OSHMAN, ROBIN GAIL

THE CHARACTERIZATION OF A NEW SINGLE STRAND DNA BINDING PROTEIN (SSB-B) ISOLATED FROM AN ESCHERICHIA COLI SSB(-) MUTANT

City University of New York PH.D. 1981

THE CHARACTERIZATION OF A NEW SINGLE STRAND DNA BINDING PROTEIN (SSB-B) ISOLATED FROM AN *ESCHERICHIA COLI* *ssb*$^-$ MUTANT

By

Robin Gail Oshman

A dissertation submitted to the Graduate Faculty in Biomedical Science in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1981

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

6/11/81
date

James G. Wetmur
Chairperson of Examining Committee

6/11/81
date

Executive Officer

Doris Bucher, Ph.D.

David Calhoun, Ph.D.

Gary W. Litman, Ph.D.

Edwin D. Kilbourne, M.D.

The City University of New York

ABSTRACT

THE CHARACTERIZATION OF A NEW SINGLE STRAND DNA BINDING PROTEIN (SSB-B) ISOLATED FROM AN *ESCHERICHIA COLI* *ssb*$^-$ MUTANT

by

Robin Gail Oshman

Advisor: James G. Wetmur, Ph.D.

A protein (SSB-B) has been isolated from an *Escherichia coli* (*E. coli*) temperature sensitive single strand DNA binding protein mutant (*ssb*$^-$) which is not isolated from the wild type strain using the same procedures. SSB-B has a molecular weight of 17,800 daltons as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The temperature sensitive and wild type *E. coli* single strand DNA binding proteins (SSB) have a molecular weight of approximately 18,500 daltons as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. SSB-B differs qualitatively from temperature sensitive and wild type *E. coli* SSB in its antigenic determinants as determined by an enzyme linked

immunosorbent assay. SSB-B differs quantitatively from E. coli wild type SSB in its ability to protect single stranded DNA in a DNase I-snake venom phosphodiesterase nuclease protection assay. SSB-B differs from wild type SSB in the nature of its binding to single stranded DNA. An SSB-B-single strand DNA complex is extremely hydrophobic and binds to most surfaces. A wild type SSB-single strand DNA complex remains in solution. The amino acid composition of SSB-B is unlike any other E. coli DNA binding protein amino acid composition published to date. None of the E. coli proteins capable of binding to single strand DNA have a molecular weight identical to SSB-B. After studying the sequence of wild type E. coli SSB, it was determined that SSB-B could not be a processed product of SSB, but must be a unique protein. Using an equilibrium dialysis assay, SSB-B was shown to have a noncooperative binding constant identical to that of wild type SSB. Electron micrographs of SSB-B-single strand DNA complexes show that under certain conditions the protein appears to bind cooperatively to single strand DNA. These complexes are more extended than wild type SSB-single strand DNA complexes. SSB-B is therefore believed to be a single strand DNA binding protein never before isolated from E. coli.

ACKNOWLEDGEMENTS

My deepest appreciation goes to Dr. James Wetmur for providing me with the opportunity to pursue my scientific curiosities in his laboratory.

I am very grateful to Dr. Kilbourne who always greeted my suggestions with an open mind. I thank him for supporting my ideas and encouraging my future pursuits.

Many thanks are due to Dr. David Calhoun who always found time to discuss my research. His ideas both stimulated further research and broadened my perspectives. I would also like to thank Dr. John Gray for patiently explaining the proper use of equipment in Dr. Calhoun's laboratory.

Thanks to Dr. Doris Bucher for providing a role model for all women scientists in this department. I have always admired her. I am sincerely grateful for the support, encouragement, and scientific discussion which she always made available. I would also like to thank Ms. Diane Forde of Dr. Bucher's laboratory for performing the amino acid analyses of my samples.

Many thanks are due to Dr. Terry Krulwich whose door was always open. I enjoyed working in her labor-

atory and serving with her on Graduate School committees. She provided me with both scientific and administrative knowledge.

Dr. Wajeed Khan has always provided stimulating conversation. With his help and that of Ms. Su-huei Lin of his laboratory, I was able to perform the ELISA discussed within.

Of course, life around here would be pretty dull without the laboratory technicians and secretaries. So here's to all of you. Thanks for your support and help.

Judy Davis deserves special thanks for sharing all of my thoughts. Good friends are difficult to find. Fortunately, fate and microbiology brought us together.

Thank you Senta Frank for always being there when I needed you. You have helped me more than you know.

Most of all I would like to express my deepest thanks to Dr. Gary Litman. He introduced me to the scientific methods of protein isolation and wrestling sharks (backstage at the aquarium). It was because of Gary and all of the people in his laboratory that I decided to pursue a scientific career.

DEDICATION

This thesis is dedicated to my husband, Barney Spivack, who endured the many years of preparation toward my degree.

This work is also dedicated to my mother, who has always encouraged me to pursue and to achieve all of my dreams, and to my father, who has always supported my goals.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

LIST OF FIGURES (CONTINUED)

CHAPTER 1

A REVIEW OF SINGLE STRAND DNA BINDING PROTEINS AND THE ROLE OF SSB IN ESCHERICHIA COLI DNA REPLICATION, RECOMBINATION AND REPAIR

A. DNA Binding Proteins

To illustrate the complexities involved with DNA maintenance, replication, recombination and repair, one merely has to note all of the classes of proteins which bind to this genetic material (see Kornberg, 1980, for an extensive review). One class of proteins recognizes and binds to specific sequences on the DNA. These include DNA polymerases, restriction endonucleases, repressor proteins and replication prepriming and priming proteins. A second major class of proteins binds nonspecifically to DNA. One group of proteins in this class binds both double stranded (ds) and single stranded (ss) DNA's. These include the DNA dependent ATPases such as repA, helicase, and the topoisomerases gyrase and ω protein. Another group in this class is the proteins which bind to dsDNA to form stoichiometric complexes such as nucleosomes. A third group of proteins, the single strand DNA binding proteins, binds preferentially to ssDNA and may promote the melting of the genome. This final group of DNA binding proteins plays an important role in DNA rep-

lication, recombination and repair and has therefore undergone extensive study in many laboratories. Isolation and characterization of one such protein, a new single strand DNA binding protein from Escherichia coli (E. coli), constitutes the work in this thesis.

The single strand DNA binding proteins are found in a wide phylogenic range. They have been isolated from cells containing bacterial and animal viruses, from bacteria, fungi, plants, normal mammalian tissue, and transformed cells (see Coleman and Oakley, 1980, for an extensive literature review).

The bacteriophage T4 gene 32 protein (P32) is the best characterized single strand DNA binding protein to date. P32 is a 35,000 dalton protein first isolated and characterized by Alberts et al. (1968). P32 binds cooperatively to ssDNA (Delius et al., 1972), possibly through electrostatic forces (Alberts and Frey, 1970). In vitro studies revealed that P32 enhanced the rate of T4 DNA synthesis 5-10 fold and that a complex formed between P32 and T4 polymerase which may be required for this increased polymerase action (Huberman et al., 1971). P32 has been sequenced (Williams et al., 1980).

The bacteriophage T7 single strand DNA binding protein has been isolated from infected E. coli cells. The T7 protein was shown to bind strongly and preferentially to ssDNA and to have a molecular weight of 31,000 daltons (Reuben and Gefter, 1973; Reuben and

Gefter, 1974). The T7 ssDNA binding protein stimulates *in vitro* DNA synthesis by T7 polymerase about 5-10 fold when partially single stranded T7 DNA is used as a template (Scherzinger et al., 1973). The stimulation is specific for T7 polymerase and has no effect if *E. coli* DNA polymerase I, II, or III, or T4 DNA polymerase is substituted (Reuben and Gefter, 1973).

Adenovirus type 2 DNA binding protein (DBP) has been isolated from infected monkey kidney cells (van der Vliet and Levine, 1973), human KB cells (Yanashita and Green, 1974), and HeLa cells (Schechter et al., 1980). DBP has a molecular weight of 72,000 daltons per protein monomer (Ariga et al., 1980), is synthesized early in infection (Shanmugan et al., 1975), self associates to form a trimeric peptide and interacts with both ss and dsDNA as measured in a filter binding assay (Schechter et al., 1980). Binding protein isolated from cells infected with a temperature sensitive (ts) adenovirus type 2 DNA binding protein mutant exhibits thermolability in DNA binding properties of the protein as determined by van der Vliet et al. (1975). When DBP is cleaved with chymotrypsin, a 44,000 dalton subunit is formed containing the carboxy end of the protein. This subunit retains the ability to bind ssDNA and complements ts DBP stimulated DNA synthesis *in vitro* (Ariga et al., 1980). This subunit is also rich in methionine and binds equally

well to ss and dsDNA (Linné and Philipson, 1980). Fowlkes et al. (1979) reported that adenovirus DBP has an affinity for dsDNA. Melting studies show that the melting temperature of poly[d(A-T)]-DBP complexes are increased relative to uncomplexed poly[d(A-T)]. The ligand must therefore bind more strongly to the helix form than to the coil form of poly[d(A-T)]. In a series of filter binding competition assays, increasing amounts of unlabeled specific restriction fragments of adenovirus DNA were added to a constant amount of labeled adenovirus DNA. Adenovirus DBP was then added. On a weight basis DBP appeared to have a higher affinity toward smaller dsDNA restriction fragments. On a molar basis, none of the dsDNA fragments competed for DBP any differently than full length adenovirus DNA. These results suggest that DBP preferentially binds to the ends of adenovirus dsDNA. Adenovirus ^{32}P-labeled dsDNA-DBP complexes were isolated in sucrose gradients, glutaraldehyde fixed, and restricted with EcoR1. The original adenovirus dsDNA termini bound significantly more DBP than any internal dsDNA regions. An adenovirus DNA replication model proposed by Lechner and Kelly (1977) suggests that initiation of adenovirus replication can initiate from either end of the genome. These experiments suggest that the adenovirus DBP may be involved with the initiation of adenovirus DNA replication by binding to the termini

of the DNA.

Banks and Spanos (1975) have isolated a 20,000 dalton ssDNA binding protein from the basidiomycete fungus Ustilago maydis using a ssDNA cellulose column. The ssDNA binding protein binds specifically to ssDNA, stimulates the rate of DNA synthesis by the Ustilago DNA polymerase and protects single stranded DNA degradation by U. maydis DNase I. A filter binding assay revealed that one molecule of the protein binds seven to ten ssDNA nucleotides in what appears to be cooperative binding.

Meiotic cells of three commercial clones of lilies were found to contain a 35,000 dalton ssDNA binding protein (Hotta and Stern, 1971a). The Lilium ssDNA binding protein is present in the nuclei of meiotic cells in the prophase stage and has a high affinity for ssDNA as exhibited in a filter binding assay. Hotta and Stern (1971b) also isolated DNA binding proteins from nuclei of rat, bull and human testis cells in meiotic prophase. The protein is not found in somatic tissues and binds specifically to denatured DNA.

Through the use of a ssDNA cellulose column, Huang et al. (1975) isolated a 24,000 dalton DNA binding protein unique to lymphocytes from chronic lymphocytic leukemia (CLL) patients. The CLL protein enhances helix-coil transition of poly[d(A-T)]. When added to human metaphase chromosomes, the CLL protein lengthens the chromatids

suggesting that the protein may act on complex chromosomes. Using quantitative immunochemical determinations, the CLL protein could not be found in lymphocyte extracts of normal individuals. This protein has not been further characterized.

Herrick, Delius and Alberts (1976) isolated a 19,000 dalton ssDNA binding protein from calf thymus (UP1). Sedimentation studies using sucrose gradients showed that each UP1 molecule occupied a seven nucleotide long site on ssDNA. This is in the same range as the E. coli single strand DNA binding protein (SSB) which occupies an eight nucleotide long site on ssDNA (Sigal et al., 1972). When UP1 was added to an in vitro solution containing calf thymus-DNA polymerase-α and native DNA partially degraded with E. coli exonuclease III, the rate of nucleotide incorporation was stimulated ten-fold. No stimulation of the calf thymus DNA polymerase was observed if the T4 gene 32 protein was substituted for UP1. Like the T4 gene 32 protein and E. coli SSB, UP1's binding to DNA is salt sensitive and thus relies in part on ionic interactions with the DNA phosphates. When UP1 is complexed to DNA there is nearly complete DNA hyperchromicity indicating that the bases are held in a nonstacked conformation. Delius et al. (1976) have not been able to demonstrate cooperative binding of UP1 to ssDNA either by sedimentation or electron microscopic

analysis, one property which is a major difference between UP1 and the two ssDNA binding proteins, T4 gene 32 protein and E. coli SSB.

A single stranded nucleic acid binding protein (HD40) was most recently isolated from the embryos of the brine shrimp Artemia salina (Marvil et al., 1980). The isolation procedure involved high salt washes of ribosomes, and passage over a ssDNA agarose column. The HD40, through a series of filter binding assays, showed a higher affinity binding for ssRNA than for ssDNA but was capable of binding both. No binding was seen with dsDNA or dsRNA. A molecular weight of 40,000 daltons was determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. The amino acid composition of HD40 is very similar to that of E. coli SSB (Weiner et al., 1975; Sancar et al., 1981) except for the presence in HD40 of dimethylarginine. HD40 protects poly(rU) and MS2 RNA from pancreatic ribonuclease and protects denatured SV40 DNA from pancreatic DNase I and snake venom phosphodiesterase. The protein exhibits no tendency for self aggregation although aggregates are formed in the presence of polynucleotide template (Nowak et al., 1980). The higher affinity for RNA and its cytoplasmic location argue against the possibility of HD40 being a DNA helix-destabilizing protein involved in replication. The T4 gene 32 protein, however, has also been shown to preferen-

tially and cooperatively bind all single stranded nucleic acids (Alberts et al., 1968). Although P32 has a higher affinity for ssDNA than for ssRNA, it has been shown in a series of rifampicin containing experiments to bind its own mRNA and thus translationally control production of P32 (Russel et al., 1971).

As is evident from this review of the literature, all ssDNA binding proteins which are isolated are not characterized using identical experimental procedures. Comparing proteins isolated from different sources therefore becomes difficult and should be done with caution. Sigal et al. (1972) first isolated the wild type E. coli single strand DNA binding protein (SSB) by DNA cellulose chromatography followed by DEAE cellulose chromatography. Molecular weight determinations using SDS polyacrylamide gel electrophoresis and amino acid sequencing (Sancar et al., 1981) showed the monomeric form of the protein to be 18,873 daltons. Molineaux et al. (1974) using sedimentation analysis with glycerol gradients, found the native form of the protein to be tetrameric. The stoichiometry of binding to ssDNA is one protein monomer per 8 nucleotides when the complex was isolated by sedimentation in a sucrose gradient (Sigal et al., 1972) and when isolated by gel filtration on Bio-Gel A5M (Weiner et al., 1975). Cooperative binding of SSB to ssDNA was first visualized in the electron microscope by Sigal et al.

(1972). E. coli SSB was combined with fd circular ssDNA. Under nonsaturating protein conditions, the fd DNA molecules either became fully saturated with SSB or had little or none bound to them, thus exhibiting the cooperative binding property of SSB. Experiments concerning the E. coli SSB's noncooperative and cooperative binding capacity, its ability to protect DNA against exonucleases, and its amino acid composition are described in Chapters 5 and 6.

Sevastopoulos et al. (1977) isolated a ts E. coli SG 1634 SSB mutant (ssb$^-$) (see Introduction of Chapter 2). Meyer et al. (1979) characterized the ssb$^-$ mutant as being defective in DNA replication, recombination and repair at nonpermissive temperatures (see Chapter 5). In attempting to isolate this ts SSB, I isolated a new single strand DNA binding protein (SSB-B). Significant quantities of SSB-B are not found in wild type E. coli cell extracts when subjected to identical isolation procedures (see Chapter 4). The characterization of SSB-B isolated from the ssb$^-$ bacteria constitutes the research described herein. A comparison of physical properties of SSB-B and SSB is also made (see Chapters 5 and 6). The biological properties of SSB are outlined below.

B. The Role of SSB in E. coli DNA Replication

DNA replication involves a host of proteins interacting to maintain the integrity of this genetic material. Although intact replication enzyme complexes have yet to be isolated, some progress has been made in defining proteins which are believed to comprise such complexes. One of the simplest replication systems, the replication of bacteriophage G4, has been reconstituted in vitro (Zechel et al., 1975). Conversion of the ss-circular DNA of bacteriophage G4 to the ds-replicative form requires only three proteins, a priming protein (dnaG), the DNA polymerase III holoenzyme (dnaE and dnaZ), and the E. coli SSB. A more complicated system of in vitro replication has been developed for ϕX174 (Kornberg, 1978) and seems to more closely resemble the replication system of E. coli. A prepriming stage exists in the ϕX174 system involving a number of proteins which interact to produce a prepriming intermediate. A primer is then synthesized by primase thus allowing elongation of the DNA by polymerase to yield a complementary strand of DNA. SSB is needed in the in vitro ϕX174 DNA replication system to cover all areas of naked ssDNA. A model for the replication fork of E. coli has been proposed by Kornberg (1978) based on the studies of bacteriophage replication. At the origin of replication a bidirectional fork appears. On the leading strand, replication advances continuously in a 5' to 3'

direction through the action of the rep protein, a ssDNA dependent ATPase which catalyzes strand separation of duplex DNA. SSB is thought to bind all ssDNA preventing renaturation during strand separation and positioning the DNA to act as a template for DNA polymerase I. On the opposite strand of DNA where discontinuous replication occurs, helicase III, a DNA dependent ATPase which catalyzes strand separation of duplex DNA in the presence of ATP, is believed to replace the rep protein, moving along the strand in a 5' to 3' direction (Yarranton et al., 1979). SSB covers all ssDNA regions on this strand too. The lagging strand contains a dnaB protein which preceeds the primase protein which transcribes the Okazaki fragments. These fragments serve as a primer for DNA synthesis by DNA polymerase III holoenzyme. DNA polymerase I removes the RNA primers attached to Okazaki fragments in a 5' to 3' direction and fills the DNA gaps with the proper deoxyribonucleotides. DNA ligase seals all 3'-hydroxyl and 5'-phosphoryl ends on both strands to form a covalently intact progeny of dsDNA. In addition to these passive roles in DNA replication, SSB has been shown to be a requirement for the ATP dependent unwinding activity of the rep protein (Yarranton and Gefter, 1979). Sigal et al. (1972) showed that SSB is capable of stimulating the rate of E. coli DNA polymerase II in synthesizing DNA on exonuclease III treated duplex bacteriophage T7 DNA. No stimulation

occurred when E. coli DNA polymerase I or III or bacteriophage T4 DNA polymerase were substituted. E. coli SSB could stimulate E. coli DNA polymerase III holoenzyme in converting G4 ssDNA to the duplex form (Weiner et al., 1975). Molineaux and Gefter (1974) discovered that SSB formed a complex with E. coli polymerase II in the absence of DNA. They therefore postulated the existence of three binding sites on the SSB molecule: i) one for itself; ii) one for ssDNA; and iii) one for DNA polymerase II. If many mutants of E. coli SSB were available, it might be possible to dissociate effects on these binding sites as Breschkin and Mosig (1977b) did for the T4 gene 32 protein (see Part C of this Chapter).

Using a poly[d(A-T)] template fidelity assay, whereby the error rate is defined as the ratio of incorrect to total correct deoxynucleotides incorporated, [^{3}H]dGTP was used to detect base misincorporation. Because E. coli polymerase III (Pol III) has been shown to be the replicative DNA polymerase in E. coli (Gefter et al., 1971), this enzyme was chosen for the assay. When SSB was added to the assay system, the rate of polymerization increased 15% to 27% and there was a 5.8 fold increase in fidelity. SSB increased the accuracy of Pol III-catalyzed DNA syntheses in a concentration dependent manner. When DNA polymerases from avian myeloblastosis virus (AMV), Novikoff hepatoma (β), calf thymus (α), and bacteriophage T4 were substituted for E. coli Pol III in this assay

system, the fidelity of poly [d(A-T)] synthesis was increased severalfold in all cases. This increase is not mediated by an increase in excision of incorrectly incorporated nucleotides because the DNA polymerases from AMV, Novikoff hepatoma (β) and calf thymus (α) lack any proofreading exonucleolytic activity (Loeb, 1974; Weissbach, 1977; Battula and Loeb, 1976). Because synthetic homopolymers and heteropolymers are potentially subject to artifactual results in fidelity measurements due to the repetitious sequences of these templates, Kunkel et al. (1979) decided to examine the effect of SSB on the fidelity of DNA polymerases using a ϕX174 fidelity assay. In this assay the ϕX174 DNA contains an amber mutation. Certain incorrect misincorporations at the amber site will produce reversion to wild type. E. coli spheroplasts are infected with this replicated ϕX174 DNA and plated on indicator bacteria. The error rate is determined from the reversion frequency for copied DNA when compared to an uncopied control. Because Pol III is incapable of copying long stretches of ssDNA (Kornberg and Gefter, 1972; Livingston et al., 1975) this DNA polymerase could not be used in this assay. SSB did increase the accuracy of ϕX174 DNA synthesis severalfold in the presence of either E. coli DNA polymerase I (Pol I), T4 DNA polymerase, AMV DNA polymerase, Novikoff hepatoma DNA polymerase-β, or acute lymphocytic leukemia DNA polymerase-α. Again the

increased fidelity through SSB addition is not due to enhanced proofreading because the accuracy of DNA polymerases without 3' to 5' exonucleases was increased to an extent similar to that of Pol I (Kunkel et al., 1979). The mechanism proposed for the increased fidelity observed is that enhanced base selection was due to a template-SSB interaction resulting in increased rigidity of the template. The polymerases could then position the substrate for correct base-pairing with the template.

By monitoring the hyperchromic shift characteristic of the helix-coil transition of DNA, Sigal et al. (1972) demonstrated that bacteriophage T4 DNA could be denatured in the presence of excess E. coli SSB at low ionic strengths. The amount of denaturation attainable was dependent upon the quantity of protein added. When examined in the electron microscope SSB was found to preferentially bind A-T rich regions of dsDNA. The interaction of SSB with other DNA replication proteins, the enhanced fidelity of DNA synthesis observed in the presence of SSB, and the ability of SSB to facilitate denaturation of DNA reveal various facets of the role which SSB plays in DNA replication.

