions contained 24 pmol of N4 SSB. *Lanes 11–14* are identical to *lanes 3–6* except that each reaction contained 60 pmol of N4 SSB. *Lanes 15–18* are identical to *lanes 3–6* except that each reaction contained 120 pmol of N4 SSB.

To identify the mechanism of stimulation, we analyzed the effect of N4 SSB on DNA synthesis by N4 DNA polymerase on ϕX174 (+) single-stranded DNA uniquely primed with ^{32}P-3′-end-labeled *Hae*III restriction Fragment 10 of ϕX174 RF DNA. Reactions containing the N4 DNA polymerase alone were analyzed directly on a 6 M urea, 8% polyacrylamide gel. Synthesis products from reactions containing N4 SSB were too long to enter the gel and were digested by the restriction enzyme *Alu*I before gel separation. In addition, these reactions were carried out in the presence of [α-^{32}P]dCTP so that the entire length of the products could be visualized.

Products of N4 DNA polymerase are very short, even when the majority of the primers have been extended (*lanes 3–6*, Fig. 11). The addition of N4 SSB has two effects on the synthetic reaction. First, the product lengths now exceed 2,000 base pairs as indicated by the appearance of *Alu*I fragments distant from the primer. Second, in reactions that contain an amount of N4 SSB that covers only two-fifths of the template

(see *lanes 7–10*), N4 SSB decreases the proportion of primers that are extended within a given time interval. Taken together, these results indicate that N4 SSB increases the processivity of the DNA polymerase. When saturating amounts of N4 SSB are present (*lanes 11–14*), all of the primers are extended.

DISCUSSION

The N4 single-stranded DNA binding protein, originally detected as the activity capable of complementing the *dbp am*33A7 *in vitro* DNA replication extract has been purified to apparent homogeneity and characterized. It is expressed at high levels during infection, and the final yield of protein in Table I corresponds to a total *in vivo* concentration of 9–18 μM or about 11,000 molecules of N4 SSB/cell. This is comparable to the levels of gene 32 protein produced during a T4 infection (5). It is not known, however, whether N4 SSB autoregulates the level of its expression.

The purified protein has a molecular weight of 31,000 and is the same size as single-stranded DNA binding proteins produced by bacteriophage T4 and T7 (5, 6). In comparison, the single-stranded DNA binding proteins produced by fd phage and *E. coli* have monomeric molecular weights of 9,700 and 19,500, respectively (3, 39). Affinity chromatography with purified T4 gene 32 protein shows that this protein can interact with a large number of both T4 and *E. coli* encoded proteins (40). In addition, Mosig *et al.* (41) have characterized a large number of allele-specific extragenic suppressors of gene 32 mutations. The larger size of the T4 single-stranded DNA binding protein may be due to additional protein domains required for additional protein-protein interactions. Whether the N4 single-stranded DNA binding protein interacts with other phage or host proteins and binds the N4 DNA polymerase is, at this point, unknown.

As with other single-stranded DNA binding proteins, N4 SSB seems to interact with single-stranded DNA through surface tyrosine residues. We have used the quenching of tyrosine fluorescence to characterize the interaction between N4 SSB and single-stranded DNA in terms of three thermodynamic parameters, n, K, and ω, as described by McGhee and Von Hippel (30). The data in Fig. 6 show that N4 SSB has different affinities for different homopolymers and hence has compositional specificity. The differences in affinity result from changes in K, the intrinsic binding constant of the protein for each of the homopolymers, and not in ω. This is consistent with observations made with fd gene 5, T4 gene 32, and recA proteins (37, 42, 43). The cooperativity amplifies the differences in K, resulting in an even greater discrimination among the different homopolymers by the N4 SSB. The relative affinities for the different homopolymers by N4 SSB are similar to those of the T4 gene 32 protein, the *E. coli* SSB protein and the recA protein of *E. coli* (42, 44, 45). A detailed molecular mechanism for this sequence specificity is unknown. This similarity suggests that either the structure of the binding domain is common to all these proteins (46) or that the strength of the interaction is determined by the structural properties of the different homopolymers in solution, or a combination of both.

The N4 SSB has a binding site size of 11 nucleotides, an intrinsic binding constant of 3.8×10^4 M^{-1} and a cooperativity of 300 when it binds single-stranded DNA in 0.22 M NaCl and at 37 °C. The N4 SSB binding site is the largest among the bacteriophage-coded, single-stranded DNA binding proteins characterized thus far. In common with these binding proteins, the N4 SSB site size is independent of salt concentration indicating a single binding mode. This is in contrast to the multiple binding modes observed when *E. coli* SSB protein interacts with DNA at different salt concentrations (47). The value of ω is intermediate to the values reported for other bacteriophage single-stranded DNA binding proteins. The T4 gene 32, fd gene V, and *E. coli* SSB proteins have cooperativity values of approximately 2,000, 200, and 420, respectively (37, 42, 48). Based on its similarity with other phage-coded, single-stranded DNA binding proteins, we propose that the N4 SSB binds with unlimited cooperativity to single-stranded DNAs. However, we cannot rule out the possibility of limited cooperativity as observed with *E. coli* SSB protein (48). The intrinsic binding constant of N4 SSB for M13 DNA is almost identical to that of the T4 gene 32 protein at both 25 and 37 °C (42, 49).