C. The Role of SSB in *E. coli* DNA Recombination and Repair

When *E. coli* DNA synthesis is impaired by chemical (mitomycin C) or physical (ultraviolet light) means, the cell has the option of repairing the nucleic acid in one of three ways (Witkin, 1976). Through the photoreactivation pathway, pyrimidine dimers are excised by a photoreactivating enzyme which splits the dimer in the presence of light. In the dark the excision repair or "short patch" pathway is used. The *uvrA*$^+$ and *uvrB*$^+$ gene products place a single strand nick in the 5' side of the dimer. DNA polymerase I then excises the faulty bases plus some bases on either side of them with the aid of *uvrC*$^+$ (Witkin, 1976), *uvrE*$^+$ (Witkin, 1976), and the *mfd*$^+$ (George and Witkin, 1974 and 1975) gene products and resynthesizes the excised section using the second DNA strand as a template. Polynucleotide ligase then seals the sugar-phosphate linkage to produce a repaired piece of DNA. These two repair processes are considered error-proof, since no significant mutagenesis occurs in the cell when repair is accomplished through these pathways. The third mechanism, SOS repair, postreplication repair, recombination, or the "long patch" pathway, is observed in bacteria which are kept in the dark and are *uvr*$^-$ mutants. This error prone pathway involves the *lexA*$^+$ and *recA*$^+$ gene products. In this case daughter strands of DNA are synthesized with gaps occurring at each unexcised dimer. Parental DNA

strands recombine at the gap sites to produce a continuous daughter strand (exact mechanism unknown). A model for regulation of this inducible SOS repair in bacteria has been proposed (Witkin, 1976; Gudas and Mount, 1977). In studying *lexA*$^-$ and *recA*$^-$ mutants a number of SOS functions were observed. These coordinated responses for improved cell survival included prophage induction, filamentous cell growth, inhibition of inducible error prone DNA repair, induction of *recA*$^+$ and inhibition of DNA degradation. It is thought that all SOS functions have evolved repressors similar enough to respond to the same inducer, and that the *recA*$^+$ and *lexA*$^+$ products are necessary for their induction and/or expression. Each SOS function is repressed (*lexA*$^+$, *recA*$^+$, undamaged cells) by its own repressor which binds to a separate operator. These repressors are all similar enough to bind the *lexA*$^+$ gene product, thereby preventing their inactivation by constitutive antirepressors. The *recA*$^+$ gene is also repressed by the *lexA*$^+$ gene product which binds to its operator site. When DNA is damaged, an inducer is released which forms a complex with the *recA*$^+$ protein. This activated complex binds the *lexA*$^+$ product allowing more *recA*$^+$ to be produced, and removing the *lexA*$^+$ from the pool of SOS repressors. The SOS repressors are now proteolytically cleaved and released from their operator sites allowing induction of all SOS functions. Baluch et al. (1980) determined that SSB was also necessary for ultraviolet (UV) mediated induction of *recA*$^+$ protein.

Using the ssb$^-$-A1 strain, UV treatment produced increased synthesis of recA$^+$ protein at 30°C but at 42°C no such effect was apparent as indicated by gel electrophoresis.

The recA$^+$ protein has been extensively studied, sequenced (Sancar et al., 1980), and found to be stimulated by E. coli SSB (McEntee et al., 1980; Shibata et al., 1980; Cassuto et al., 1980). In studies using recA$^+$ protein alone, McEntee et al. (1979) made the following observations. RecA$^+$ protein binds duplex DNA cooperatively only in the presence of nucleotide triphosphates. Binding of recA$^+$ protein to ssDNA is not cooperative and does not require nucleotide triphosphates. When ATP is hydrolyzed the recA$^+$ protein is released from the DNA. Shibata et al. (1979a, 1979b), using the ATP analog, adenosine 5'-O-(3-thiotriphosphate) determined how recA$^+$ protein catalyzes the homologous pairing of single stranded and duplex DNA to form a D-loop. In the model, recA$^+$ forms a stable complex with ssDNA in the presence of ATP. The recA$^+$ protein, stimulated by ssDNA, unwinds duplex DNA to enable a search for homology. The ssDNA need not be homologous to promote the binding of dsDNA by recA$^+$ protein. Homologous pairing of ssDNA with duplex DNA and detachment of recA$^+$ protein entails hydrolysis of ATP. McEntee et al. (1980), Shibata et al. (1980) and Cassuto et al. (1980) then suggested based on the following findings that SSB participates in generalized recombination, and therefore repair, by

aiding recA$^+$. SSB increases the rate and extent of strand assimilation into homologous duplex DNA in the presence of recA$^+$ and ATP (McEntee et al., 1980; Cassuto et al., 1980). SSB enhances the formation of a complex between recA$^+$ protein and duplex DNA in the presence of homologous or heterologous ssDNA (McEntee et al., 1980). SSB reduces the rate and extent of ATP hydrolysis catalyzed by recA$^+$ protein in the presence of ssDNA (McEntee et al., 1980). SSB reduces the high concentration of recA$^+$ protein required for strand assimilation when forming D-loops by preventing the formation of non-productive recA$^+$-ssDNA complexes (McEntee et al., 1980; Shibata et al., 1980). Homologous DNA was required for the formation of D-loops (Shibata et al., 1980). The ts mutant SSB was less effective than wild type SSB in stimulating strand assimilation which strongly suggests that SSB participates in general recombination in vivo (McEntee et al., 1980).

Christiansen and Baldwin (1977) demonstrated that E. coli SSB catalyzed DNA reassociation in a polyamine dependent reaction. DNA strand reassociation is a second order reaction whose rate is a function of temperature, counter-ion type, concentration, DNA complexity, and DNA strand length (Wetmur and Davidson, 1968). Using intact λ DNA, 2 mM spermidine or spermine, and an excess of E. coli SSB, a 5000 fold catalysis in DNA renaturation was observed at 37^{o}C. The reaction followed second order

kinetics and the rate increased with the square root of DNA length just as does the high temperature high salt DNA renaturation in the absence of E. coli SSB. These results imply that SSB could therefore play an important independent role in base pair recognition in vivo.

Sequence studies suggest (Sancar et al., 1980) that the amino terminal portion of $recA^{+}$ protein binds proteins because it contains three cysteines and two histidines, amino acids that are part of the active sites of most proteases. The carboxy terminus which contains 75% of all basic amino acids and 80% of the aromatic amino acids may interact with DNA through ionic and/or stacking interactions. The opposite organization occurs with the bacteriophage T4 gene 32 protein (Breschkin and Mosig, 1977a).In this case the amino terminus binds the gene 32 protein to DNA while the carboxy terminus binds and inactivates nucleases thus protecting DNA from degradation. The amino terminus of the gene 32 protein also binds to proteins which initiate DNA replication and recombination. Even though the amino acid sequence of E. coli SSB is known, the distribution of function of this protein is unknown.

CHAPTER 2

ORIGIN AND CHARACTERIZATION OF THE E. COLI STRAINS KLC 436 AND KLC 438

A. Origin and Genetic Characterization of the E. coli Strains

INTRODUCTION

The wild type E. coli D10 SSB was first isolated and characterized by Sigal et al. in 1972 (see Chapter 1, Section A). Isolating a temperature sensitive ssb^- mutant to further characterize the function of this protein proved to be a difficult task and could only be accomplished with the aid of a computer-controlled device (nicknamed "Cyclops") capable of screening E. coli colonies for their ability to grow on nutrient agar at 30°C and 41°C after being treated with nitrosoguanidine (Sevastopoulos et al., 1977). Of the 1.4 million colonies screened, 2266 were temperature sensitive mutants, and of these, 110 were defective in DNA synthesis but not in protein synthesis. To identify ts mutants defective in DNA replication (dna_{ts} mutant), all strains exhibiting a ts phenotype were screened for incorporation of radioactive leucine and radioactive thymine in vivo at the nonpermissive temperature, 41°C. E. coli mutants defective

only in [^{3}H] thymidine incorporation were grouped into three classes: i) "quick stop" mutants (a protein involved with elongation of DNA during replication is defective), ii) "reduced rate" mutants (DNA synthesis continues at at reduced rate due to a partially incapacitated replication system), and iii) "slow stop" mutants (a protein involved with initiation of DNA synthesis is defective). The map positions of the $\underline{dna}_{ts}$ genes were roughly defined through the transfer of temperature-resistant and other control wild type alleles from an Hfr (male) strain to the female $\underline{dna}_{ts}$ strains. More accurate mapping of the genes was achieved through bacteriophage P1 generalized transduction. Of the 110 $\underline{dna}_{ts}$ mutants, only one of thirty "quick stop" mutants isolated proved to be defective in SSB.

Meyer et al. (1979) characterized this $\underline{dna}_{ts}$ mutant as having a defective SSB using the extracts from the $\underline{ssb}^-$ cells in an *in vitro* G4 replication assay (see Chapter 5). Glassberg et al. (1979) then mapped the *ssb* gene between *uvrA* and *melA* at 90.8 minutes on the *E. coli* K12 genetic map through bacteriophage P1 generalized transduction. Using a bacteriophage P1 grown on strain SG 1635 ($\underline{mal}^+\underline{ssb}^-$) for generalized transduction, *E. coli* strain KLC 436 ($\underline{mal}^+\underline{ssb}^-$) was constructed by Vales et al. (1980) from *E. coli* strain KLC 434 ($\underline{mal}^-\underline{ssb}^+$), selecting for $\underline{mal}^+$ recombinants. The $\underline{mal}^+$ marker is located between 90 and 91 minutes on the *E. coli* chromosome

(Bachmann et al., 1976) and thus would be cotransduced with the ssb gene. The E. coli ssb$^+$ strain KLC 438 (mal$^+$ssb$^+$) was a mal$^+$ derivative of E. coli strain KLC 434 and was used as an isogenic wild type control strain in all bacterial characterization experiments (see Table 1 for a list of the bacterial strains used).

B. Physiologic and Phenotypic Characterization of the Temperature Sensitive E. coli KLC 436 Strain

INTRODUCTION

Having constructed the E. coli ssb$^-$ mutant strain KLC 436, Vales et al. (1980) then went on to examine the phenotypic and physiologic characteristics of this dna$_{ts}$ strain. Microscopic observation revealed that the ts mutant became extensively filamentous at nonpermissive temperatures (Vales et al., 1980). The generation time of the ts ssb$^-$strain was 5% to 10% longer than the isogenic E. coli wild type KLC 438 strain at 30^{o}C. The ts ssb$^-$strain exhibited reduced survival on L-broth agar plates incubated at 42^{o}C relative to its survival at 30^{o}C (Vales et al., 1980). UV survival studies of the ts ssb$^-$ strain showed increased sensitivity to UV irradiation when compared to the wild type KLC 438 strain (Vales et al., 1980). In order to classify the strains of bacteria received by this laboratory, the morphological,

TABLE 1

BACTERIAL STRAINS

E. coli K12

Strains	Relevant Genotype	Source
SG 1634	*ssb*	Sevastopoulos et al. (1977)
KLC 434	F^- *mal mel thy rha*	Vales et al. (1980)
KLC 436	F^- *ssb mel thy rha*	Vales et al. (1980)
KLC 438	F^- *mel thy rha*	Vales et al. (1980)

UV sensitivity, generation time and temperature sensitivity criteria were examined.

MATERIALS AND METHODS

Bacteria

E. coli K12 bacteria, strains KLC 438 and KLC 436, were received from Dr. J. Chase of Albert Einstein College of Medicine (see Table 1 for relevant genotype).

Morphology

Bacteria were grown overnight in L-broth at 30°C. Samples were removed, mounted on slides, and examined at 100X magnification in the light microscope. The bacteria were then placed in a shaking waterbath at 42°C for 1 hour and reexamined. These cells were gram stained positive (i.e., not destained) to give better contrast when photographed.

Growth rate

One liter of an overnight culture of bacteria grown to an increase of 75 *ΔKlett units, as determined with a Klett-Summerson photoelectric colorimeter, was inoculated into nine liters of L-broth plus thymidine (see Materials and Methods in Chapter 3 on growing large scale batches of bacteria) and incubated at room temperature. Ten milliliter

*ΔKlett = Klett units of media plus bacteria - Klett units of media alone

samples were removed at one hour intervals to determine the ΔKlett unit reading.

UV sensitivity (Miller, 1972)

Bacteria were grown in L-broth plus thymidine at 30°C to mid-log, centrifuged, and resuspended in 10 ml of 0.1M $MgSO_4$. Five ml of this sample was placed in a small glass petri dish and spun with a stirring bar to prevent shielding. The ultraviolet General Electric germicidal lamp G8T5 was placed 25 cm from the dish to yield an exposure of 20 ergs/sec/mm^2 as measured by a model IL570 International light radiometer. Samples of 0.1 ml were removed at 15, 30, 60, 80, 100, 120, and 150 seconds. Each irradiated suspension was titered in dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}, plated on L-broth plates, and incubated at 30°C overnight. Colonies surviving irradiation were counted the next day.

Quantitating survival of the ts mutant at the non-permissive temperature

Bacteria grown to mid-log were titered in dilutions of 10^{-2}, 10^{-4}, 10^{-5}, and 10^{-6}. Samples of 0.1 ml of each dilution was plated in duplicate and incubated at 30°C and 42°C. Colonies were counted the next morning.

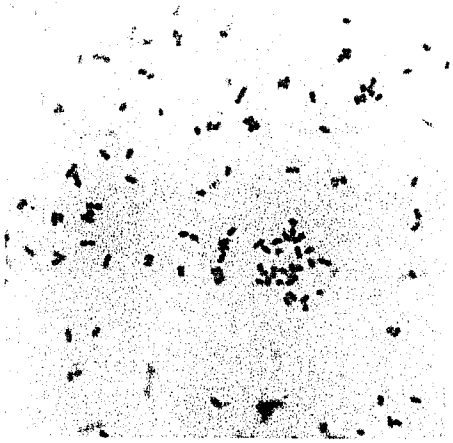
RESULTS AND DISCUSSION

1. Morphology of the *E. coli* KLC Strains

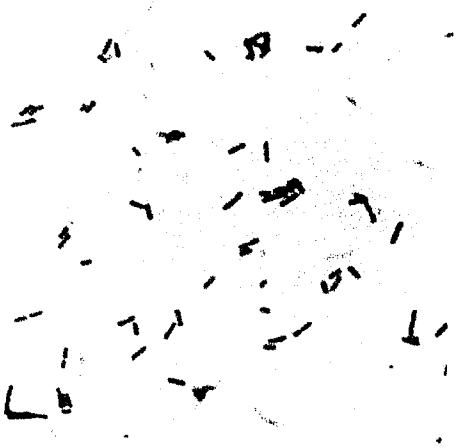
An overnight culture of the wild type *E. coli* KLC 438 cells grown in L-broth at 30°C exhibited the normal morphology of *E. coli*. The temperature sensitive mutant, *E. coli* KLC 436, in contrast, showed a tendency toward filamentation under the same conditions (see Figure 1, A and B). When these cultures were shifted to 42°C for one hour and examined, the wild type strain remained the same while the temperature sensitive strain became extremely filamentous (see Figure 1, C and D).

As discussed in Part C of Chapter 1, filamentous cell growth is believed to be an inducible SOS repair function. Witkin (1967) proposed that the synthesis of a septum-inhibiting protein may be governed by a repressor which responds to an inducer synthesized only when DNA replication is interrupted. Using a strain of *E. coli* containing a mutation in the *lon* gene, Johnson (1977) determined that the *lexA*$^+$ function is required for a filamentous response. A mutation in the *lon* gene results in inhibition of septum formation following exposure of cells to agents (e.g., ultra-violet irradiation) which inhibit DNA replication (Howard-Flanders et al., 1964). A *lexA*$^+$*lon*$^-$ strain of *E. coli* was able to form filaments after exposure

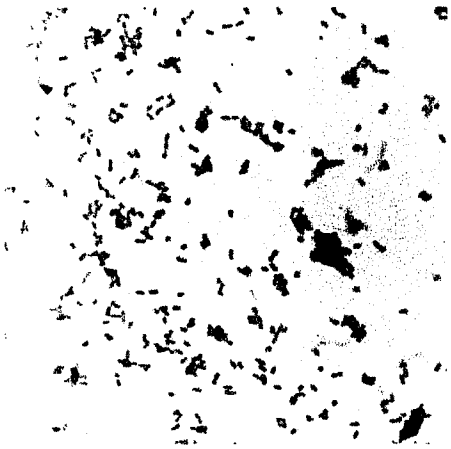
Figure 1. The morphologic effect on the dna_{ts} mutant E. coli strain KLC 436 after shifting to nonpermissive temperatures. Wild type E. coli KLC 438 (A) and the dna_{ts} mutant E. coli strain KLC 436 (B) were grown overnight in L-broth plus thymidine at 30°C. The bacteria were then placed in a shaking waterbath for one hour at 42°C and reexamined (C and D, respectively)
Oil objective = 100X magnification. (See Materials and Methods for mounting and staining procedures)



A



B



C

D

to UV irradiation. A *lexA*$^-$*lon*$^-$ strain was not able to form filaments under the same conditions. In this case the *lexA*$^-$ gene product may no longer be susceptible to proteases which would free the *lexA* protein from repressors. This would no longer allow the repressors to be proteolytically cleaved, thus derepressing SOS induced functions such as filamentation formation. The fact that *ssb*$^-$ mutants are extremely filamentous at nonpermissive temperatures implies that DNA replication is being inhibited (in this case due to a faulty SSB) and SOS functions, one of which is filamentation, are being induced (see Chapter 1, Section C for a model of induction of SOS functions).

2. Growth Rate

According to Vales et al. (1980) the generation time of the *dna*$_{ts}$ mutant *E. coli* KLC 436 when grown at 30^{o}C was 5% to 10% longer than the wild type strain under the same conditions. When the cells were incubated at room temperature in this laboratory, the growth rate of the *E. coli* KLC 436 strain was calculated to be 50% less than that of the wild type strain. The lower temperature, the difficulty in properly aerating large carboys filled with medium, and the differences in calculating quantities of cells (Vales used colony forming units, ΔKlett was used here), account for the discrepancy in growth rates. At any rate, the ts strain did show a

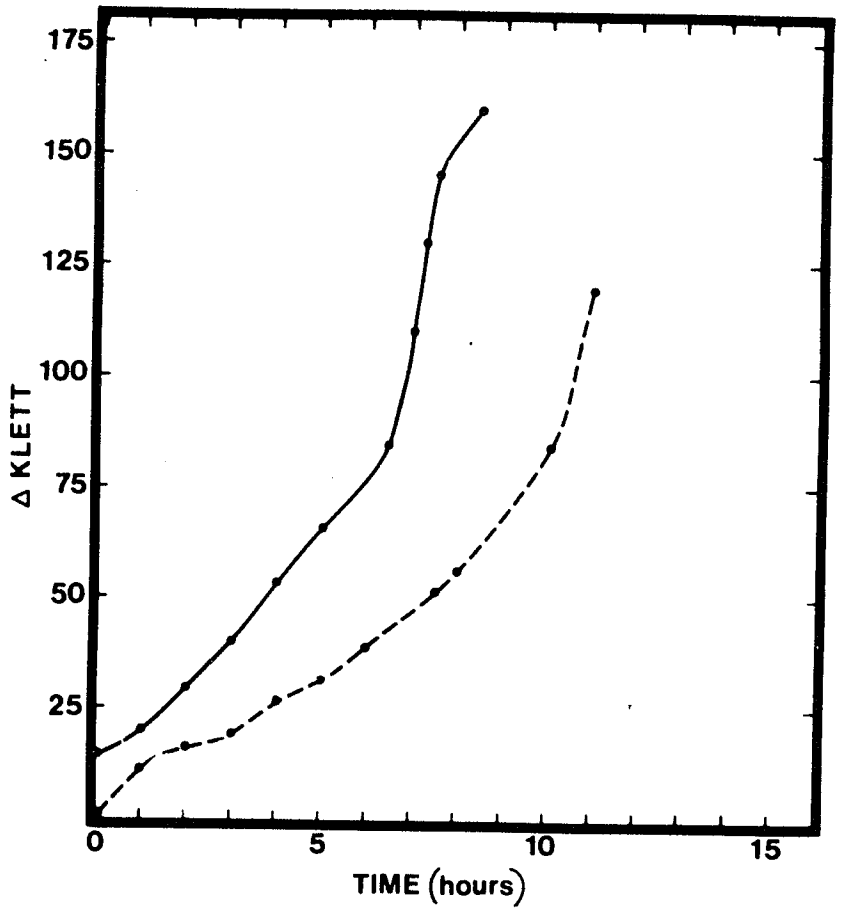
reduced rate in cell growth. Growth curves for E. coli KLC 436 and E. coli KLC 438 are shown in Figure 2.

3. Temperature Sensitivity

Bacteria were grown in L-broth plus thymidine to an absorbance (A_{600}) of 0.9, serially diluted and plated onto L-plates plus thymidine. These plates were incubated at 30°C and 42°C overnight. The colonies were counted the next day. At a dilution of 10^{-4} or below, the E. coli KLC 436 strain did not grow at 42°C. At 30°C the temperature sensitive strain behaved like the wild type E. coli KLC 438 strain (data not shown).

The following reasons may account for the ts SSB's inactivity at high temperatures. First, the ts SSB's conformation may change due to the temperature change. This may in turn affect the protein's interaction with other DNA replication proteins or affect the protein's ability to interact with DNA. Alternatively, the protein may be more susceptible to proteolytic enzymes which render it inactive at the high temperature. A third possibility is that the protein can no longer interact with itself to form a proper tetramer or to bind cooperatively to single stranded DNA. Although the exact mechanism is not known, one does know that the already synthesized ts SSB is itself altered immediately upon raising the tem-

Figure 2. Growth curve of the wild type E. coli strain KLC 438 (——————) and the E. coli ssb$^-$ mutant strain KLC 436 (— — — — —) grown in L-broth plus thymidine at room temperature (25^{o}C). Growth rate was determined by noting the doubling time in ΔKlett units.


175
150
125
100
75
50
25
Δ KLETT
0
5
10
15
TIME (hours)

perature. This is in agreement with this mutant's "quick stop" DNA replication phenotype.

4. Ultraviolet Sensitivity

Twenty milliliters of bacteria were grown to a cell density of 1 X 10^9 cells/ml (A_{600}=0.6, ΔKlett=100). The cells were spun down, resuspended in 40 ml of 0.1 M $MgSO_4$ and placed on ice to prevent further growth. The cell suspension was then serially diluted, plated, and incubated at 30°C. Five milliliters of the remaining suspension were placed in a small glass petri dish. The mixture was stirred to prevent shielding and exposed to UV light set at 20 ergs/sec/mm^2. Samples of 0.1 ml were removed at various times (between 15 and 150 seconds), diluted, plated, and grown overnight at 30°C. Table 2 shows that at a comparable dilution of 10^{-3}, the E. coli KLC 436 strain is about 20 fold more UV sensitive than the wild type E. coli KLC 438 strain. This finding is in agreement with the result reported by Vales et al. (1980).

The UV sensitivity noted here suggests that wild type SSB is in some way required for the maintenance or repair of DNA after UV treatment. Although these UV experiments were performed at the permissive temperatures of 30°C, the ts SSB does not function to the extent of the wild type protein.

TABLE 2

SENSITIVITY OF ESCHERICHIA COLI STRAINS KLC 438 AND KLC 436 TO ULTRAVIOLET LIGHT EXPOSURE (20 ergs/sec/mm^2)

Strain	Dilution	Duration of UV Light Exposure		
		0 sec	15 sec	30 sec
KLC 438	10^{-2}	ND	TMTC	TMTC
KLC 436		ND	389	2
KLC 438	10^{-3}	ND	217	20
KLC 436		ND	10	0
KLC 438	10^{-4}	887	71	0
KLC 436		695	0	0
KLC 438	10^{-5}	146	14	0
KLC 436		103	0	0

The values represent colony forming units.
TMTC = too many to count
ND = not determined

CHAPTER 3

GROWING LARGE SCALE BACTERIAL CULTURES

INTRODUCTION

Because E. coli SSB comprises only 0.1% of the total bacterial proteins (Sigal et al., 1972), it was realized that large batches of E. coli KLC 436 must be grown to isolate a substantial amount of protein. In order to utilize the least amount of medium yielding a high density of bacteria (3L yields 300 gm wet weight), a fermentor was used to grow the cells. Rotating the cells in a 30°C waterbath provided the proper temperature for growth and allowed the maximum aeration of the cells by providing a greater surface area (Sadler et al., 1976).

A second method used to grow large batches of cells was to sterilize large volumes of medium in 10 liter carboys. Air was introduced through air filters attached to spargers which were lowered into the carboys. This method presented greater chance for contamination, and, due to the large volume, took longer to centrifuge.

MATERIALS AND METHODS

Origin of wild type E. coli K12

In order to avoid the time required to grow large batches of wild type cells, E. coli K12 was ordered in 1000 gram amounts from Grain Processing, Muscatine, Iowa. These cells were grown to mid-log in enriched medium, frozen and shipped.

Media

In all fermentor experiments, cells were grown in 3.5 liters of 1% Bactotryptone, 2% Yeast extract, 0.5% glucose (SLBH) plus 0.1 M potassium phosphate, pH 7.3. Thymidine (30 mg) was included.

Large scale batches of E. coli KLC 436 cells were grown in 10 liter carboys containing 1% Bactotryptone, 0.5% Yeast extract, 0.5% NaCl, 0.005% thymidine, and 0.1% glucose at pH 7.3 (L-broth plus thymidine).

Continuous flow centrifugation

The Beckman continuous flow system was used to spin down the cells. The medium containing cells was siphoned into a rotor at 250 ml/min. The rotor speed was 16,000 rpm. Batches of bacteria isolated in this way were frozen at -70^{o}C (Revco) for future use.

RESULTS AND DISCUSSION

1. Growing E. coli KLC 436 in a Fermentor

E. coli KLC 436 was grown overnight at 30°C in 50 ml of SLBH plus 0.1 M potassium phosphate, pH 7.3. A 1 ml sample of this stock was used to inoculate 200 ml of SLBH plus 0.1 M potassium phosphate, pH 7.3 the next day. This 200 ml culture was incubated at 30°C for 21 hours and added to 3 liters of SLBH plus 300 ml of potassium phosphate, pH 7.3 to yield a final concentration of 0.1 M. This was placed in a rotating fermentor with the waterbath temperature adjusted to 30°C. The bacterial density rose from a ΔKlett of 16.5 to 260 in 2 hours and remained at that density whether given oxygen, or more thymidine (see Table 3). Two attempts at using this procedure failed to increase the yield of the bacteria. The bacteria exhibited an extremely filamentous morphology at the end of each run. A final yield of 5 gm wet weight was insufficient for all of the experiments planned. Wild type E. coli grows to high density under these conditions (data not shown).

The reasons for the failure of the ssb$^-$ mutant bacterial growth under these conditions are not known.

2. Growing E. coli KLC 436 in Carboys

To compensate for the fermentor problem, 10 liter carboys were used to grow the ssb$^-$ bacteria. One liter

TABLE 3

GROWTH OF KLC436 IN A HIGH DENSITY FERMENTOR

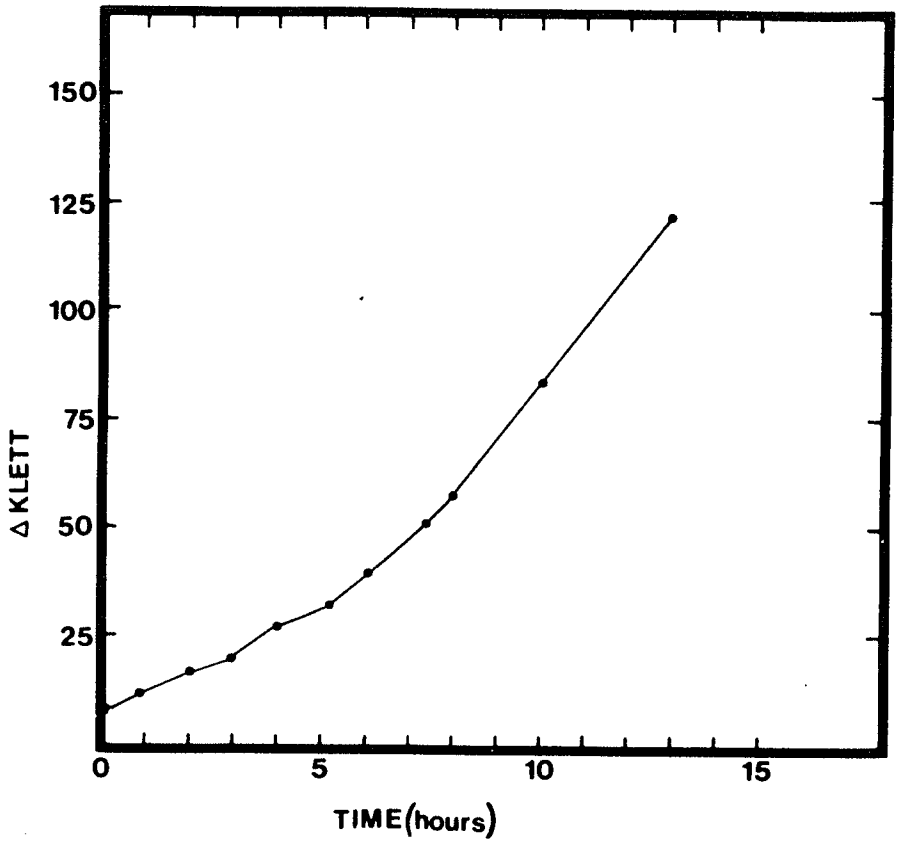
Δt(hrs)	ΔKlett	°C	LPM	Aeration	RPM	4% KOH	pH
0	16.5	30	7	Air	280	20 ml	7.30
1	210	30	7	Air	280	20 ml	7.32
2	258	30	7	Air	280	20 ml	7.31
3	264	30	5	O_2	280	——	7.31
3.5	264	30	7	Air	280	——	
7	280	30	7	Air	280	——	

LPM = liters per minute
RPM = rotations per minute

of L-broth plus thymidine was inoculated with E. coli KLC 436 and grown to a ΔKlett of 60 to 75. The culture was refrigerated overnight and used to inoculate 9 liters of L-broth plus thymidine the next morning. Air was infused through air filters and sterilized spargers. Five carboys were prepared per run. When the carboys reached a ΔKlett of 120 to 155, the cells were spun down using the Beckman continuous flow system. These cells are in mid-log phase (see Figure 3). Samples were taken from each carboy and placed at 30°C and 42°C to ensure the purity and temperature sensitivity of the bacteria. The yield per 5 carboys was approximately 100 grams of cells, wet weight. The cell paste was stored at -70°C.

A problem occurred when the 9 liters of media was prepared the day before the run. A spore forming bacteria (probably a Bacillus strain) grew up in the media overnight. To rectify this problem, 8 liters of water were sterilized for 1 hour to destroy all spores and allowed to cool overnight. At the time of bacterial cell inoculation, 10X L-broth plus thymidine was added to the carboys.

Figure 3. Growth curve of E. coli KLC 436 grown in L-broth plus thymidine at room temperature (25°C).


150
125
100
75
50
25
Δ KLETT
0
5
10
15
TIME(hours)

CHAPTER 4

ISOLATION OF SINGLE STRAND DNA BINDING PROTEINS SSB AND SSB-B

INTRODUCTION

Sigal et al. (1972) first isolated the wild type E. coli SSB using the technique of DNA cellulose chromatography (Alberts et al., 1968) followed by DEAE cellulose chromatography. A further development in protein isolation eliminated the use of both of these columns. Through a number of ammonium sulfate cuts, Meyer et al. (1980) succeeded in isolating a fraction of cell extract enriched with SSB. This fraction was placed over a blue dextran Sepharose column and washed with Tris buffer containing steps from 0.1 M to 1.5 M NaCl until the protein eluted. This system may be used to isolate both the wild type and temperature sensitive SSB.

The preferred method of protein isolation in this laboratory has evolved into a combination of the aforementioned procedures. Cell extracts were placed over a ssDNA cellulose column and washed with a Tris buffer containing steps from 0.05 M to 2 M NaCl. The 2 M NaCl SSB-containing fraction was dialyzed into a 0.05 M NaCl containing Tris buffer and passed over a blue Sepharose column. This column contains a cibacron blue

F3G-A dye covalently coupled to Sepharose CL-6B as opposed to Meyer's et al. (1980) column which consists of blue dextran 2000 coupled to Sepharose 4B. For some reason the SSB has a higher binding affinity for the cibacron blue dye and could be removed only through denaturation with 4 M Guanidine-HCl buffer (4 M Gu-HCl, 0.1 mM EDTA, 20 mM sodium phosphate, pH 5.56, and 10% glycerol) (Schneider and Wetmur, to be published). The activity of the protein was restored when dialyzed from Gu-HCl buffer into a low salt Tris-HCl buffer (50 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 7.6). Activity of the SSB protein was determined quantitatively using a nuclease protection assay (see Chapter 5). Purity of the protein was determined qualitatively through SDS polyacrylamide gel electrophoresis (see text in this Chapter). Blue Sepharose group affinity chromatography proved ideal for isolating the wild type SSB to 95% purity. Purification of the ts SSB was attempted using the same procedure. The ts SSB fails to bind the ssDNA cellulose column (Chase, private communication). A new single strand DNA binding protein (SSB-B) from the E. coli ssb^- mutant was isolated using the same protein purification procedure as used for the wild type SSB.

MATERIALS AND METHODS

Chemicals, reagents, and enzymes

Materials were obtained as follows: deoxycholic acid sodium salt, Sigma; guanidine hydrochloride (Gu-HCl) (ultra-pure), Schwarz-Mann; lysozyme, Worthington Biochemicals; deoxyribonuclease I (DNase I), Sigma; cellus 410 cellulose powder, Bio-Rad; blue Sepharose CL-6B, Pharmacia.

Buffers

Lysis buffer consisted of 50 mM NaCl, 1 mM Na_2EDTA, 20 mM Tris-HCl, pH 7.6, 10% glycerol, 10 mg DNase I, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 40 mg lysozyme and a final deoxycholate concentration of 0.06%. Buffer A contained 50 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 7.6, and 10% glycerol. Gu-HCl buffer was 4 M Gu-HCl, 0.1 mM EDTA, 20 mM sodium phosphate, pH 5.56, and 10% glycerol.

Cell lysis

Approximately 250 gm of cell paste was resuspended in 300 ml of lysis buffer. The mixture was stirred overnight at $4^{o}C$. The partially lysed cells were then passed two times through a French Pressure Cell Press at 14,000 pounds per square inch and incubated at $10^{o}C$ for 4 hours. The lysate was spun at 24,000 rpm for 1 hour at $4^{o}C$ in a Beckman SW27 rotor. The supernate was

dialyzed into Buffer A. After extensive dialysis the extract was centrifuged at 24,000 rpm for 1 hour with the same rotor at 4°C. The supernatant was now ready to be placed over a DNA cellulose column.

DNA cellulose

Purification of cellex 410: Particles were sieved to greater than 200 mesh and washed with boiling ethanol (80°C) to remove all traces of pyridine. Further purification involved successive washes with 100 mM NaOH, 1 mM EDTA, 10 mM HCl and water until the effluent was neutral. The cellulose was then lyophilized to dryness.

Addition of DNA: Calf thymus DNA (Sigma), suspended in Tris-EDTA buffer (1 mM EDTA, 10 mM Tris, pH 7.4) at 2 mg/ml, was boiled for 15 minutes and immediately cooled in an ice water bath. Clean, dry cellex was added to the DNA (1 gm cellulose/6 mg DNA). The mixture was air dried for one week, ground to a fine powder, and then lyophilized overnight. The DNA cellulose was resuspended in 20 volumes of Tris-EDTA buffer per 5 grams cellulose and placed at 4°C overnight. After washing and decanting twice, an 18.2 cm X 2.6 cm column (Pharmacia) was poured and equilibrated in Buffer A at 4°C.

Blue Sepharose

Blue Sepharose (Pharmacia) was washed with distilled

water and resuspended in Buffer A. A 25 cm X 1.5 cm column was poured and placed at 4°C.

SDS 7½% to 15% polyacrylamide gel electrophoresis

Slab gels with a 3% stack (15 cm X 3 cm X 0.13 cm) and 7½% to 15% gradient resolving gel (15 cm X 20 cm X 0.13 cm) were prepared according to the method of Maizel (1971). Electrophoresis was carried out at 100 volts for 18 hours. The gels were stained (0.2% coomassie brilliant blue (Kodak), 50% methanol, 7% glacial acetic acid) for 1 hour at 37°C. The gels were then destained at room temperature in a 10% glacial acetic acid, 1% glycerol solution until all background stain was removed.

Sample preparation for gel: 10 µg to 50 µg of protein was precipitated with a 7X volume of 99 mol% pure acetone (Fisher). The precipitate was re-suspended in 25 µl of sample buffer (20 µl of 2% SDS, 1% β-mercaptoethanol plus 5 µl 50% glycerol, 0.25% bromphenol blue), boiled for two minutes and loaded onto the gel.

RESULTS AND DISCUSSION

The first step in isolating the wild type SSB and SSB-B proteins was pumping the E. coli extract at 30 ml/min over a ssDNA cellulose column. The column was washed successively with Buffer A containing 0.05 M NaCl,

0.3 M NaCl and 2.0 M NaCl. Fractions were collected (100 drops/tube) and read at an absorbance of 280 nm. In comparing the wild type (Figure 4) and ts (Figure 5) E. coli ssDNA cellulose chromatographs, the following is observed. Peaks A and B of the wild type elution correspond to peaks B and D of the ts elution respectively. Wild type peak A and ts peak B appear to contain approximately the same amount of protein. The yield of protein seen in wild type peak B and ts peak D vary from preparation to preparation. The wild type peak B contained less material than usual in this preparation. The 2.0 M NaCl plus Buffer A eluted fraction contains proteins which strongly bind to ssDNA, including the wild type SSB. When examined on SDS polyacrylamide gel, these fractions do show differences between the wild type and ts extracts. Figure 6, lane 2, is the 2.0 M NaCl plus Buffer A eluted fraction of the wild type E. coli extract over ssDNA cellulose. There are 27 bands present. Figure 7, lane 3, is the 2.0 M NaCl plus Buffer A eluted fraction of the ts E. coli extract over ssDNA cellulose. There are 25 bands present. When the migratory distances of the bands are compared, it is found that 16 bands of the wild type extract migrate to points not found in the ts extract lane. Fourteen bands of the ts extract migrate to points where no corresponding

Figure 4. Elution profile of wild type E. coli K12 cell extract from a ssDNA cellulose column. Whole extract (500 ml) was pumped over an 18.2 cm X 2.6 cm column at 30 ml/min. 3 ml samples were collected per tube. Peak A represents the 0.3 M NaCl plus buffer A elution of the column. Peak B represents the 2.0 M NaCl plus buffer A elution of the column. Fractions between the arrows were pooled and examined on SDS polyacrylamide gel. The entire procedure was performed at 4°C.

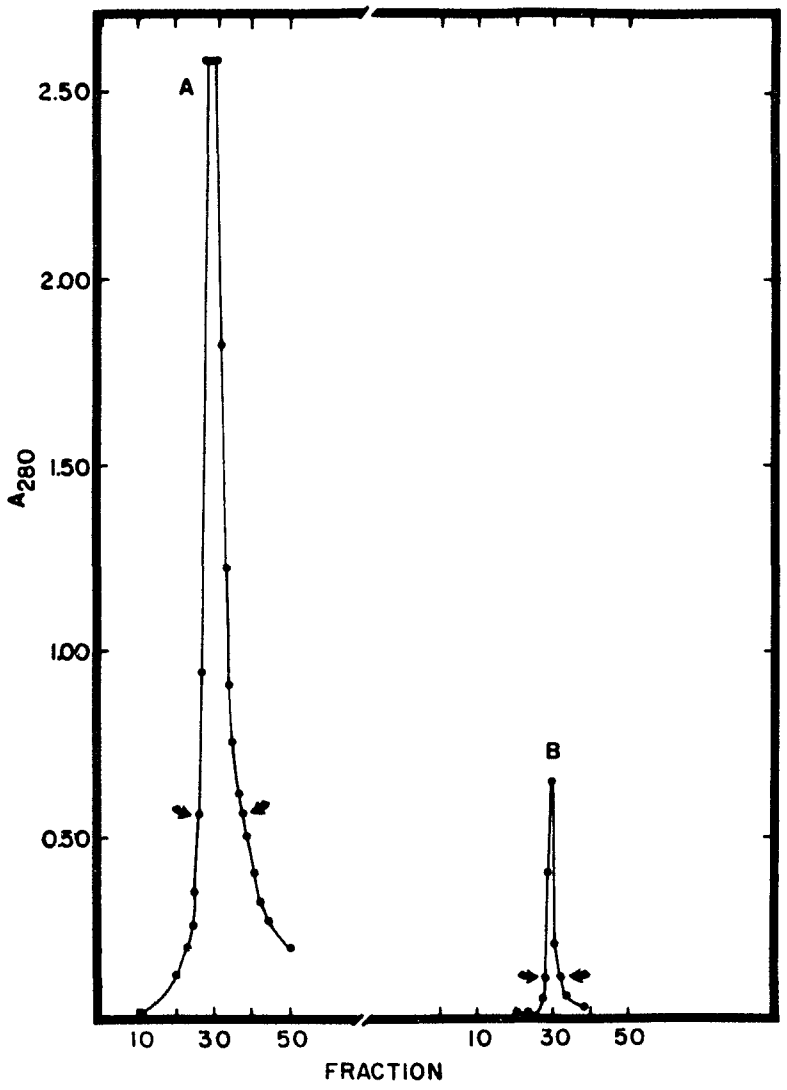

2.50
2.00
1.50
1.00
0.50
A_{280}
A
B
10
30
50
10
30
50
FRACTION

Figure 5. Elution profile of ts E. coli KLC 436 cell extract from a ssDNA cellulose column. Whole extract (500 ml) was pumped over an 18.2 cm X 2.6 cm column at 30 ml/min. 3 ml samples were collected per tube. Peaks A and B represent the buffer A plus 0.3 M NaCl elution of the column. Peaks C and D represent the buffer A plus 2.0 M NaCl elution of the column. Fractions between the arrows were pooled and examined on SDS polyacryamide gel. The entire procedure was performed at $4^{o}C$.

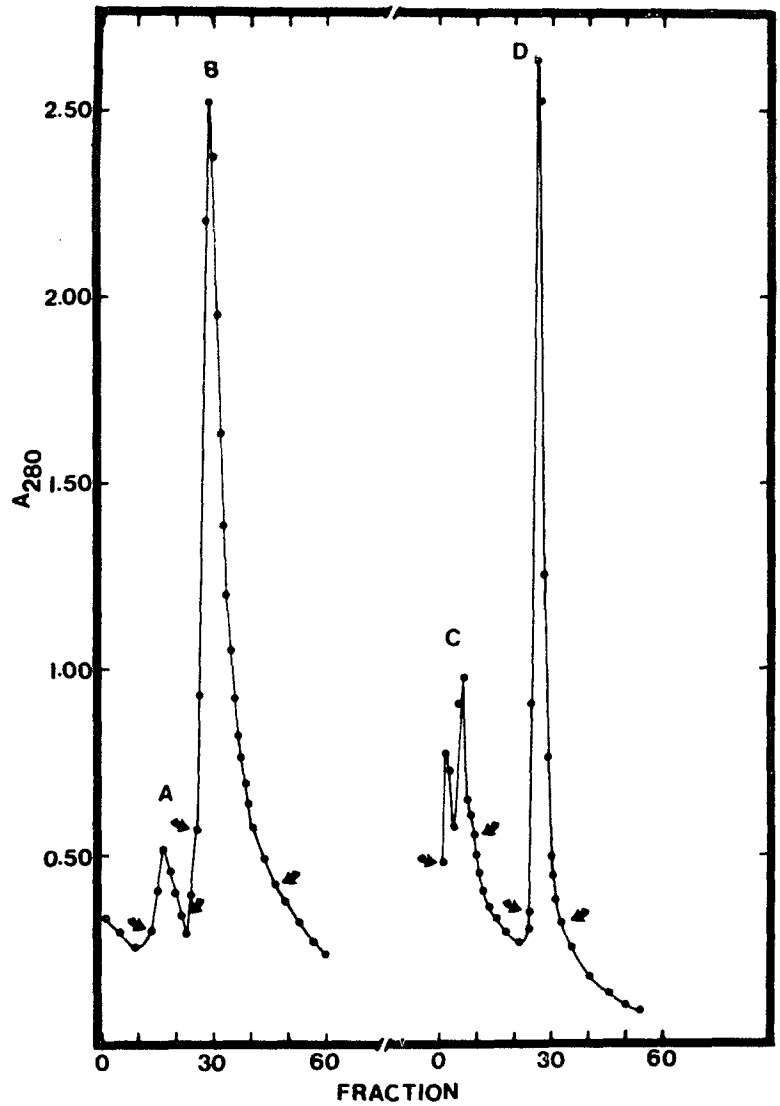

A
B
C
D
2.50
2.00
1.50
1.00
0.50
A_{280}
0
30
60
0
30
60
FRACTION

Figure 6. SDS 7½% to 15% polyacrylamide gel electrophoresis profile of wild type E. coli K12 extract eluted from a ssDNA cellulose column and a blue Sepharose column. Protein (50 µg) from each pooled sample (see Figures 4 and 9) was prepared according to the procedure described in Materials and Methods. The samples were loaded onto an SDS polyacrylamide stack gel and run at 75 volts for 30 minutes. The voltage was then raised to 100 and electrophoresis was continued for 18 hours.