Considering the *in vivo* concentration of N4 SSB, its binding affinity and larger binding site size, and assuming *in vivo* concentrations of single-stranded DNA in N4 infections is comparable to T4 infections, we can predict that there is sufficient N4 SSB to saturate all the single-stranded DNA present during an N4 infection. The binding parameters at 0.22 M NaCl (Fig. 7) assumed to reflect the intracellular *E. coli* salt concentration were used in these calculations. The major intracellular cationic and anionic species present in *E. coli* are potassium and glutamate, respectively (48). We expect that replacing potassium for sodium would have little effect on the binding affinity of N4 SSB for single-stranded DNA. However, changing the nature of the anion present can have a strong effect on the binding affinity of many proteins for DNA (42, 43, 51). Fig. 6 shows that N4 SSB has a lower affinity for single-stranded DNA when chloride has been replaced with bromide. Therefore, we expect that N4 SSB has an even greater affinity for single-stranded DNA *in vivo* than what we calculate from our *in vitro* results.

N4 SSB is able to lower the melting transition of poly(dA-dT)·poly(dA-dT) by at least 60 °C. The stoichiometry associated with denaturation of poly(dA-dT)·(dA-dT) is expected from the mechanism by which single-stranded DNA binding proteins denature DNA. Other proteins that denature duplex DNA, such as helicase I or the RecBC protein of *E. coli*, accomplish it through catalytic mechanisms in which one protein molecule is able to denature more than one DNA molecule (52). The hyperchromicity associated with melting duplex DNA can be attributed to unstacking of the bases when the DNA is in a single-stranded form. The total amount of hyperchromicity shown in Fig. 8 is higher than one would expect from melting an equivalent amount of poly(dA-dT)·poly(dA-dT). The increased hyperchromicity could be due to an unusual protein-DNA interaction that increases the extinction coefficient of single-stranded DNA or to an increase in the unstacking of the DNA bases. Support for the latter idea comes from the observation that the N4 SSB increases the fluorescence of etheno-M13 DNA (29) 2-fold upon binding (data not shown).

The large shift in the melting transition of poly(dA-dT)·poly(dA-dT) induced by N4 SSB and the fact that N4 SSB cannot enhance the rate of renaturation of natural DNA indicate that the N4 SSB should also be able to shift the melting transition of natural DNA. However, this is not the case. The T4 gene 32 protein is also unable to melt native DNA despite the fact that the reaction is thermodynamically favorable (53). It has been suggested that there is a kinetic block to this process that is released when 48 amino acids are removed from the carboxyl terminus of the T4 phage gene 32 protein by protease treatment (54) and N4 SSB binding to DNA may be similarly controlled.

In vivo and *in vitro* experiments have shown that single-

stranded DNA binding proteins are involved in stimulating DNA replication, recombination, and repair (1, 11). N4 SSB is able to specifically stimulate the N4 DNA polymerase by increasing the processivity of the polymerase. There are two possible ways this can be achieved. First, N4 SSB can maintain the template strand in a conformation that is preferred by the N4 DNA polymerase but not by other DNA polymerases. The increase in processivity could reflect tighter binding of the polymerase to the altered structure of the template DNA. Alternatively, the stimulation could be due to specific protein-protein interactions between N4 SSB and the polymerase resulting in a tighter association of the polymerase to the DNA template. Formosa *et al.* (40) have shown a direct physical association between the T4 phage gene 32 protein and the T4 coded DNA polymerase strongly suggesting that protein-protein interactions are responsible for stimulation of T4 DNA polymerase by T4 gene 32 protein.

As mentioned above, N4 SSB is one of the larger prokaryotic single-stranded DNA binding proteins purified to date and this increased size may be a reflection of the number of proteins that it interacts with *in vivo*. We have preliminary evidence of a physical and functional association between N4 SSB and *E. coli* RNA polymerase. This enzyme is required for the synthesis of N4 late RNAs. Some *E. coli* RNA polymerase coelutes with N4 SSB on the single-stranded DNA cellulose column. Moreover, N4 phage mutant in N4 SSB do not synthesize late RNAs. Finally, N4 SSB enhances the rate of *in vitro* transcription by RNA polymerase holoenzyme on N4 late promoters but not *E. coli* promoters.[1] To identify other genes required for N4 DNA replication we are constructing a N4 SSB affinity column to purify host or N4-encoded proteins from N4-infected cell lysates that specifically bind to it.

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[1] N. Y. Baek, unpublished work.