Lane 1: 0.3 M NaCl plus buffer A elution of ssDNA cellulose column

Lane 2: 2.0 M NaCl plus buffer A elution of ssDNA cellulose column

Lane 3: Hemoglobin molecular weight markers

Lane 4: 4 M Gu-HCl buffer elution of blue Sepharose column (ovalbumin added to facilitate precipitation of SSB)

Lane 5: Wild type SSB marker (ovalbumin added to facilitate precipitation of SSB)

Lane 6: 2.0 M NaCl plus buffer A elution of blue Sepharose column

Lane 7: Precipitate formed in 2.0 M NaCl plus buffer A elution of blue Sepharose column

Lane 8: Precipitate formed in 4 M Gu-HCl buffer elution of blue Sepharose column

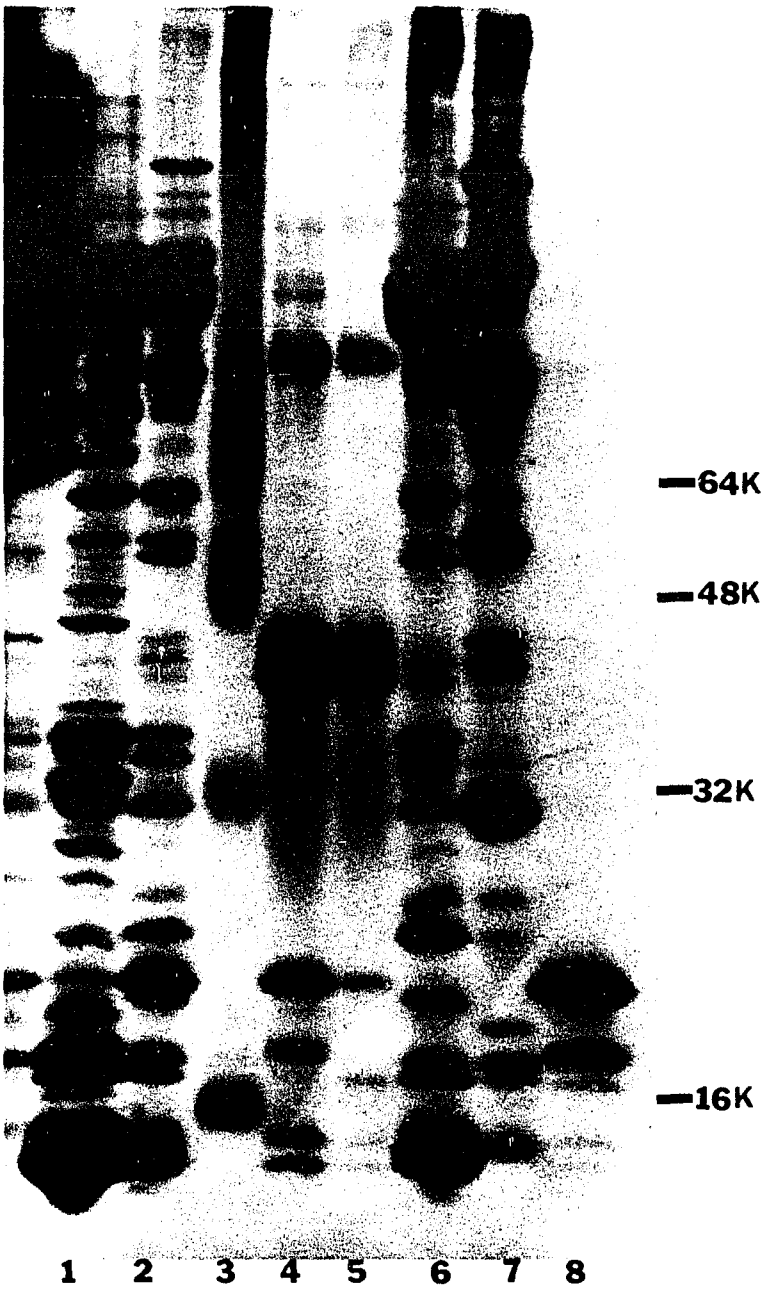

64K
48K
32K
16K
1 2 3 4 5 6 7 8

Figure 7. SDS 7½% to 15% polyacryamide gel electrophoresis profile of ts E. coli KLC 436 extract eluted from a ssDNA cellulose column and blue Sepharose column. Protein (50 µg) from each pooled sample (see Figures 5 and 10) was prepared according to the procedure described in Materials and Methods. The sample was loaded onto an SDS 3% polyacrylamide stack gel and run at 75 volts for 30 minutes. The voltage was then raised to 100 and electrophoresis continued for 18 hours.

Lane 1: Precipitate from 2.0 M NaCl plus buffer A elution of ssDNA cellulose column

Lane 2: Precipitate from 0.3 M NaCl plus buffer A elution of ssDNA cellulose column

Lane 3: 2.0 M NaCl plus buffer A elution of ssDNA cellulose column

Lane 4: 2.0 M NaCl plus buffer A elution of blue Sepharose column

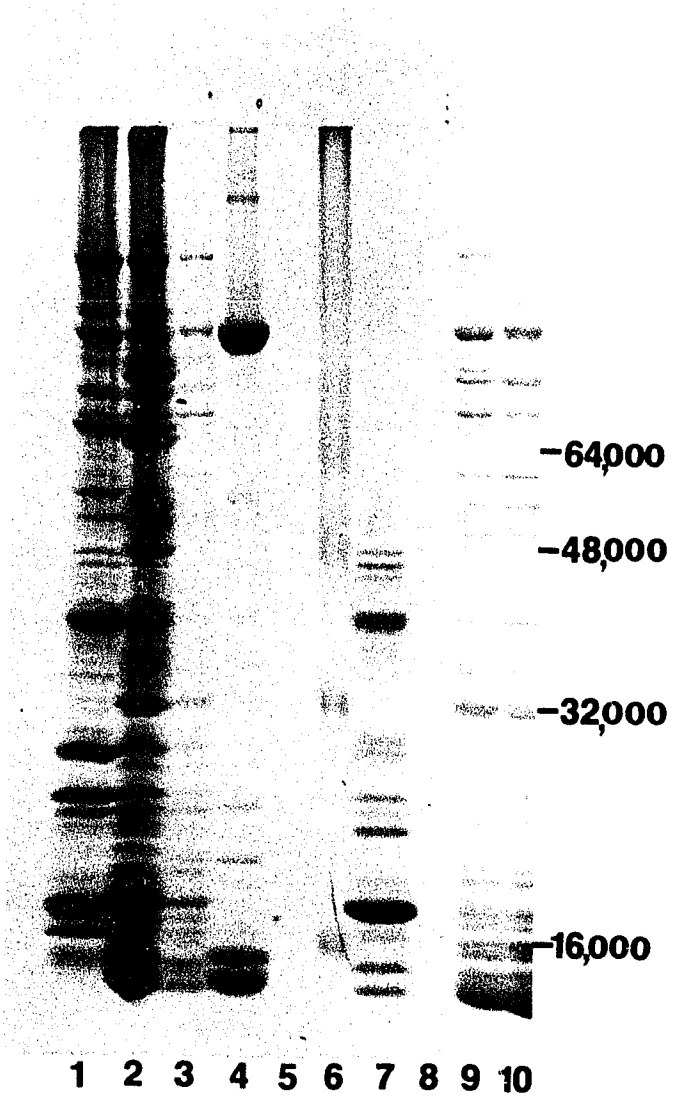
Lane 5: Blank

Lane 6: Hemoglobin molecular weight markers

Lane 7: 4 M Gu-HCl buffer elution of blue Sepharose column

Lane 8: Blank

Lanes 9 and 10: 0.3 M NaCl plus buffer A elution of ssDNA cellulose column


-64,000
-48,000
-32,000
-16,000
1 2 3 4 5 6 7 8 9 10

band is found in the wild type extract lane. To account for the observed differences, one may hypothesize that the ts ssb$^-$ mutant, unlike the wild type strain, is underproducing certain proteins and overproducing other ssDNA binding proteins. It is also possible that the ts ssb$^-$ mutant is producing other defective proteins which are not binding tightly to ssDNA. Most important to note is that there is no band in the 2.0 M NaCl in Buffer A eluted ts fraction (Figure 7, lanes 1 and 3) which corresponds to the wild type SSB.

Prior to this work, β-mercaptoethanol had been included in all buffers. A dextran sulfate-bovine serum albumin in Buffer A rinse of the ssDNA cellulose column prior to a 0.3 M NaCl plus Buffer A elution had also been part of the protocol. Examining the protein purification procedures by polyacrylamide gel electrophoresis, it was discovered that these two steps could be eliminated. Figure 8 represents the elution profile of wild type E. coli K12 whole cell extract over a ssDNA cellulose column. β-mercapto-ethanol was excluded from all buffers. Lanes 10 and 11 of Figure 8 are examples of E. coli K12 whole cell extract loaded onto the gel. Most of these proteins pass through the column. Washing the column with Buffer A (Figure 8, lane 3) removes the weakly adhering

Figure 8. SDS 7½% to 15% polyacrylamide gel electrophoresis profile of wild type *E. coli* K12 extract eluted from a ssDNA cellulose column. Protein (50 µg) from pooled samples (chromatograph not shown) was prepared according to the procedure described in Materials and Methods. The sample was loaded onto an SDS 3% polyacrylamide stack gel and run at 75 volts for 30 minutes. The electrophoresis was continued at 100 volts for 18 hours.

Lane 1: Molecular weight markers

Lane 2: Precipitate from 2.0 M NaCl plus buffer A elution of ssDNA cellulose column

Lane 3: Buffer A wash of ssDNA cellulose column

Lane 4: Hemoglobin molecular weight markers

Lane 5: 0.3 M NaCl plus buffer A elution of ssDNA cellulose column

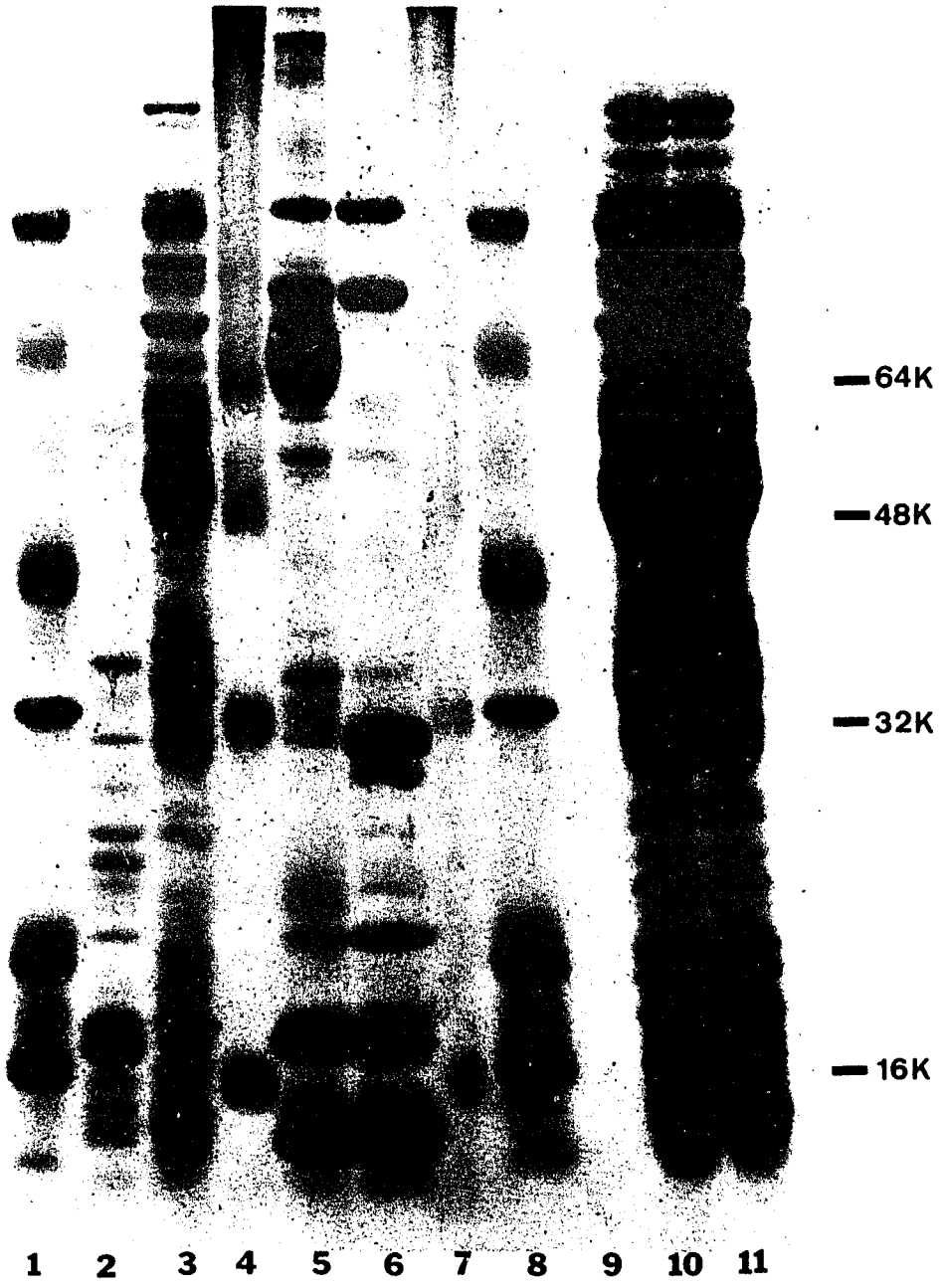
Lane 6: 2.0 M NaCl plus buffer A elution of ssDNA cellulose column

Lane 7: Hemoglobin molecular weight markers

Lane 8: Molecular weight markers

Lane 9: Blank

Lanes 10 and 11: *E. coli* whole extract


64K
48K
32K
16K
1 2 3 4 5 6 7 8 9 10 11

proteins. This particular column was then washed with 0.36 µg/ml dextran sulfate plus 100 µg/ml bovine serum albumin in Buffer A. A 0.3 M NaCl in Buffer A rinse followed (Figure 8, lane 5). In Figure 6 the dextran sulfate rinse is eliminated. Figure 6, lane 1 shows that the 0.3 M NaCl in Buffer A rinse elutes 40 bands as compared to 20 bands when the column first receives a dextran sulfate rinse (Figure 8, lane 5). When the column is then rinsed with 2.0 M NaCl in Buffer A, 15 bands are eluted from the dextran sulfate treated column (Figure 8, lane 6), while 31 bands are eluted from the nontreated column (Figure 6, lane 2). These fractions were then dialyzed into Buffer A and placed over a blue Sepharose column. This column was then washed with Buffer A, 2.0 M NaCl in Buffer A (Figure 6, lane 6) and 4 M Gu-HCl buffer (Figure 6, lane 4). The wild type SSB is highly enriched in the 4 M Gu-HCl buffer eluted fraction (the band seen in Figure 6, lanes 4 and 5 above the 21,000 dalton band for SSB is a 45,000 dalton ovalbumin band). Fifty micrograms of ovalbumin was added to the sample to facilitate precipitation of the gel sample. A more precise picture of the purified protein can be seen in Figure 6, lane 8. Ovalbumin was not added to this sample. Because the excess protein eluted in the non-dextran sulfate treated column was eliminated when the sample passed

over a blue Sepharose column, there was no need to add an extra dextran sulfate rinse step to the ssDNA cellulose elution.

Prior to this work, a DEAE cellulose column was used before the blue Sepharose column. As is evident from the gels (Figure 6, lane 8), this step was also shown to be unnecessary. An attempt was made to eliminate the ssDNA cellulose column and simply pass the E. coli extract directly over a blue Sepharose column. Too many proteins in the cell extract had as high an affinity for blue Sepharose as SSB (data not shown). The best method to date for isolating wild type SSB is the ssDNA cellulose, blue Sepharose column combination.

After the procedure of going directly from a ssDNA cellulose column to a blue Sepharose column was worked out for the wild type E. coli K12 protein, an attempt was made to isolate the ts E. coli mutant SSB in the same manner. Instead, a different ssDNA binding protein, SSB-B was isolated.

Figures 9 and 10, respectively, are chromatographs from blue Sepharose columns of the wild type E. coli K12 and ts E. coli KLC 436 samples previously eluted from a ssDNA cellulose column with 2.0 M NaCl in Buffer A. Peaks A, B, and C of the wild type elution correspond to peaks A, B, and C of the ts elution.

Figure 9. Elution profile of wild type E. coli K12 protein sample over a blue Sepharose column. Wild type E. coli K12 eluted from a ssDNA cellulose column with 2.0 M NaCl plus buffer A (see Figure 4, Peak B) was dialyzed into buffer A and placed over a 25 cm X 1.5 cm blue Sepharose column. The flow rate was 20 ml/hr. The sample volume was 3 ml/tube. Peak A represents loading the sample onto the column and eluting with buffer A. Peak B represents the 2.0 M NaCl plus buffer A elution of the column. Peak C represents the 4 M Gu-HCl buffer elution of the column. Fractions between the arrows were pooled and examined on SDS polyacrylamide gel. The entire procedure was performed at $4^{o}C$.

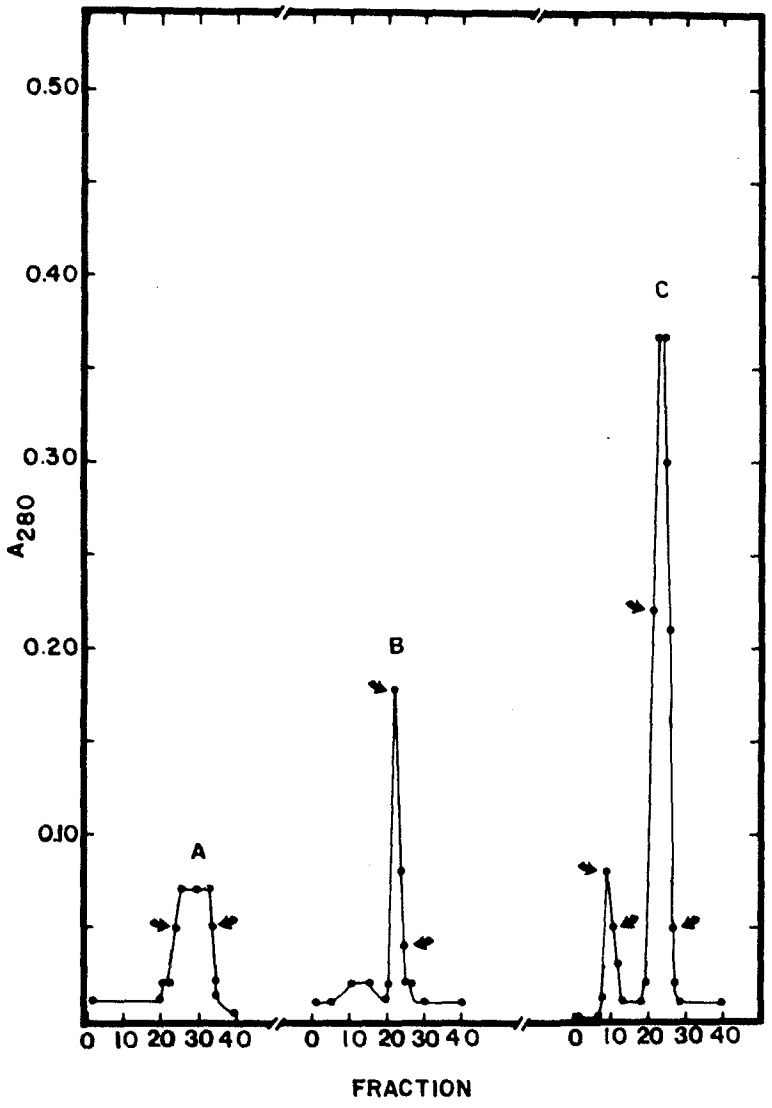
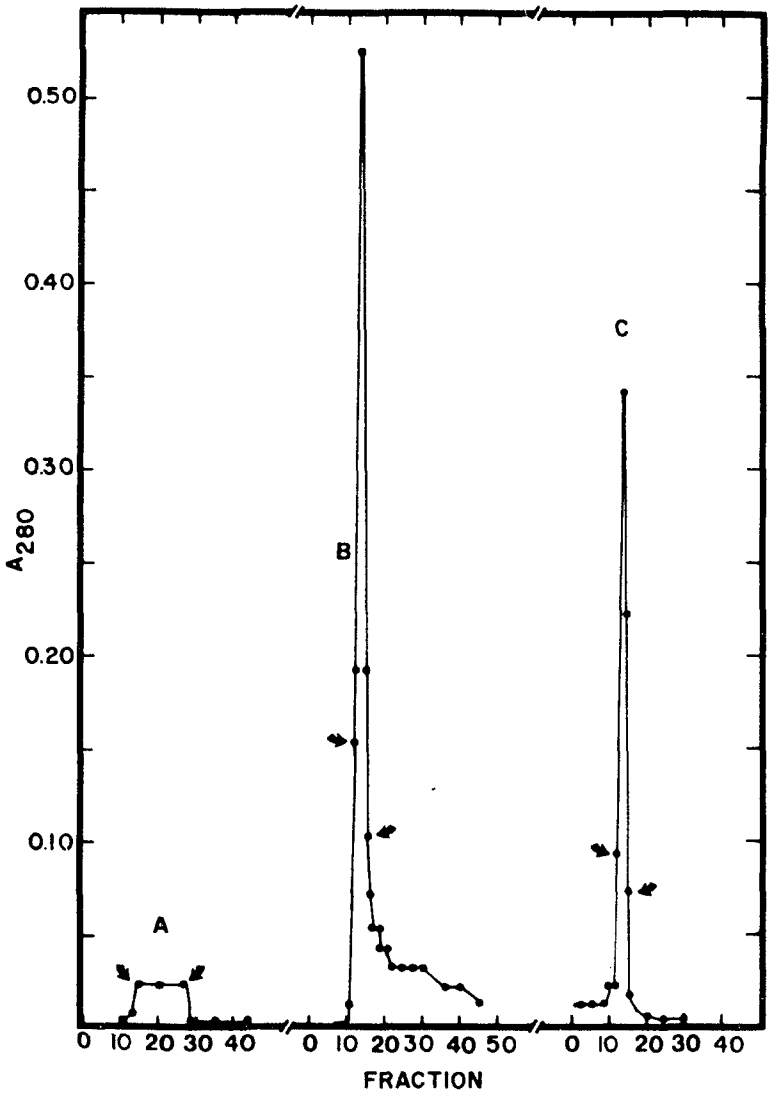

0.50
0.40
0.30
0.20
0.10
A_{280}
A
B
C
0 10 20 30 40
0 10 20 30 40
0 10 20 30 40
FRACTION

Figure 10. Elution profile of the ssb⁻ E. coli KLC 436 protein sample over a blue Sepharose column. The ssb⁻ E. coli KLC 436 eluted from a ssDNA cellulose column with 2.0 M NaCl plus buffer A (see Figure 5, Peak D) was dialyzed into buffer A and placed over a 25 cm X 1.5 cm blue Sepharose column. The flow rate was 20 ml/hr. The sample volume was 4 ml/tube. Peak A represents loading the sample onto the column and eluting it with buffer A. Peak B represents the 2.0 M NaCl plus buffer A elution of the column. Peak C represents the 4 M Gu-HCl buffer elution of the column. Fractions between the arrows were pooled and examined on SDS polyacrylamide gel. The entire procedure was performed at $4^{o}C$.


0.50
0.40
0.30
0.20
0.10
A_{280}
A
B
C
0 10 20 30 40
0 10 20 30 40 50
0 10 20 30 40
FRACTION

Peak B represents the 2.0 M NaCl in Buffer A wash of the blue Sepharose column. When examined on SDS polyacrylamide gel, this fraction contains many E. coli proteins (Figure 6, lane 6; Figure 7, lane 4). Figure 11 depicts the results of the final purification step. As is evident from the gel, the wild type SSB (lane 1) was not found in the SSB-B fraction isolated from the ts E. coli KLC 436 (lane 2), or vice versa. When calculated, the wild type SSB had a molecular weight of 21,000 daltons while the predominant SSB-B protein had a molecular weight of approximately 18,000 daltons. A molecular weight of 21,000 daltons for wild type SSB does not agree with the 18,873 molecular weight determined by sequencing the protein (Sancar et al., 1981). Other laboratories calculated a 22,000 dalton molecular weight for SSB (Sigal et al., 1972; Molineaux et al., 1974; and Anderson and Coleman, 1975). This discrepancy may be the result of using different polyacrylamide gel electrophoresis systems and different molecular weight markers to determine the molecular weight of SSB. A number of other proteins which strongly bind to blue Sepharose are co-isolated with SSB-B (Figure 11, lane 2). These may be other proteins produced by the ssb$^-$ mutant to compensate for the lack of a wild type SSB. Ultraviolet absorption spectra of the wild type SSB shown in Figure 12 were identical to

the spectra of the SSB-B sample (data not shown). Further characterization of the SSB-B was needed before any statements could be made about the protein.

Figure 11. SDS 7½% to 15% polyacrylamide gel electrophoresis of SSB and SSB-B samples. Protein (50 µg) was prepared according to the procedure described in Materials and Methods. The samples were loaded onto an SDS 3% polyacrylamide stack gel and run at 75 volts for 30 minutes. Electrophoresis was continued at 100 volts for 18 hours.

Lane 1: 4 M Gu-HCl elution of the blue Sepharose column containing the wild type *E. coli* K12 sample

Lane 2: 4 M Gu-HCl elution of the blue Sepharose column containing the *ssb*⁻ *E. coli* KLC 436 sample

Lane 3: Hemoglobin molecular weight markers

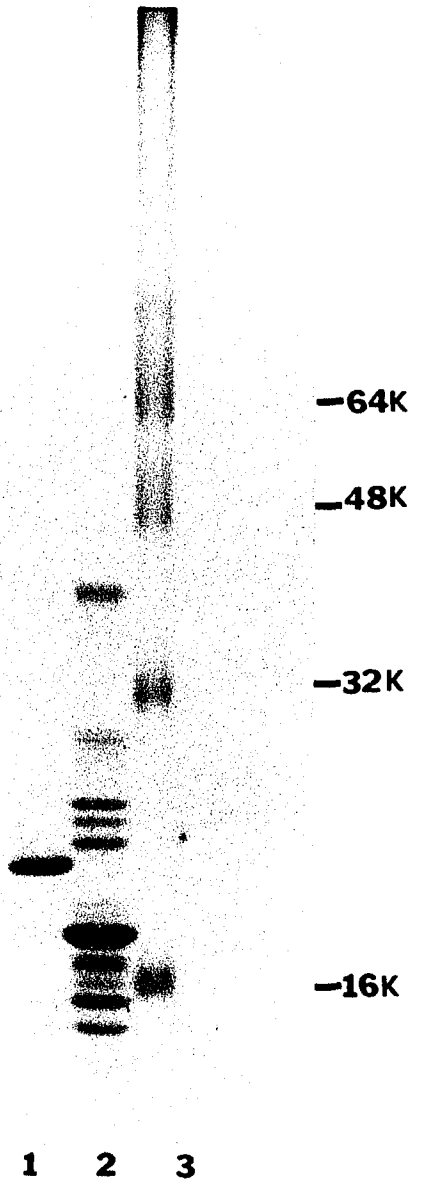
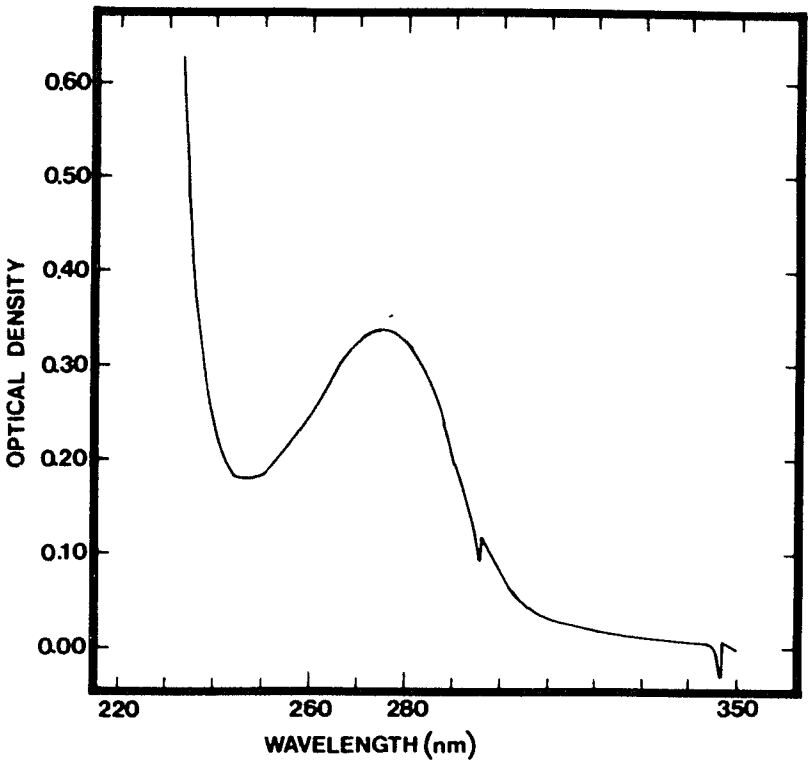

64K
48K
32K
16K
1
2
3

Figure 12. Ultraviolet absorption spectrum of wild type SSB.


0.60
0.50
0.40
0.30
0.20
0.10
0.00
OPTICAL DENSITY
220
260
280
350
WAVELENGTH (nm)

CHAPTER 5

CHARACTERIZATION OF SINGLE STRAND DNA BINDING PROTEINS SSB AND SSB-B

INTRODUCTION

Wild type E. coli SSB has been isolated from a number of different strains of E. coli (see Table 4). Details of determining SSB's tetrameric form and molecular weight, its stoichiometry of binding to ssDNA and its interaction with other DNA binding proteins have been discussed in Chapter 1. Amino acid analysis of wild type E. coli SSB (Weiner et al., 1975; Anderson and Coleman, 1975) revealed a characteristic amino acid composition (see Table 5). An unusually high proportion of glycine and glutamine and/or glutamic acid was found in the protein. When SSB was acid hydrolyzed and performic acid oxidized, cysteine oxidation products were not detected. It is interesting to note that other DNA binding proteins lack cysteine (E. coli H protein, Hubscher et al., 1980; E. coli HU protein, Laine et al., 1980; A. salina HD40 protein, Marvil et al., 1980; SSB-B, this report). Based on the amino acid sequencing of SSB, Sancar et al. (1981) divided the protein into three domains. The first 105 residues of the amino terminus contain 79% of all

TABLE 4

STRAINS OF ESCHERICHIA COLI USED TO CHARACTERIZE THE WILD TYPE AND TEMPERATURE SENSITIVE SSB

E. coli Strain	Reference
B (wild type)	Anderson and Coleman (1975) Christiansen and Baldwin (1977) Kunkel et al. (1979) Weiner et al. (1975)
B (temperature sensitive)	Johnson (1977) Vales et al. (1980)
D10 (wild type)	Sigal et al. (1972)
K12 (wild type)	Molineux et al. (1974) Molineux and Gefter (1974) Ruyechan and Wetmur (1975) Ruyechan and Wetmur (1976)
K12 (temperature sensitive)	Sevastopoulos et al. (1977) Meyer et al. (1979) Glassberg et al. (1979) Meyer et al. (1980) Vales et al. (1980) Whittier and Chase (1980)

TABLE 5

AMINO ACID COMPOSITIONS OF SSB AND SSB-B

Amino Acid	DNA Sequence(1)	*Amino Acid Analyses SSB(1)	SSB(2)	SSB(3)	SSB(4)	SSB-B(4)
Cys	0	0.3	0.9	0	0	0
Asx	16	16.3	16.2	16	14.1	12.8
Thr	9	8.4	13.3	11	¶9.8	¶9.0
Ser	11	9.6	11.8	13	9.4	6.2
Glx	28	27.2	26.0	30	25.2	19.2
Pro	12	11.5	10.0	9	¶13.0	¶7.8
Gly	28	27.9	24.5	34	28.6	16.7
Ala	13	13.3	13.9	17	12.9	23.0
Val	13	12.6	12.9	8	11.0	15.1
Met	5	4.1	4.9	9	4.7	5.1
Ileu	5	4.8	5.3	3	5.2	7.3
Leu	8	8.5	9.0	6	9.5	11.0
Tyr	4	4.4	4.4	4	3.8	2.2
Phe	4	4.3	3.9	4	5.5	5.6
His	1	1.3	2.6	1	2.6	5.3
Lys	6	6.2	6.1	8	6.9	10.4
Arg	10	9.5	8.1	9	¶10.6	¶14.6
Trp	4	4.6	2.6	3	ND	ND

*Data are numbers of amino acids based on a molecular weight of 18,873 daltons for SSB and 17,800 daltons for SSB-B.

¶Amino acids determined through a system utilyzing ninhydrin with sodium acetate and citric buffers.

ND = not determined

1. Sancar et al. (1981)
2. Anderson and Coleman (1975)
3. Weiner et al. (1975)
4. This work

charged amino acids found in the protein. This region is predicted to have a high region of secondary structure (α-helix and β-pleated sheet). It is predicted that this domain may bind to ssDNA via ionic interactions. The next 60 residues of the protein contain only two charged amino acids and are devoid of α-helix and β-pleated sheet. This long coil region is believed to have some secondary structure (β-bends). The 12 remaining carboxy terminal residues contain a negatively charged, α-helical region. Amino acid analysis of the SSB-B protein is discussed in this Chapter.

In order to measure the binding activity of SSB to ssDNA, Ruyechan and Wetmur (1976) developed a nuclease protection assay. Wild type E. coli SSB protects ssDNA from snake venom phosphodiesterase and DNase I cleavage (Molineaux and Gefter, 1975). Ruyechan and Wetmur (1976) reported that the amount of ssDNA protected from nuclease digestion was dependent upon the amount of wild type SSB present. When ssDNA was covered from 6% to 92% with SSB, the percent of protection observed was linear. Stable protein-DNA complexes were found to occur in less than 1 minute. Based on the same principle that SSB protects ssDNA from nucleases, Meyer et al. (1980) used an S1 nuclease digestion assay to test the abilities of wild

type SSB and ts SSB to bind ssDNA. A four-fold higher concentration of purified ts SSB was needed to protect the ssDNA to the same extent as purified wild type SSB. Both of these nuclease protection assays were used to detect the binding capacity of SSB-B to ssDNA (see text of this Chapter).

A second method of examining SSB's ability to bind ssDNA is the filter binding assay developed by Whittier and Chase (1980). Sonicated, denatured and labeled bacteriophage T7 DNA adheres to the filters in this assay only when complexed with protein. Presaturating the nitrocellulose filter with denatured salmon sperm DNA prevented noncomplexed ssDNA from binding the filter when high ionic strength buffers were used. This allowed Whittier and Chase to examine E. coli SSB in crude extracts for the protein's ssDNA binding capacity. Proteins which weakly bind to ssDNA would not interfere due to inhibition of their binding ssDNA in high ionic strength buffer. Antibody prepared to wild type SSB was mixed with homogeneous SSB and added to [^{3}H]ssDNA. Binding of this ssDNA to the filters was inhibited 87% in a ½X SSC buffer (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.4). Anti-SSB antibody was then added to a crude extract of wild type E. coli prior to the addition of [^{3}H]ssDNA. In this assay, binding of [^{3}H]ssDNA to the

filters was inhibited 74% in ½X SSC. When assayed in 3X SSC buffer, antibody to wild type SSB inhibited both crude extract and homogeneous wild type SSB-[^{3}H]ssDNA complexes from binding ssDNA saturated filters by 98%. Crude extract from the ts ssb$^-$ E. coli KLC 436 mutant was combined with [^{3}H]ssDNA. When assayed in ½X SSC buffer on ssDNA saturated filters, four-fold less ssDNA was retained on the filters than when the same amount of wild type extract was combined with [^{3}H]ssDNA. When the ability of ts extract proteins to bind ssDNA was assayed in 3X SSC buffer, fifty-fold less [^{3}H]ssDNA was retained on the ssDNA saturated filters as compared to the amount of ssDNA retained when wild type E. coli was used. The reason for the decreased protein-ssDNA complex formation when the ts extract is assayed in 3X SSC buffer may be that the ts SSB is eluted from ssDNA in the higher salt buffer. Curiously enough, the activity of the ts extract was not inhibited by anti-SSB antibody in the ½X SSC assay. Using a radioimmunoassay, Meyer et al. (1979) was able to detect ts SSB in ts extract fractions using antibody to wild type SSB. It seems likely that rather than measuring ts SSB-ssDNA complexes, Whittier and Chase may have been measuring ssDNA complexed to SSB-B and other KLC 436 proteins in ½X SSC buffer. As will be

discussed in this chapter, antibody to wild type SSB does not bind to SSB-B.

Zechel et al. (1975) developed the following *in vitro* DNA synthesis system which is often used to determine the replication activity of SSB. Bacteriophage G4 circular ssDNA is converted to the double stranded replicative form in the presence of three proteins: the *E. coli* DNA polymerase III holoenzyme (*dnaZ* and *dnaE*), priming protein (*dnaG*) and *E. coli* SSB. Crude cell extracts of wild type *E. coli* cells were found to support the replication of G4 in this assay system (Meyer et al., 1979). If the crude cell extract from the ts *E. coli ssb*$^-$ mutant was used, no replication of G4 was seen even when 20 times more total protein was used. Incubation temperatures between 20^{o}C and 37^{o}C made no difference. If purified wild type SSB was added to the ts SSB crude extract mixture, activity was restored (Meyer et al., 1979). Purified ts SSB was able to support G4 DNA replication at 30^{o}C. However, a higher level of the ts SSB was needed (2 µg of ts SSB gave the same activity as 0.5 µg of the wild type SSB). At 42^{o}C the ts SSB was much less active than the wild type SSB in the G4 assay. Four times more ts SSB than wild type SSB was needed to achieve 10% to 25% of the activity of wild type SSB (Meyer et al., 1979). It would have been in-

structive to attempt to use SSB-B in the G4 assay system. Unfortunately this assay was not available when experiments in SSB-B characterization were performed.

MATERIALS AND METHODS

Iodination of DNA (Orosz and Wetmur, 1974)

Sonicated calf thymus DNA (Sigma) was dialyzed into filtered 7.2 M $NaClO_4$ (Frederick Smith Chem. Co.). Six hundred micrograms of DNA was combined with 3.3×10^{-5} M KI and 1 µCi of ^{125}I. $TlCl_3$ and NaOAc, pH 5.0 were then added to a final molarity of 9×10^{-4} and 0.1 respectively. The mixture was incubated at 60°C for 15 minutes after which it was ice quenched to stop the reaction. The sample was dialyzed into 500 ml of phosphate buffer (0.4 M NaCl, 0.2 mM EDTA, 10 mM sodium phosphate, pH 6.86) overnight. The next day the entire flask was incubated at 60°C for 2 hours. The sample was then extensively dialyzed into a buffer of choice.

DNase I-snake venom phosphodiesterase nuclease protection assay

Buffer: 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM Tris-HCl, pH 7.6

Nuclease Buffer: 50 mM NaCl, 10 mM $MgCl_2$,

10 mM Tris-HCl, pH 7.6, 100 µg/ml (1 unit* per 90 µl buffer) DNase I (Sigma) and 10 µg/ml snake venom phosphodiesterase (Sigma).

Procedure: Protein concentrations were varied to give a protein to DNA mass ratio of 2:1, 4:1, 6:1, and 10:1. A ratio of 8:1 should yield 100% protein coverage of DNA and therefore result in complete protection. Buffer was added to yield a final volume of 95 µl. ^{125}I-labeled and sonicated calf thymus DNA was boiled for 1-2 minutes and placed on ice. Each tube had 0.25 µg of labeled DNA in 5 µl aliquots. The mixture was incubated for 15 minutes at 4°C. Ten microliters of each sample was added to 90 µl of nuclease buffer and placed at 37°C for 30 minutes. One hundred microliters of cold 0.2 M Na_2EDTA plus NaOH, pH 7.9 was used to stop the reaction. Ten microliter samples were placed on thin layer chromatography polygram strips (Brinkmann Instruments). The strips were placed in a chromatography tank containing 1 M HCl. When the HCl reached the top of the strip, the strip was removed and dried. After the strips dried, they were cut in half and counted in a Beckman Biogamma counter. The following formula was used to calculate the amount of ssDNA protected from nuclease

*1 unit = the amount of nuclease capable of degrading 50 ng of DNA in one minute at 25° C.

digestion:

$$\% \text{ protection} = \frac{\text{counts remaining on bottom of strip}}{\text{bottom counts plus top counts}} \times 100$$

S1 nuclease protection assay

S1 nuclease buffer: 300 mM NaCl, 1 mM zinc acetate, 50 mM sodium acetate, pH 4.6, and 2 units of S1 nuclease (Miles).

Procedure: This assay followed the procedure of the DNase I snake venom phosphodiesterase assay substituting S1 nuclease for the two nucleases and S1 nuclease buffer for the nuclease buffers in the nuclease protection assay.

Immunization of rabbits

On day zero rabbits received intramuscular injections of 100 µg purified wild type E. coli K12 SSB in complete Freund's adjuvant. Rabbits were boosted intravenously with 100 µg of protein 40 and 76 days later. Small bleeds (1 ml) were taken on day zero and day 22. A large bleed (10 ml) was taken on day 47. The rabbits were exsanguinated on day 85.

Enzyme linked immunosorbent assay (ELISA)

The antibody assay using an enzyme-labeled anti-globulin procedure followed that of Voller et al. (1976). Alkaline phosphatase labeled antiglobulin was used.

Absorbance of contents of each well was read in a spectrophotometer at a wavelength of 400 nm.

Immunodiffusion

Immunodiffusion plates (Hyland, pattern D) were used for all experiments. Samples were loaded and allowed to diffuse overnight at room temperature.

Amino acid analysis

Samples from preparatory gels: One hundred microliters each of wild type SSB and SSB-B were electrophoresed on an SDS 7½% to 15% polyacrylamide gel (15 cm X 20 cm X 0.4 cm) for 20 hours at 100 volts. Precautions were taken to prevent any protein contamination of the gel. The gel was stained with 0.2% coomassie brilliant blue (Kodak), 50% methanol and 7% glacial acetic acid for 1 hour at 37^{0}C. The gel was then destained using 7% glacial acetic acid until all background stain was removed. The protein bands were cut out of the gel. Two cm X one cm (diameter) SDS 5% polyacrylamide tube gels were poured. These gels were loaded onto a Bio-Rad tube gel apparatus and had 40 volts/tube passed through them for 30 minutes. Dialysis bags were attached to the bottom of each tube. Fresh Tris-glycine buffer (0.06% Tris, 2.88% glycine, 0.01% SDS (Bio-Rad)) was added and the cut out bands were layered above the new gels. The gels were run at 40 volts/tube for 24 hours. The dialysis

bags were removed. The samples were dialyzed into 10% glacial acetic acid and then hydrolyzed for either 24 or 72 hours. Analysis for amino acid composition was done using O-Phthaldehyde as a detection agent in a fluorometric microbore amino acid analyzer (Lee et al., 1979).

Samples not gel purified: Protein samples were dialyzed directly into 10% glacial acetic acid. These were then analyzed by the same procedure as gel eluted samples.

RESULTS AND DISCUSSION

1. Amino Acid Analysis

Using a highly sensitive fluorescent detecting amino acid analyzer, micromolar quantities of proteins could be analyzed and compared. Whether protein samples were used directly or electroeluted from SDS polyacrylamide gels, their compositions were identical. The wild type SSB analyzed here agreed with that published by Anderson and Coleman (1975), Weiner et al. (1975), and Sancar et al. (1981) (see Table 5). As was found with the published compositions, the wild type SSB contained no cysteines. When SSB-B was similarly analyzed, the following was observed (see Table 5). Although SSB-B also contained no cysteines, the amino acid composition looked very different from that of

wild type SSB. Upon examination of the wild type SSB protein sequence (Sancar et al., 1981), it was realized that SSB-B could not be a cleavage product of SSB. Removing bases from the amino or carboxy terminus of wild type SSB could not fully account for the observed difference in amino acid composition. SSB-B does appear to have an amino acid composition similar to an E. coli histone-like protein, H-protein (see Table 6). These proteins differed, however, in molecular weight (Hubscher et al., 1980). SSB-B does share a molecular weight similar to that of an E. coli histone-like protein (BH1) isolated by Varshavsky et al. (1977). Further characterization of BH1 is necessary before any comparisons can be made. Other E. coli histone-like proteins (HU-1 and HU-2) showed no similarities to SSB-B in amino acid composition (see Table 6). SSB-B may therefore be a histone-like protein which is overproduced by the E. coli ts ssb^- mutant.

2. Antibody Assays

Antibody to purified wild type E. coli SSB was raised in rabbits. To check the antigenic activity of the rabbit serum, immunodiffusion plates were used. A definite precipitin line formed when wild type SSB (50 µl of a 0.1 mg/ml sample) was run against whole immune serum. SSB-B showed no reaction in this assay (data not shown). Believing that a precipitin

TABLE 6

AMINO ACID COMPOSITIONS OF SSB, SSB-B AND OTHER DNA BINDING PROTEINS

Amino Acid	*Amino Acid Analyses					
	† SSB(1)	SSB-B(2)	H(3)	HU-1(4)	HU-2(4)	HD40(5)
Cys	0	0	0	0	0	0
Asx	9.0	8.9	7.6	9.9	8.2	10.5
Thr	5.1	¶4.5	4.5	5.5	7.7	3.9
Ser	6.2	4.3	4.3	5.3	4.9	5.1
Glx	15.8	10.9	11.6	7.2	11.8	11.4
Pro	6.8	¶3.9	4.8	2.4	2.4	4.6
Gly	15.8	11.6	9.7	9.1	6.7	19.1
Ala	7.4	12.2	11.3	21.0	16.4	7.3
Val	7.4	10.4	8.3	6.3	7.8	5.5
Met	2.8	3.5	0	0.9	1.1	1.5
Ileu	2.8	5.1	6.5	7.7	5.6	3.6
Leu	4.5	7.6	7.2	5.7	7.6	3.8
Tyr	2.3	1.5	0.4	0	0	5.6
Phe	2.3	3.9	1.3	3.3	3.2	4.1
His	0.6	3.5	1.7	0	1.0	1.8
Lys	3.4	7.2	10.7	10.2	12.0	7.7
Arg	5.7	¶7.3	9.9	5.1	3.7	2.2
Trp	2.3	ND	0	0	0	1.0
Dimethyl Arg						1.4

*Data are numbers of amino acids calculated as mole%

† DNA sequence

¶ Amino acids determined through a system utilyzing ninhydrin with sodium acetate and citric buffers

ND= not determined

1. Sancar et al. (1981)
2. This study
3. Hubscher et al. (1980)
4. Laine et al. (1980)
5. Marvil et al. (1980)

assay was not sensitive enough to detect common determinants between the two proteins, an ELISA was done. Antibody to wild type SSB showed a titer of 6400. SSB-B with a titer of less than 50, showed no reaction above background (see Table 7). This finding suggested that the wild type SSB and SSB-B may indeed have nothing in common except their ability to strongly bind ssDNA and blue Sepharose. This antigenic unrelatedness confirms the results of the amino acid analysis which suggested that SSB and SSB-B are different proteins. The next step was to determine if, like wild type SSB, SSB-B was capable of protecting ssDNA from nuclease degradation.

3. Nuclease Protection Assays

a. DNase I - snake venom phosphodiesterase nuclease protection assay

An assay used to measure the cooperative binding activity of wild type SSB is the nuclease protection assay. Ruyechan and Wetmur (1976) found that wild type E. coli SSB protects ssDNA from snake venom phosphodiesterase and DNase I cleavage (see Introduction of this Chapter for more details).
To test the binding activity of SSB-B to ssDNA, the following experiment was performed. Protein varying in concentrations from 0.5 µg to 2.5 µg was incubated with 0.25 µg of iodinated ssDNA at 4^{0}C for fifteen minutes.

TABLE 7

ENZYME LINKED IMMUNOSORBENT ASSAY OF SSB AND SSB-B

Rabbit Anti-SSB Serum Dilutions	Antigen (100 ng/well)		
	SSB	SSB-B	Prebleed
1/50	1.7	0.29	0.45
1/100	1.7	0.29	0.32
1/200	1.7	0.22	0.22
1/400	1.69	0.18	0.15
1/800	1.42	0.13	0.11
1/1600	0.71	0.11	ND
1/3200	0.45	0.09	ND
1/6400	0.29	0.08	ND
1/12800	0.19	0.07	ND
1/25600	0.12	0.03	ND

The values represent spectrophotometer readings (A_{405}).

ND = not determined

The nucleases were then added and the mixtures incubated at either 4°C, 30°C, or 37°C for 30 minutes. The reaction was stopped with the addition of cold 0.2 M EDTA plus NaOH, pH 7.9. Ten microliter samples were removed and run up thin layer chromatography strips. The strips were dried, cut in half, and counted in a Beckman Biogamma counter. Any acid soluble counts running up the strip were considered unprotected ssDNA. The results are shown in Figure 13. Wild type SSB protected DNA at all temperatures while the SSB-B protected DNA at 4°C only. A control experiment was run to show that proteins which strongly bind ssDNA, other than SSB, do not exhibit this protecting ability (Figure 14). Wild type proteins were eluted with high salts from a ssDNA cellulose column (Figure 4, peak B). This fraction was then passed over a blue Sepharose column and eluted with 2.0 M NaCl in buffer A (Figure 9, peak B). This protein fraction was then used in a protection assay. SSB is removed from blue Sepharose in 4 M Gu-HCl only (see Chapter 4). These proteins gave no observable protection at any of the three temperatures over a range of protein concentrations from 0.5 µg to 5.0 µg (see Figure 14).

At this point it appeared as though SSB-B was capable of protecting DNA at 4°C. To further

Figure 13. The ability of SSB and SSB-B to protect ssDNA in a DNase I snake venom phosphodiesterase nuclease protection assay. Protein, varying in concentration from 0.5 µg to 2.5 µg, was incubated with 0.25 µg of sonicated ^{125}I-labeled ssDNA at 4°C for 15 minutes. The nucleases were added and the mixtures were incubated at either 4°C (SSB ○ , SSB-B ●), 30°C (SSB □ , SSB-B ■) or 37°C (SSB ☆ , SSB-B ★) for 30 minutes. The reaction was stopped with the addition of cold 0.2 M EDTA, pH 7.9. (see Materials and Methods for calculation of percent protection).

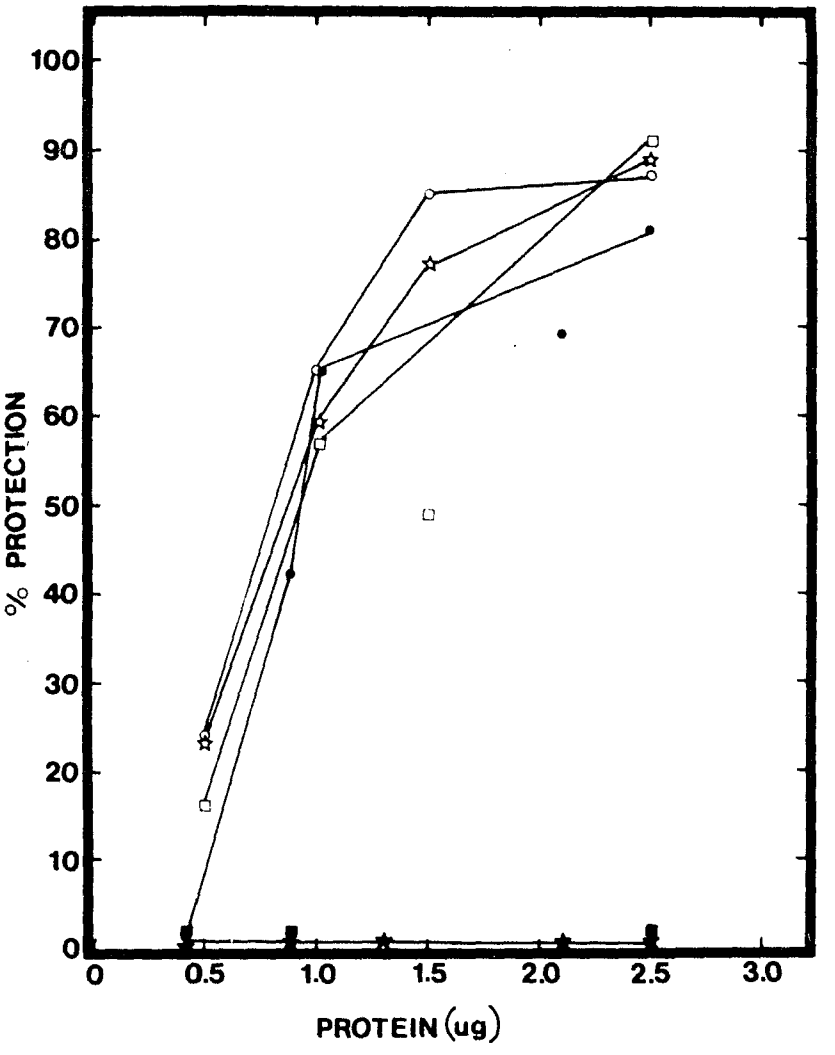
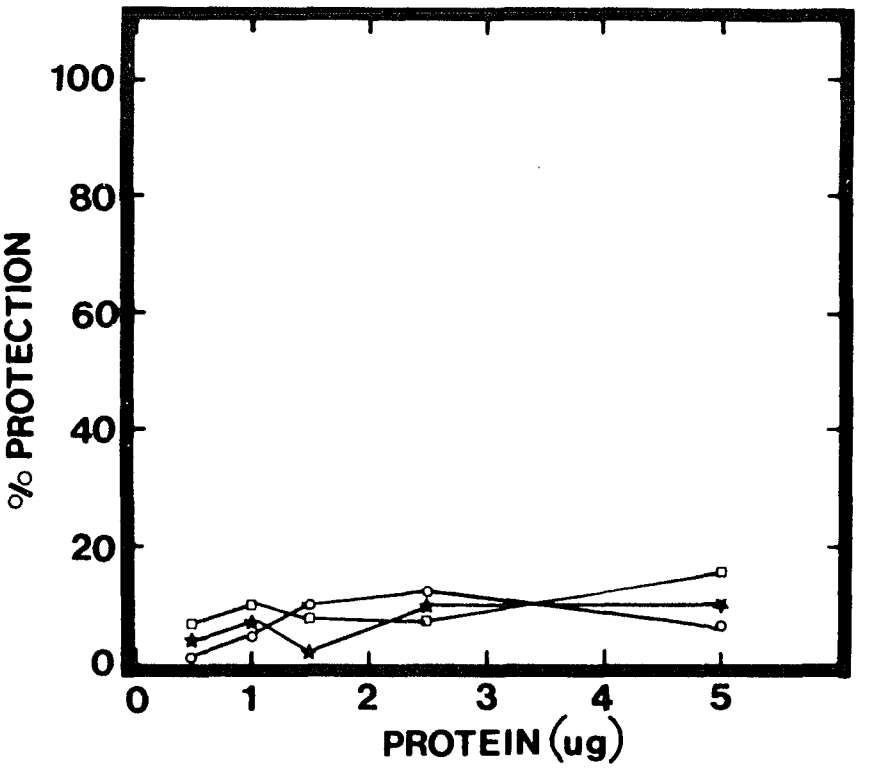

100
90
80
70
60
50
40
30
20
10
0
% PROTECTION
0
0.5
1.0
1.5
2.0
2.5
3.0
PROTEIN (ug)

Figure 14. The ability of proteins which strongly bind ssDNA, other than SSB, to protect ssDNA in a DNase I snake venom phosphodiesterase nuclease protection assay. Protein, varying in concentration from 0.5 µg to 2.5 µg, was incubated with 0.25 µg of sonicated ^{125}I-labeled ssDNA at 4°C for 15 minutes. The nucleases were added and the mixtures were incubated at 4°C (O), 30°C (★) or 37°C (□) for 30 minutes. The reaction was stopped with the addition of cold 0.2 M EDTA, pH 7.9 (see Materials and Methods for calculation of percent protection).


100
80
60
40
20
0
% PROTECTION
0
1
2
3
4
5
PROTEIN (ug)

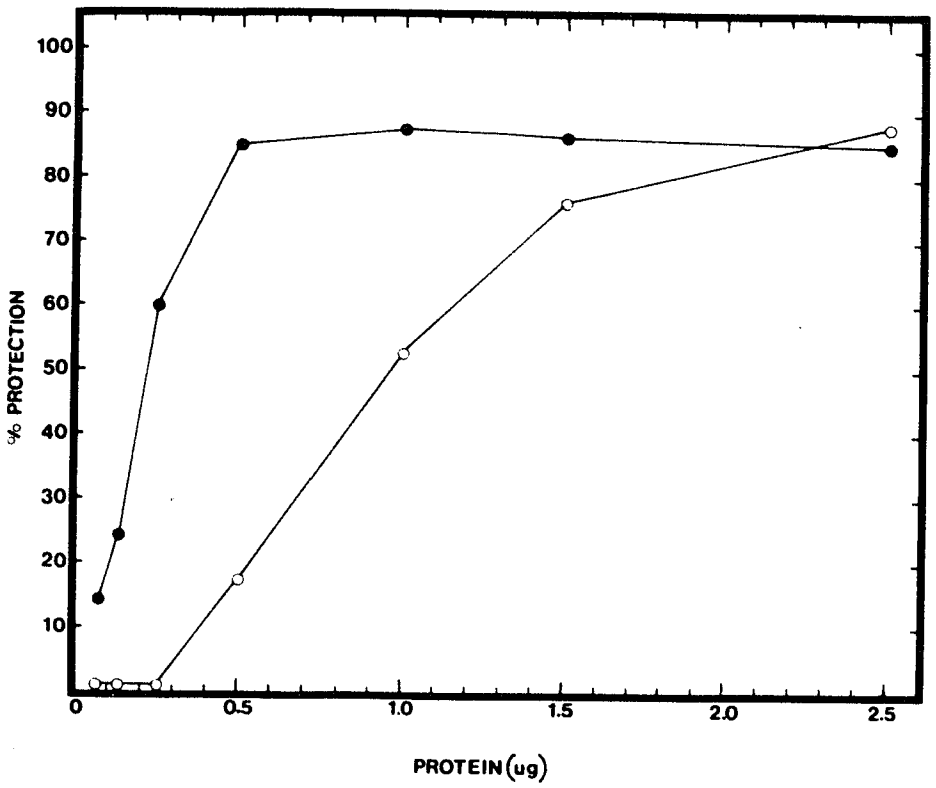
test the ability of SSB-B to protect DNA, an S1 nuclease protection assay was performed and compared to a published assay of the same nature (Meyer et al., 1980).

b. S1 nuclease protection assay

This assay called for preincubating the protein with sonicated ^{125}I-labeled ssDNA at 4^{o}C for 10 minutes. Afterwards, S1 nuclease was added and the mixture was incubated at 37^{o}C for 15 minutes. The rest of the experiment followed the DNase I snake venom phosphodiesterase nuclease protection assay protocol. As can be seen in Figure 15, SSB-B seemed to be more efficient than wild type SSB in protecting ssDNA even at 37^{o}C. Meyer et al. (1980) reported that the ts SSB isolated from the E. coli ssb^{-} mutant was ineffective in protecting ssDNA against S1 nuclease digestion at the concentration at which wild type SSB completely protected the ssDNA. Four-fold higher concentrations of ts mutant SSB were needed to protect the ssDNA. One reason for the ability of SSB-B to protect ssDNA from S1 disgestion and not protect ssDNA from DNase I digestion at 37^{o}C may be the way in which SSB-B binds ssDNA. This result is discussed further in the summary and conclusion chapter.

The evidence presented here distinguishes

Figure 15. The ability of SSB and SSB-B to protect ssDNA in an S1 nuclease protection assay. Protein, varying in concentration from 0.075 µg to 2.5 µg, was incubated with 0.25 µg of sonicated ^{125}I-labeled ssDNA at 4°C for 10 minutes. S1 was added and the mixture was incubated at 37°C for 15 minutes. The reaction was stopped with the addition of cold 0.2 M EDTA, pH 7.9. (see Materials and Methods for calculation of percent protection). Open circles (○) represent SSB and closed circles (●) represent SSB-B.


100
90
80
70
60
50
40
30
20
10
% PROTECTION
0
0.5
1.0
1.5
2.0
2.5
PROTEIN (ug)

SSB-B from wild type SSB in the following manner. These two proteins differ in their amino acid compositions. SSB and SSB-B differ in antigenic determinants and differ in their ability to protect ssDNA in a DNase I snake venom phosphodiesterase nuclease protection assay. When compared to other DNA binding proteins described in the literature, SSB-B has an amino acid composition similar to *E. coli* H-protein and a molecular weight similar to BH1 protein. To further characterize the binding of SSB-B to ssDNA, equilibrium dialysis experiments were performed. Through electron microscopy, SSB-B-ssDNA complexes were visualized. The results of these last two experiments are described in the next chapter.

CHAPTER 6

BINDING OF SSB AND SSB-B TO SINGLE STRAND DNA AND OLIGONUCLEOTIDES

INTRODUCTION

The wild type E. coli SSB is capable of two types of binding: i) an isolated binding to ssDNA resulting in a noncooperative initiation reaction, and ii) binding adjacent to an already occupied site resulting in a cooperative propagation reaction. By determining these binding constants one can better understand how SSB interacts with ssDNA and can thereby devise a model for DNA-protein interaction. Similar measurements of SSB-B were studied to compare the mechanisms of ssDNA-protein interactions.

Cooperative binding of the wild type SSB was first visualized in the electron microscope by Sigal et al. (1972). Combining wild type E. coli SSB with fd circular ssDNA under nonsaturating protein conditions, some DNA molecules became fully saturated with SSB while others had little or none bound to them. Ruyechan and Wetmur (1975) determined the cooperative binding constant for wild type E. coli K12 SSB as follows. SSB was incubated with bacteriophage λ ssDNA and the complex was visualized using the

electron microscope. To convert the length of cooperatively bound protein ($\bar{\ell}$) to the number of ssDNA sites occupied ($\bar{\nu}$) the following was taken into account: i) the protein binds in a tetrameric form to 32 nucleotides and ii) spacing of 1.8 Å/base exists in the DNA-protein complex (Sigal et al., 1972). Using a statistical mechanical model the cooperative binding constant (K_2) was mathematically determined under the following conditions. It was mathematically shown that when the fractional number of sites occupied was equal to 50% ($\frac{\bar{\nu}}{N}$ =0.5, where N=number of possible SSB binding sites per DNA molecule), the following relation existed during cooperative binding:

$$\bar{\ell} = \sqrt{\frac{K_2}{K_1}}$$

where $\bar{\ell}$ = average length of an uninterrupted ssDNA-protein complex

K_1= noncooperative binding constant

K_2= cooperative binding constant

Employing this method has yielded a cooperative binding constant on the order of 10^{11} l/mol for wild type E. coli SSB.

The noncooperative binding constant (K_1) for wild type E. coli SSB was determined through a series of equilibrium dialysis experiments by Ruyechan and Wetmur (1976). Several concentrations of ^{125}I-labeled

oligomers, $d(pCpT)_{6-9}$ and $d(pCpT)_4$, were separately dialyzed against a constant amount of wild type E. coli SSB. When equilibrium was reached the samples were removed from their chambers on either side of a dialysis membrane and counted (see Materials and Methods of this Chapter). The Scatchard equation (Scatchard, 1951)

$$\bar{\nu}/[O] = nK_1 - \bar{\nu}K_1$$

where $[O]$ = free oligomer concentration

K_1 = noncooperative binding constant

$\bar{\nu}$ = number of ssDNA binding sites occupied

n = total number of binding sites per protein molecule

was used to analyze the data. After plotting $\bar{\nu}/[O]$ vs $\bar{\nu}$ it appeared as though two binding sites per tetramer bound each oligomer. The binding constant, K_1, for $d(pCpT)_{6-9}$ equalled 3.25×10^5 1/mol/site and for $d(pCpT)_4$ was 2×10^5 1/mol/site. The difference in binding constant is due to the number of ways in which a protein site can interact with the oligonucleotide.

Collating the information from the cooperative and noncooperative binding experiments, it was suggested that an anitial relatively weak interaction

occurred between a single SSB molecule and a small number of DNA bases followed by a strong cooperative process. The work presented in this chapter examines the ability of SSB-B to bind cooperatively and non-cooperatively to ssDNA.

MATERIALS AND METHODS

Electron microscopy

Various ratios of ϕX174 viral DNA (Bethesda Research Laboratories) to SSB and to SSB-B were made using a buffer (40 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.6) to achieve all necessary dilutions. The samples were then incubated at $4^{o}C$ for 1 hour or 24 hours or $37^{o}C$ for 24 hours. One microgram of cytochrome C (5 µl) was added to 5 µl of sample and 5 µl of this mixture was placed directly onto a parlodion coated copper grid. The drop was touched with a piece of filter paper to remove excess sample and stained (Davis et al., 1971) in a 5×10^{-5} M uranyl acetate 90% ethanol solution for thirty seconds. The grid was then placed in petroleum ether to remove the aqueous phase and allowed to dry (Method 1).

A second method of mounting the sample onto the grid was an adaptation of a procedure published by Wu and Davidson (1975). Glass slides were coated with parafilm and placed in a petri dish containing a

moist piece of filter paper. A 20 µl drop of sample prepared as above was placed onto the parafilm. The petri dish was covered, and the entire sample was incubated at $4^{o}C$ for 1 or 24 hours or at $37^{o}C$ for 1 hour. Twenty microliters of cytochrome C (0.05 µg) was added to each sample drop and allowed to incubate for 10 minutes. Parlodion grids were touched to the surface of the sample drop and either stained directly or touched to a piece of filter paper and then stained as described. Each drop was used once. The samples were then shadowed using carbon-platinum pellets (Ladd) in an Edwards E12E Evaporating Unit while rotating with an angle of 82^{o}. A JEOL JEM-100CX electron microscope was used to study all samples.

Radioiodination of ssDNA oligomers

Single stranded DNA oligomers $d(pCpA)_3$, $d(pCpA)_{6-9}$, and $d(pCpT)_{6-9}$ were purchased from Collaborative Research Inc., Waltham, Mass. Iodination of these oligomers was performed according to the method of Orosz and Wetmur (1974). ^{125}I (0.2 to 1.0 µCi, Amersham) was added to a freshly made solution containing 6×10^{-4} M $TlCl_3$, 2×10^{-4} M KI, 0.1 M acetate buffer, pH 5.0, and one of the above oligomers present at a phosphate concentration of about 2×10^{-4} M. This mixture was incubated at

60°C for 20-30 minutes. The reaction was ice quenched and passed over a 1 cm X 40 cm Sephadex G-10 column at 4°C to separate the oligomer from the inorganic salts. The eluted oligomer was then made 0.1 M in phosphate buffer, pH 6.0 and heated for an additional 3-5 hours at 60°C to complete the iodination reaction. The sample was passed over a second 1 cm X 40 cm Sephadex G-10 column at 4°C. Water was used instead of buffer to elute the oligomer.

Equilibrium dialysis

The apparatus for this experiment consisted of two Plexiglas acrylic blocks with six shallow cylindrical depressions (6 mm X 2 mm) cut into each (Ruyechan and Wetmur, 1976). Once attached, these 50 µl chambers were separated by a wet cellulose 15,000 dalton molecular weight cutoff dialysis membrane (Spectrapor). SSB or SSB-B was injected into one chamber and an ^{125}I-labeled ssDNA oligomer was injected on the other side of the dialysis membrane. After equilibrium was reached (1 to 2 weeks at 4°C), 40 µl samples were removed from each chamber and placed into 10 ml of a dioxane based scintillation cocktail. The samples were counted in a Beckman LS 9000 Liquid Scintillation Counter. All of the experiments were carried out in a 0.04 M NaCl, 0.01 M Tris, pH 7.6 buffer.

RESULTS AND DISCUSSION

1. Visualization of Protein-ssDNA Complexes

Mounting DNA onto grids using the Kleinschmidt technique exposes the sample to high salt buffers. To maintain preformed protein-DNA complexes in this high salt buffer, one glutaraldehyde fixes the sample prior to mounting. Glutaraldehyde fixation involves exposure of the sample to high temperature incubation. Because of the possibility that these conditions may interfere with SSB-B-ssDNA complexes, a different method of mounting samples onto grids had to be devised. An attempt was therefore made at placing the sample directly onto a grid. Various conditions of incubation time, temperature, and protein concentrations were tried. Viral ϕX174 DNA was combined with protein at a protein to DNA mass ratio of either 10:1 or 100:1. These samples were either incubated at $4^{o}C$ for 1 or 24 hours or at $37^{o}C$ for 24 hours (see Table 8). Immediately before mounting, cytochrome C was added to the sample. A sample volume of 5 µl was applied to each parlodion coated copper grid. The sample was then uranyl acetate stained and carbon-platinum shadowed to yield greater contrast. Under all conditions the ϕX174 samples which contained no protein remained collapsed and appeared as little balls of

TABLE 8

INCUBATION CONDITIONS OF VIRAL ϕX174 DNA PLUS SSB OR SSB-B IN PREPARATION FOR MOUNTING (METHOD 1) AND ELECTRON MICROSCOPE VISUALIZATION

*Protein:ϕX174	Temperature	Time	Figures	
			SSB	SSB-B
10:1	37°C	24 hours	17	20
10:1	4°C	1 hour	NS	NS
10:1	4°C	24 hours	NS	NS
100:1	4°C	1 hour	NS	NS
100:1	4°C	24 hours	18	19

* SSB or SSB-B protein to DNA mass ratio

NS = not shown

DNA (see Figure 16). When the wild type SSB was incubated with viral ϕX174 DNA at either a 10:1 or 100:1 protein to DNA mass ratio, a combination of partially or completely opened circles formed under all conditions (Figures 17 and 18). When the SSB-B was incubated for 24 hours at 4°C with viral ϕX174 DNA, the following occurred. At a protein to DNA mass ratio of 100:1, one observed that aggregates of ϕX174 DNA were produced (Figure 19). When incubated at 37°C for 24 hours at a protein to DNA mass ratio of 10:1 similar aggregates were produced (Figure 20). These aggregates appear to involve intramolecular and intermolecular binding of ϕX174-SSB-B complexes. These complexes differ from ϕX174 alone (Figure 16) in the greater rigidity demonstrated by the contours of the complexed molecules.

SSB-B is not aggregated in the absence of ssDNA. Had this occurred, an increase in turbidity of any SSB-B would result in an increase in absorbance. When SSB-B combines with ssDNA, the complex becomes hydrophobic and adheres to all surfaces. This is observed in the noncooperative binding equilibrium dialysis experiments (see the next section of this Chapter). Because most ϕX174-SSB-B complexes adhere to various surfaces, those complexes seen by electron microscopy represent only those complexes remaining

Figure 16. Viral ϕX174 DNA mounted using Method 1. Magnification is 1.98×10^4.

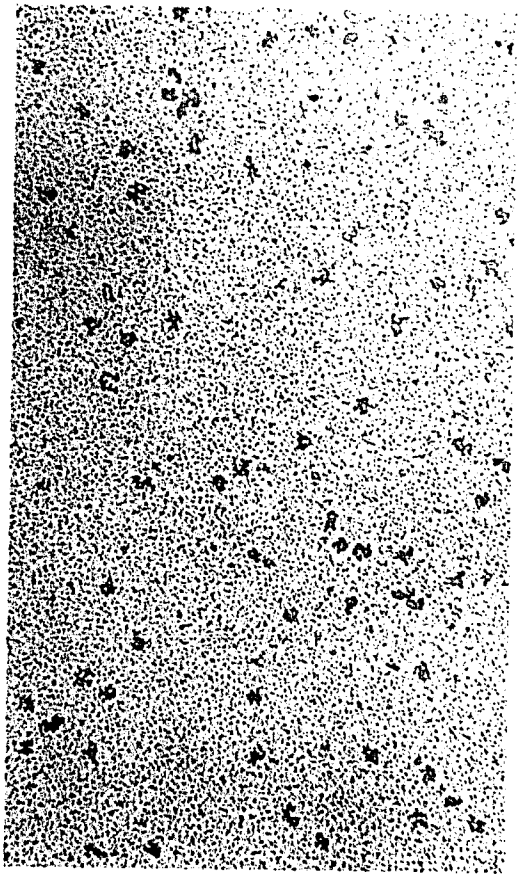


Figure 17. Viral ϕX174-SSB complexes mounted using Method 1. The protein to DNA mass ratio was 10:1. Incubation conditions were 24 hours at 37^{0}C. Magnification is 1.98×10^{4}.

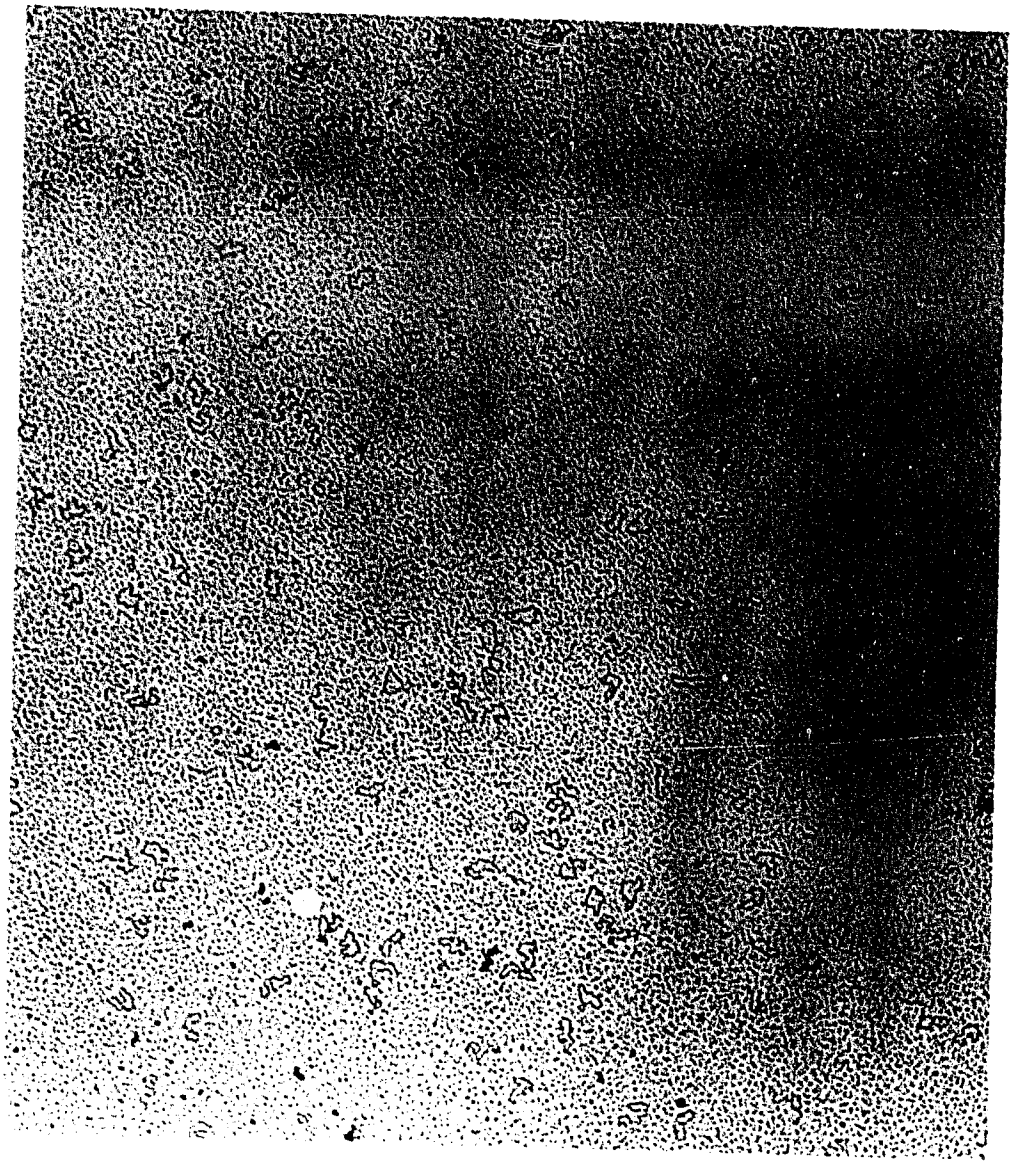


Figure 18. Viral ϕX174-SSB complexes mounted using Method 1. The protein to DNA mass ratio was 100:1. Incubation conditions were 24 hours at $4^{o}C$. Magnification is 1.98×10^{4}.

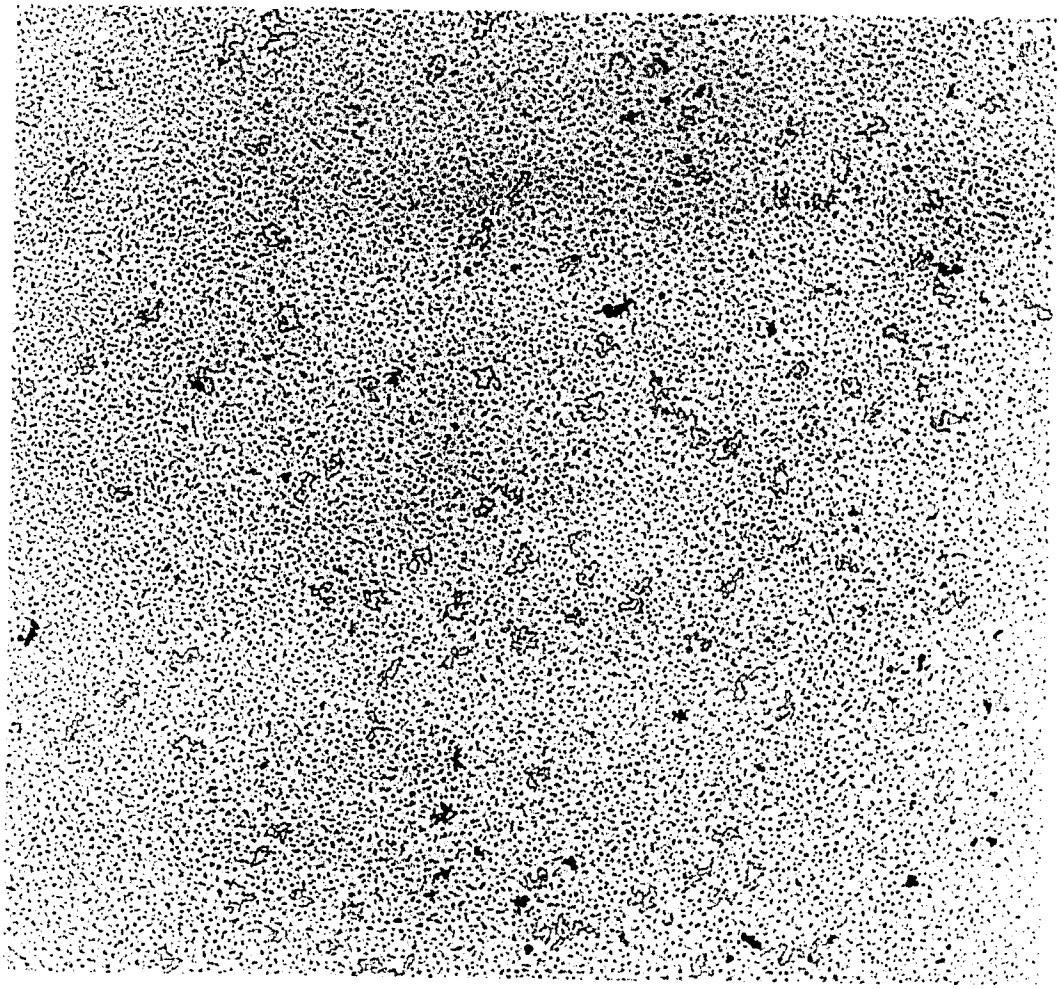


Figure 19 . Viral ϕX174-SSB-B complexes mounted using Method 1. The protein to DNA mass ratio was 100:1. Incubation conditions were 24 hours at $4^{o}C$. Magnification is 1.98 X 10^{4}.

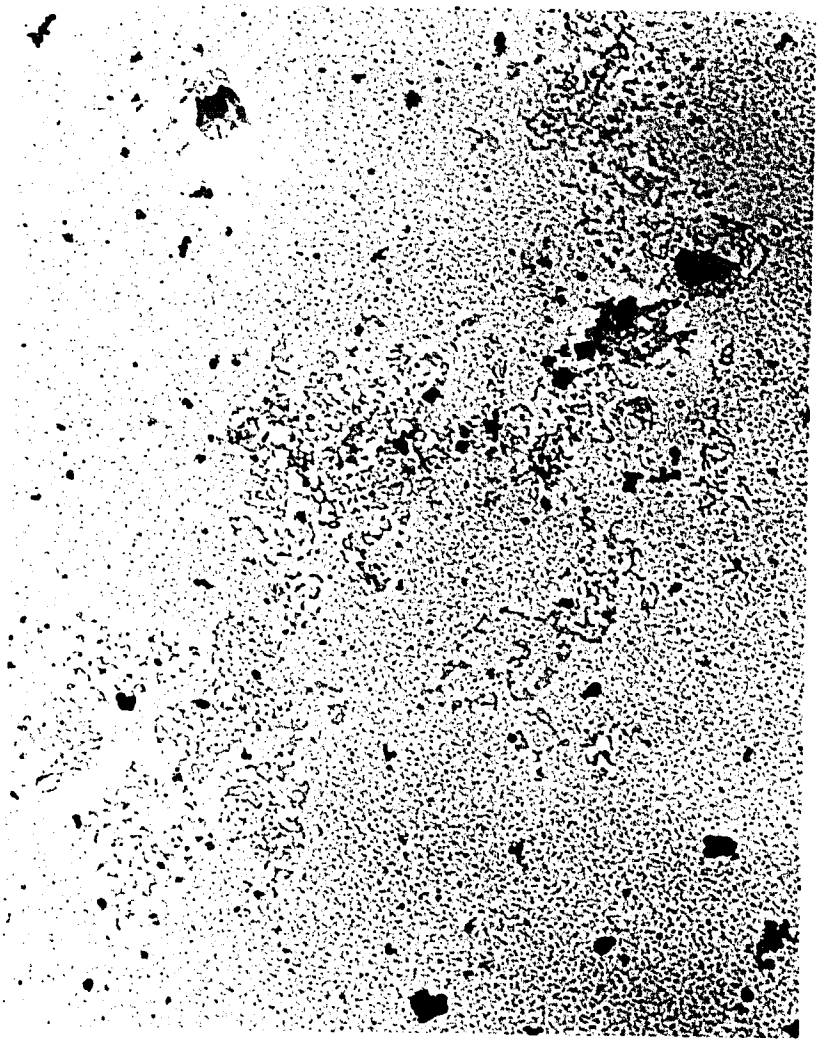
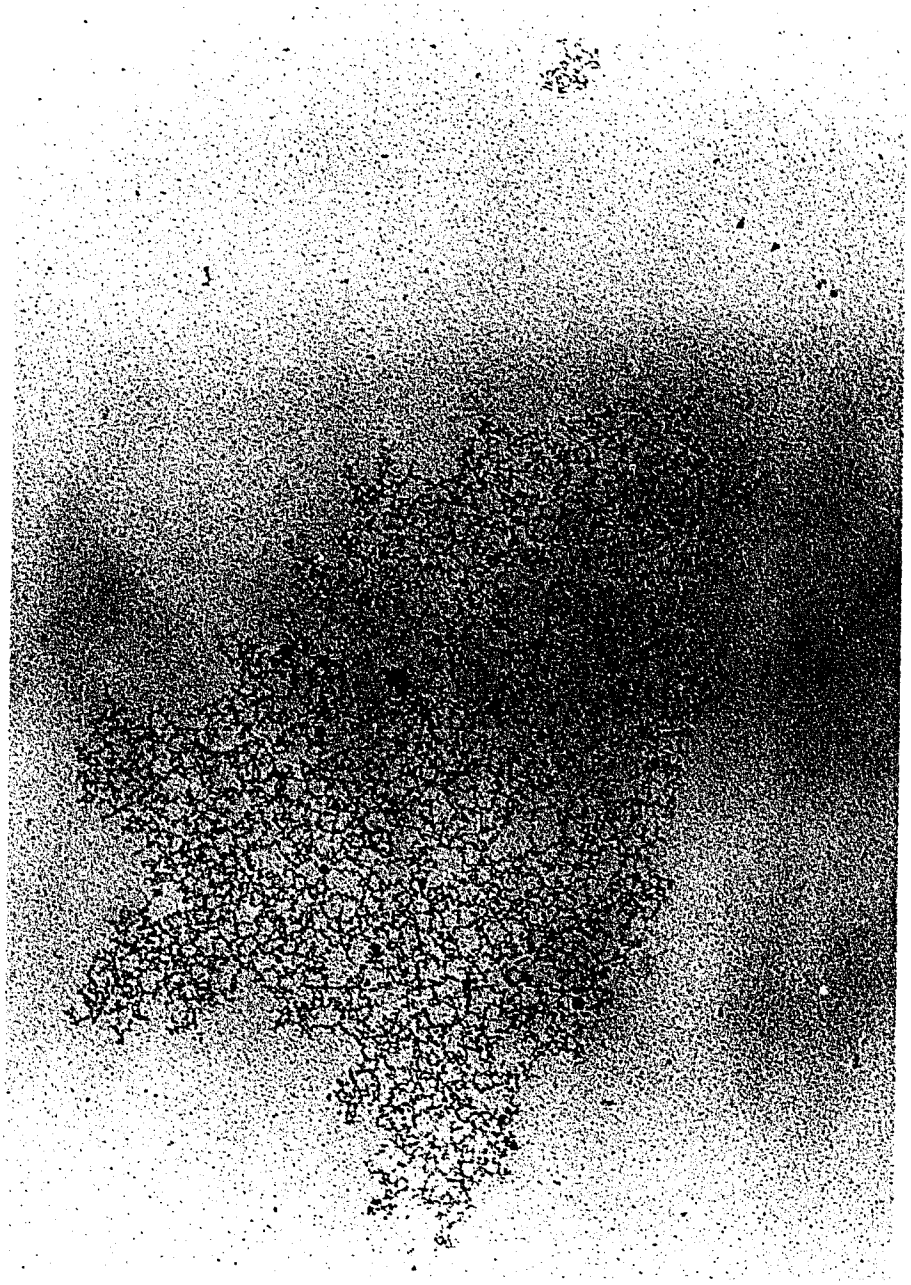


Figure 20. Viral ϕX174-SSB-B complexes mounted using Method 1. The protein to DNA mass ratio was 10:1. Incubation conditions were 24 hours at $37^{o}C$. Magnification is 1.98×10^{4}.



in solution. Detection of the ϕX174-SSB-B complexes was therefore very difficult. To circumvent the many transfer steps which expose the ϕX174-SSB-B complexes to additional surfaces, another procedure for mounting these complexes was developed. The procedure was based on that of Wu and Davidson (1975). Samples in 40 µl droplets were incubated directly on pieces of parafilm at either 4°C for 1 or 24 hours or 37° for 1 hour (see Table 9). Cytochrome C was added to these droplets and a parlodion coated grid was touched to the surface. As in the previous procedure, viral ϕX174 DNA alone remained a collapsed circle under all conditions (see Figure 21). When viral ϕX174 DNA was combined with wild type SSB at a protein to DNA mass ratio of 20:1, open circles were seen under all conditions (see Figure 22). When SSB-B was combined with the viral DNA at a protein to DNA mass ratio of 20:1 and incubated at 4°C for 1 or 24 hours, aggregated ϕX174 DNA SSB-B complexes were seen (see Figure 23). Unlike the previous procedure, many more complexes were visualized using this improved mounting technique. ϕX174 DNA and SSB-B in a protein to DNA ratio of 20:1 were incubated at 37°C for one hour. This resulted in circles of various degrees of extension (see Figure 24). The circumference of these circles measured 1.64 microns (3Å/base). Wild type SSB complexed to

TABLE 9

INCUBATION CONDITIONS OF VIRAL ϕX174 DNA PLUS SSB OR SSB-B IN PREPARATION FOR MOUNTING (METHOD 2) AND ELECTRON MICROSCOPE VISUALIZATION

*Protein:ϕX174	Temperature	Time	Figures	
			SSB	SSB-B
20:1	37°C	1 hour	22	24
20:1	4°C	1 hour	NS	NS
20:1	4°C	24 hours	NS	23

*SSB or SSB-B protein to DNA mass ratio

NS = not shown

Figure 21. Viral ϕX174 DNA mounted using Method 2. Magnification is 1.98×10^4.

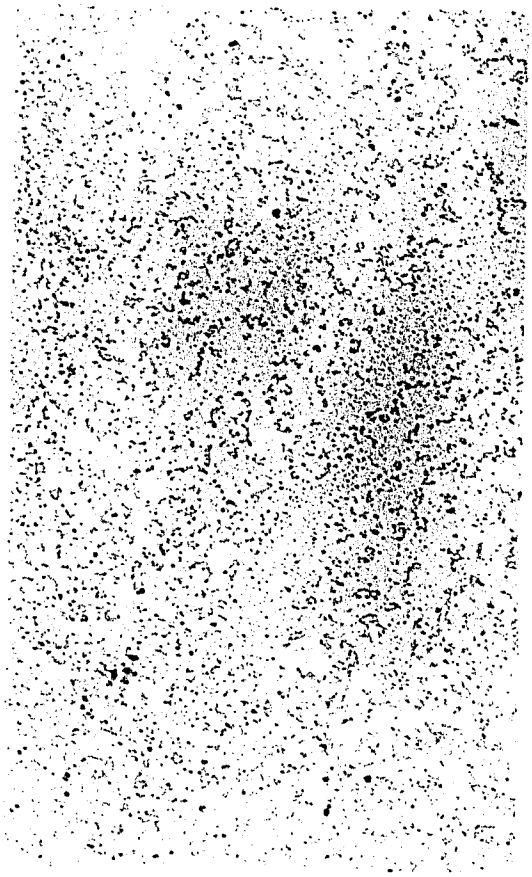


Figure 22. Viral ϕX174-SSB complexes mounted using Method 2. The protein to DNA mass ratio was 20:1. Incubation conditions were 1 hour at $37^{o}C$. Magnification is 1.98×10^{4}.

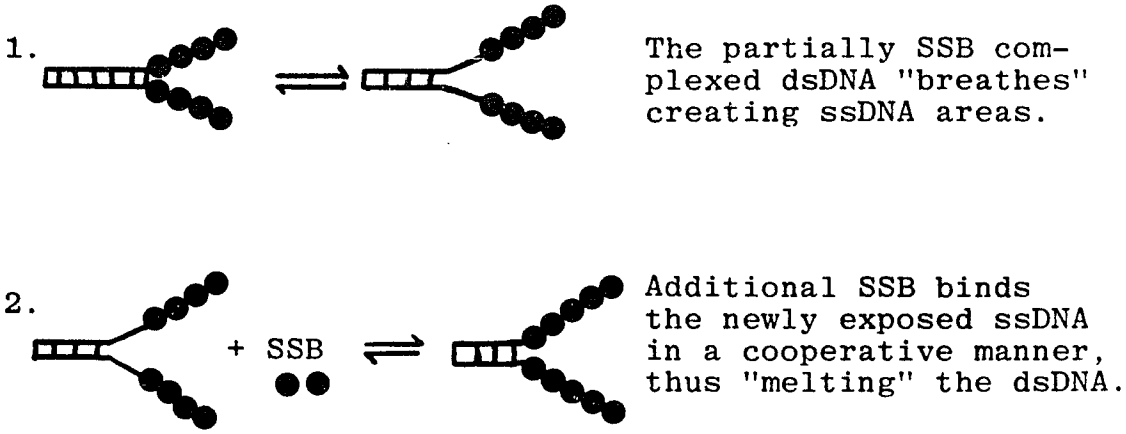
Figure 23. Viral ϕX174-SSB-B complexes mounted using Method 2. The protein to DNA mass ratio was 20:1. Incubation conditions were 24 hours at $4^{o}C$. Magnification is 1.98×10^{4}.

Figure 24. Viral ϕX174-SSB-B complexes mounted using Method 2. The protein to DNA mass ratio was 20:1. Incubation conditions were 1 hour at 37°C. Magnification is 1.98×10^4.

ϕX174 under the same conditions (see Figure 22) resulted in circle circumferences averaging 0.54 microns (1Å/base). The SSB-ssDNA complexes appear smaller than the 1.8Å/base complexes seen by Sigal et al. (1972) because of the different mounting conditions and support films used. The longer contour length exhibited by the ϕX174-SSB-B complex implies not only that this protein binds DNA cooperatively but also that the configuration of binding differs from that of wild type SSB.

2. Theoretical Proof that SSB-B Binds Single Stranded DNA in a Cooperative Manner

Using the following model and the electron microscope data, one may prove that SSB-B binds cooperatively to ssDNA. The thermodynamics of this type of coupled equilibrium is described by Crothers (1972). Wild type SSB "melts" dsDNA as follows:



For step 1: $\Delta G = RT \ln S$ (1) per base pair

$$\text{where } S = e^{\frac{-\Delta H}{R}\left(\frac{1}{T_m} - \frac{1}{T}\right)} \quad (2)$$

ΔG and ΔH are the free energy and enthalpy of base pair formation respectively.

T_m and T are the melting and experimental temperatures respectively.

S is the equilibrium constant for base pair formation.

R is the gas constant.

For step 2:

$$\Delta G = \frac{-RT}{M} \ln K_2 C \quad (3) \quad \text{where}$$

K_2 is the cooperative binding constant.

C is the free protein concentration.

M is the number of base pairs corresponding to a protein binding site.

Combining equations 1 and 3:

$$\Delta G_{Total} = -RT\left(\frac{\ln K_2 C}{M} - \ln S\right) \quad (4)$$

According to the laws of thermodynamics, the melting reaction will occur if $\Delta G_{Total} < 0$ or

$$\frac{\ln K_2 C}{M} \geq \ln S$$

A similar phenomenon occurs when denatured DNA is combined with wild type SSB at a low temperature (relative to T_m). The protein molecules "extend" the ssDNA. The extension of ssDNA produced by the SSB can occur in the absence of protein when incubated at a temperature, $T \geq (T_m - 25)^oC$. In the experiment described above, SSB-B was combined with viral ϕX174 DNA in a low salt buffer at 37°C. This resulted in the formation of extended DNA molecules (see Figure 24). According to Marmur and Doty (1962), the T_m of this DNA in the absence of protein is 81°C when incubated in a similar low salt buffer. Substituting 37°C for T in the $T \geq (T_m - 25)^oC$ equation, an incubation temperature of 56°C in the absence of protein would result in viral ϕX174 extended to the same extent as that seen in Figure 24. Because the experiments were done at 37°C, 19°C below the $T_m - 25^oC$ limit, the observed extended SSB-B-ssDNA complexed molecules were

not a result of high incubation temperatures. The extension of viral ϕX174 DNA by SSB-B at 37°C is thus similar to the "melting" of dsDNA by SSB described in equations (1) and (2) when S=2. At 19°C below T_m, S=2 for the melting process in question. This means that to see extended DNA requires $K_2C > 2^M$. Because C_{SSB-B} is approximately 2 X 10^{-6} M (monomer) than K_2>5 X 10^5 (2^M).For all values of M, K_2 is larger than the corresponding K_1 values calculated in the noncooperative binding experiments (see Table 10 in the following section). It can therefore be concluded that SSB-B binds cooperatively to ssDNA.

3. Determination of the SSB-B Non-Cooperative Binding Constant

Equilibrium dialysis experiments were prepared as follows. In all wild type SSB assays, a concentration based on A_{280} of 1.4 X 10^{-6} M tetramer was used. For all SSB-B assays, four times that amount of protein was used (i.e., 5.6 X 10^{-6} M SSB-B) because it is not known if oligomeric forms of SSB-B exist. These two different protein concentrations were chosen to allow the same number of protein molecules to potentially bind the same number of oligomer binding sites should both proteins behave in a similar manner.

The oligomers dialyzed against the proteins were either $d(pCpA)_3$, $d(pCpA)_{6-9}$ or $d(pCpT)_{6-9}$. These oligomers were ^{125}I-labeled. Several concentrations of each oligomer were used (1×10^{-6} M to 7.5×10^{-8} M phosphate). Solution conditions for all experiments were 40 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.6. Each oligomer concentration was run in triplicate. Incubation time was from 1 to 2 weeks at $4^{\circ}C$ depending on the length of time required for the particular oligomer to reach equilibrium. The data generated was used to determine the noncooperative binding constant through the Scatchard equation:

$$\frac{\bar{\nu}}{[O]} = nK_1 - \bar{\nu}K_1$$

(see Introduction for explanation) Some manipulations had to be made to detect the full amount of counts on the SSB-B side of the two chambers. The SSB-B became hydrophobic after binding the DNA oligomers and adhered to the dialysis membrane and walls of the chamber. Each chamber had to be washed with 10% SDS to remove adherent counts. The dialysis membranes were also counted. Taking these corrections into account, the SSB-B bound the oligonucleotides with the same affinity, nK, as that of the wild type

SSB (see Table 10). As the length of the oligonucleotides is increased, stronger binding is observed (see Table 10). This increase is probably due to the increase in the number of ways SSB and SSB-B can bind to longer oligomers.

The cooperative binding experiments using the electron microscope and the noncooperative binding experiments described above indicate that SSB-B is capable of binding ssDNA. SSB and SSB-B have noncooperative binding constants which are virtually identical. Both proteins bind cooperatively to ssDNA at 37^{o}C although their configuration of binding differs. This evidence indicates that SSB-B, like SSB, may interact with DNA <u>in vivo</u>.

TABLE 10

NONCOOPERATIVE BINDING CONSTANTS (nK_1 in M^{-1}) OF SSB AND SSB-B

Oligomer	SSB(1)	SSB(2)	SSB-B(2)	*Theoretical
$d(pCpT)_2$	$0.33x10^5$	ND	ND	$0.6x10^5$
$d(pCpT)_3$	$2.40x10^5$	ND	ND	$1.8x10^5$
$d(pCpA)_3$	$1.10x10^5$	$2.0x10^5$	$3.0x10^5$	$1.8x10^5$
$d(pCpT)_4$	$4.00x10^5$	ND	ND	$3.0x10^5$
$d(pCpA)_{6-9}$	ND	$16x10^5$	$13x10^5$	$7.2x10^5$
$d(pCpT)_{6-9}$	$6.50x10^5$	$6.0x10^5$	$6.0x10^5$	$7.2x10^5$

*The theoretical calculation assumes that only four nucleotides interact with each SSB tetramer with $nK_1 = 0.6x10^5$. Differences in the other binding constants is statistical.

ND = not determined

1. Ruyechan and Wetmur (1976)
2. This study

CHAPTER 7

SUMMARY AND CONCLUSION

The original purpose of this study was to isolate and characterize a temperature sensitive SSB from an *E. coli ssb*$^-$ mutant. Instead, a new *E. coli* single strand DNA binding protein was isolated. This protein, SSB-B, has been characterized and compared with SSB.

The *ssb*$^-$ "quick stop" temperature sensitive DNA replication mutant was received from Dr. J. Chase of the Albert Einstein College of Medicine. The mutant was characterized and compared to an isogenic *E. coli* K12 wild type strain. At nonpermissive temperatures (42^{o}C), the ts mutant has a filamentous morphology and ceases to replicate. At permissive temperature (30^{o}C), the ts mutant has a reduced growth rate, is UV sensitive and will not grow to high cell density in a fermentor. The filamentous morphology and UV sensitivity are attributable to impaired SOS function(s). Cell density limitation in the fermentor remains unexplained. The inability to grow at nonpermissive temperatures is attributable to a ts SSB. A possible explanation for the "quick stop" phenotype is that, as a result of an amino acid substitution or deletion in ts SSB, a conformational change in the protein's quaternary

structure may occur upon increasing the temperature to a nonpermissive temperature. This change could render the protein incapable of binding to DNA, to itself, and/or to other proteins.

Meyer et al. (1980) and Chase (personal communication) enriched for the ts SSB by subjecting cell extracts to a series of ammonium sulfate cuts. The semi-purified fraction containing the ts SSB was placed over a blue dextran-Sepharose column. The ts SSB was eluted with a high salt buffer along with a number of other proteins. This fraction was then boiled, thus aggregating the contaminating proteins. The result was a highly enriched ts SSB fraction. The ts SSB so isolated migrated to the same molecular weight position as the wild type SSB on an SDS polyacrylamide gel (approximately 18,500 daltons). In this laboratory, E. coli cell extracts were placed over a ssDNA cellulose column. The wild type SSB containing fraction was eluted with a high salt buffer and placed onto a blue Sepharose column. Wild type SSB had to be denatured with a 4 M Gu-HCl buffer to elute it from the column. When the ts extract was subjected to the same treatment, a number of proteins were coisolated. The major component, SSB-B, migrated at an approximately 18,000 dalton position on an SDS polyacrylamide gel. The wild type

SSB isolated in this laboratory migrated at a 21,000 dalton position on the same gel, indicating a 30 amino acid difference between SSB and SSB-B. SSB-B was not identical to the ts SSB isolated by Meyer et al. (1980) or Chase et al. (1980). In fact, it is now known that the ts SSB does not bind tightly enough to ssDNA cellulose to isolate it in this way (Chase, personal communication). SSB-B was therefore a different protein produced by the ts mutant.

SSB and SSB-B were compared using a number of different techniques including nuclease protection assays. Meyer et al. (1980) showed that ts SSB did not protect ssDNA against S1 endonuclease at 37°C. SSB-B was able to protect the ssDNA against S1 endonuclease at 37°C to the same extent as wild type SSB. SSB-B also protected ssDNA from DNase I and snake venom phosphodiesterase digestion when incubated at 4°C. In contrast, SSB-B was not able to protect ssDNA at 37°C in the DNase I and snake venom phosphodiesterase nuclease protection assay. SSB-B was able to protect ssDNA from S1 digestion and not from DNase I-snake venom phosphodiesterase digestion at 37°C. A reason for the selective protection observed may be a reflection of the specificity of the particular endonuclease used. DNase I will recognize both ss and dsDNA as a substrate

and will cleave an average chain length of four DNA bases (Kornberg, 1980, p. 332, Table 10-4). S1 recognizes only ssDNA as a substrate (Sutton, 1971) and cleaves an average chain length of five DNA bases (Dodgson and Wells, 1977). SSB-B may be interacting with ssDNA at 37°C in a way which no longer allows the ssDNA to be recognized as a substrate by S1.

Another interesting property of SSB-B is that after it binds ssDNA, the complex becomes extremely hydrophobic and adheres to all surfaces, including siliconized glass. This hydrophobicity was most pronounced in the noncooperative binding experiments in which the SSB-B-ssDNA complexes stuck to the dialysis membrane and plexiglas chambers. The wild type SSB showed none of these characteristics. Hydrophobic regions of the protein may be exposed after binding ssDNA and thus confer this property to the complex.

Thus, SSB-B differs from SSB in molecular weight, hydrophobic nature after complexing with ssDNA, and in ability to bind and protect ssDNA against endonucleases as measured by nuclease protection assays.

Using antibodies raised to wild type SSB, an ELISA showed that no common determinants existed between SSB-B and wild type SSB. The ts SSB shares common determinants with wild type SSB (Meyer et al., 1980). Amino acid analysis proved that a composition

difference existed between SSB and SSB-B. SSB-B had fewer glutamic acids and/or glutamines, fewer glycines, fewer arginines, and more histidines than the wild type SSB. Having reviewed the amino acid sequence of wild type SSB (Sancar et al., 1981), there is no possibility that SSB-B is a cleavage product of SSB. Removing the carboxy or amino terminus of the wild type SSB would not yield anything that resembled SSB-B. Only the E. coli histone-like DNA binding proteins share similarities in molecular weight (BH1 protein) and in amino acid composition (H-protein) with SSB-B (see Table 6).

Through a series of equilibrium dialysis experiments, noncooperative binding constants for SSB and SSB-B were determined. The binding constants for both proteins were identical. Unlike the SSB complexed oligonucleotides, the SSB-B complexes became extremely hydrophobic and adhered to all surfaces. The proteins were then combined with viral ϕX174 DNA and visualized through the electron microscope. When the protein and ssDNA were incubated at 4°C, SSB-B formed protein-ssDNA aggregates while SSB cooperatively bound ϕX174 to produce individual open circles. When the incubation temperature was raised to 37°C, both SSB and SSB-B cooperatively bound ϕX174 DNA and produced individual open circles. However, differences in the contour lengths of these

circles was observed. SSB-B-ϕX174 circles were extended to a greater extent than SSB-ϕX174 circles. This implies that the configuration of binding of SSB-B to ssDNA differs from that of wild type SSB.

The differences observed between SSB and SSB-B; i.e., i) molecular weight, ii) antigenic determinants, iii) amino acid composition, iv) ability to protect ssDNA at 37°C in a DNase I snake venom phosphodiesterase nuclease protection assay, v) property changes after binding ssDNA, and vi) the different configuration of binding of the proteins to ssDNA lead one to state that a new and different ssDNA binding protein has been isolated from the ts *E. coli* *ssb*$^-$ mutant.

Significant amounts of SSB-B were not found in wild type *E. coli* K12 extracts when subjected to isolation procedures used to obtain the protein from *ssb*$^-$ mutant *E. coli* K12 bacteria. A possible model used to explain this observation is the following. In wild type bacteria, SSB would cover most ssDNA gaps. In the *ssb*$^-$ mutant bacteria, a faulty ts SSB may bind ssDNA improperly even at permissive temperatures, leaving ssDNA gaps. The resulting unprotected ssDNA may bind SSB-B leading either to overproduction or slower degradation of SSB-B. The

true *in vivo* function of SSB-B, however, remains unknown.

REFERENCES

Alberts, B.M., Amodio, F.J., Jenkins, M., Gutmann, E.D., Ferris, F.L. (1968) Cold Spring Harbor Symp. Quant. Biol. 33, 289-305.

Alberts, B.M., and Frey, L. (1970) Nature 227, 1313-1318.

Anderson, R.A. and Coleman, J.E. (1975) Biochemistry 14, 5485-5491.

Ariga, H., Klein, H., Levine A.J., Horwitz, M.S. (1980) Virology 101, 307-310.

Bachmann, B.J., Low, K.B., Taylor, A.L. (1976) Bact. Rev. 40, 116-167.

Baluch, J., Chase, J.W., and Sussman, R. (1980) J. Bacteriol. 144, 489-498.

Banks, G.R. and Spanos, A. (1975) J. Mol. Biol. 93, 63-77.

Battula, N., and Loeb, L.A. (1975) J. Biol. Chem. 251, 982-986.

Breschkin, A.M. and Mosig, G. (1977a) J. Mol. Biol. 112, 279-294.

Breschkin, A.M., and Mosig, G. (1977b) J. Mol. Biol. 112, 295-308.

Cassuto, E., West, S.C., Mursalim, J., Conlon, S., Howard-Flanders, P. (1980) Proc. Natl. Acad.

Sci. USA 77, 3962-3966.

Chase, J.W., Whittier, R.F., Auerbach, J., Sancar, A., and Rupp, W.D. (1980) Nucleic Acids Res. 14 3215-3227.

Christiansen, C. and Baldwin, R.L. (1977) J. Mol. Biol. 115, 441-454.

Coleman, J.E., and Oakley, J.L. (1980) CRC Crit. Rev. Biochem. 8, 247-289.

Crothers, D.M. (1971) Biopolymers 10, 2147-2160.

Davis, R.W., Simon, M., and Davidson, N. (1971) Methods in Enzymology (Grossman, L. and K. Moldane, eds.) 21D, 413-428, Academic Press, New York.

Delius, H., Mantell, N.J., Alberts, B. (1972) J. Mol. Biol. 67, 341-350.

Dodgson, J. and Wells, R.D. (1977) Biochemistry 16 2374-2379.

Fowlkes, D.M., Lord, S.T., Linne, T., Pettersson, U., Philipson, L. (1979) J. Mol. Biol. 132, 163-180.

Gefter, M.L., Hirota, Y., Kornberg, T., Weschler, J.A., Barnoux, C. (1971) Proc. Natl. Acad. Sci. USA 68, 3150-3153.

George, D.L. and Witkin, E.M. (1974) Molec. gen. Genet. 133, 283-291.

George, D.L., and Witkin, E.M. (1975) Mutation Res. 28, 347-354.

Glassberg, J., Meyer, R.R. and Kornberg, A. (1979) J. Bacteriol. 140, 14-19.

Gudas, L.J. and Mount, D.W. (1977) Proc. Natl. Acad. Sci. USA 74, 5280-5284.

Herrick, G., Delius, H., and Alberts, B. (1976) J. Biol Chem. 251, 2142-2146.

Hotta, Y., and Stern, H. (1971a) Develop. Biol. 26, 87-99.

Hotta, Y., and Stern, H. (1971b) Nature New Biol. 234, 83-86.

Howard-Flanders, P., Simson, E., and Theriot, L. (1964) Genetics 49, 237-246.

Huang, A.T., Riddle, M.M. and Koons, L.S. (1975) Cancer Research 35, 981-986.

Huberman, J.A., Kornberg, A., and Alberts, B.M. (1971) J. Mol. Biol. 62, 39-52.

Hubscher, U., Lutz, H., and Kornberg, A. (1980) Proc. Natl. Acad. Sci. USA 77, 5097-5101.

Johnson, B.F. (1977) Molec. Gen. Genet. 157, 91-97.

Kornberg, A. (1978) Cold Spring Harbor Symp. Quant. Biol. 43, Part 1, 1-9.

Kornberg, A. (1980) DNA Replication, W.H. Freeman and Company, San Francisco.

Kornberg, T. and Gefter, M.L. (1972) J. Biol. Chem. 247, 5369-5375.

Kunkel, T.A., Meyer, R.R., Loeb, L.A. (1979) Proc. Natl. Acad. Sci. USA 76, 6331-6335.

Laine, B., Kmiecik, D., Sautiere, P., Biserte, G., and Cohen-Solal, M. (1980) Eur. J. Biochem. 103, 447-461.

Lechner, R.L. and Kelly, T.J., Jr. (1977) Cell 12, 1007-1020.

Lee, H.M., Forde, M.D., Lee, M.C., Bucher, D.J. (1979) Anal. Biochem. 96, 298-307.

Linne, T., Philipson,L. (1980) Eur. J. Biochem. 103, 259-270.

Livingston, D.M., Hinkle, D.C., and Richardson, C.C. (1975) J. Biol. Chem. 250, 461-469.

Loeb, L.A. (1974) in The Enzymes (P.D. Boyer, ed.), 10, 173-209, Academic Press, New York.

Maizel, J.V. (1971) Methods in Virology (Maramorsch, K. and Koprowski, H., eds), 5, 179-246, Academic Press, New York.

Marmur, J., and Doty, P. (1962) J. Mol. Biol. 5, 109-118.

Marvil, D.K., Nowak, L., Szer, W. (1980) J. Biol. Chem. 255, 6466-6472.

McEntee, K., Weinstock, G.M., Lehman, I.R. (1979) Proc. Natl. Acad. Sci. USA 76, 2615-2619.

McEntee, K., Weinstock, G.M., Lehman, I.R. (1980) Proc. Natl. Acad. Sci. USA 77, 857-861.

Meyer, R.R., Glassberg, J. and Kornberg, A. (1979) Proc. Natl. Acad. Sci. USA 76, 1702-1705.

Meyer, R.R., Glassberg, J., Scott, J.V., and Kornberg, A. (1980) J. Biol. Chem. 255, 2897-2901.

Miller, J. (1972) Experiments in Molecular Genetics, N.Y. Cold Spring Harbor Laboratory.

Moise, H., Hosoda, J. (1976) Nature 259, 455-458.

Molineux, I.J., Friedman, S., and Gefter, M.L. (1974) J. Biol. Chem. 249, 6090-6098.

Molineux, I.J. and Gefter, M.L. (1974) Proc. Natl. Acad. Sci. USA 71, 3858-3862.

Molineux, I.J. and Gefter, M.L. (1975) J. Mol. Biol. 98, 811-825.

Molineux, I.J., Pauli, A., and Gefter, M.L. (1975) Nucleic Acids Research 2, 1821-1837.

Nowak, L., Marvil, D.K., Thomas, J.O., Boublik, M., Szer, W. (1980) J. Biol. Chem. 255, 6437-6478.

Orosz, J.M. and Wetmur, J.G. (1974) Biochemistry 13, 5467-5475.

Reuben, R.C., Gefter, M.L. (1973) Proc. Natl. Acad. Sci. USA 70, 1846-1850.

Reuben, R.C., Gefter, M.L. (1974) J. Biol. Chem. 249, 3843-3850.

Russel, M., Gold, L., Morrissett, H., O'Farrell, P.Z. (1976) J. Biol. Chem. 251, 7263-7270.

Ruyechan, W.T. and Wetmur, J.G. (1975) Biochemistry 14, 5529-5534.

Ruyechan, W.T. and Wetmur, J.G. (1976) Biochemistry 15, 5057-5064.

Sadler, J.R., Miwa, J., Maas, P., Smith, T. (1974) Lab. Pract. 23, 642-643.

Sancar, A., Stachelek, C., Koningsberg, W., Rupp, W.D. (1980) Proc. Natl. Acad. Sci. USA 77, 2611-2615.

Sancar, A., Williams, K.R., Chase, J.W., Rupp, W.D. (1981) Proc. Natl. Acad. Sci. USA, in press.

Scatchard, G. (1951) Ann. N.Y. Acad. Sci. 51, 660-672.

Schechter, N.M., Davies, W., Anderson, C.W. (1980) Biochemistry 19, 2802-2810.

Scherzinger, E., Litfin, F., Jost, E. (1973) Molec. gen. Genet. 123, 247-262.

Sevastopoulos, C.G., Wehr, C.T., and Glaser, D.A. (1977) Proc. Natl. Acad. Sci. USA 74, 3485-3489.

Shanmugam, G., Bhaduri, S., Arens, M., Green, M. (1975) Biochemistry 14, 332-337.

Shibata, T., DasGupta, C., Cunningham, R.P., Radding, C.M. (1979a) Proc. Natl. Acad. Sci. USA 76 1638-1642.

Shibata, T., Cunningham, R.P., DasGupta, C., Radding, C.M. (1979b) Biochemistry 18, 5100-5104.

Shibata, T., DasGupta, C., Cunningham, R.P., Radding, C.M. (1980) Proc. Natl. Acad. Sci. USA 77, 2606-2610.

Sigal, N., Delius, H., Kornberg, T., Gefter, M.L., Alberts, B. (1972) Proc. Natl. Acad. Sci. USA 69, 3537-3541.

Sutton, W.D. (1971) Biochem. Biophys. Acta 240, 522-531.

Vales, L.D., Chase, J.W., Murphy, J.B. (1980) J. Bacteriology 143, 887-896.

van der Vliet, P., Levine, A.J. (1973) Nature New Biol. 246, 170-173.

van der Vliet, P., Levine, A.J., Ensinger, M.J., and Ginsberg, H.S. (1975) J. Virol. 15, 348-354.

Varshavsky, A.J., Nedospasov, S.A., Bakayev, V.V., Bakayeva, T.G. and Georgiev, G.P. (1977) Nucleic Acids Res. 4, 2725-2745.

Voller, A., Bidwell, D.E. and Bartlett,A. (1976) Bull. World Health Organ. 53, 55-65.

Weiner, J.H., Bertsch, L.L., and Kornberg, A. (1975) J. Biol. Chem. 250, 1972-1980.

Weissbach, A. (1977) Annual Rev. Biochem. 46, 25-47.

Wetmur, J.G. and Davidson, N. (1968) J. Mol. Biol. 31, 349-370

Whittier, R.F. and Chase, J.W. (1980) Anal. Biochem. 106, 99-108.

Williams, K.R., LoPresti, M.B., Setoguchi, M., and Konigsberg, W.H. (1980) Proc. Natl. Acad. Sci. USA 77, 4614-4617.

Witkin, E.M. (1967) Proc. Natl. Acad. Sci. USA 57, 1275-1279.

Witkin, E.M. (1976) Bacteriol. Rev. 40, 869-907.

Wu, M., and Davidson, N. (1975) Proc. Natl. Acad. Sci. USA 72, 4506-4510.

Yamashita, T., and Green, M. (1974) J. Virol. 14, 412-420.

Yarranton, G.T., Das, R.H., Gefter, M.L. (1979) J. Biol. Chem. 254, 12002-12006.

Yarranton, G.T. and Gefter, M.L. (1979) Proc. Natl. Acad. Sci. USA 76, 1658-1662.

Zechel, K., Bouche, J.P. and Kornberg, A. (1975) J. Biol. Chem. 250, 4684-4689.