Schneider, Robert Jay

THE KINETICS OF TRANSFER OF ESCHERICHIA COLI SINGLE-STRAND DNA BINDING PROTEIN BETWEEN SINGLE-STRANDED DNA MOLECULES

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THE KINETICS OF TRANSFER OF *ESCHERICHIA COLI* SINGLE-STRAND DNA BINDING PROTEIN BETWEEN SINGLE-STRANDED DNA MOLECULES

by

Robert J. Schneider

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ABSTRACT

THE KINETICS OF TRANSFER OF ESCHERICHIA COLI
SINGLE-STRAND DNA BINDING PROTEIN BETWEEN
SINGLE-STRANDED DNA MOLECULES
by Robert J. Schneider
Advisor: James G. Wetmur, Ph.D.

Very little is known about the kinetics and mechanism of exchange of Escherichia coli K12 single-strand binding protein (SSB) among single-stranded DNA molecules. The studies presented in this dissertation were designed to quantitate the kinetics of the exchange reaction and explore the possible mechanisms by which transfer of SSB may take place. The studies may be broadly grouped into three sets of experiments. These consist of the detection of SSB transfer using a nitrocellulose filter binding assay, detection using electron microscopic visualization of SSB bound and unbound DNA molecules, and detection using sucrose gradient sedimentation analysis of interacting DNA molecules.

The binding of SSB to a ^{3}H-labelled 375 nucleotide single-stranded DNA was detected using a nitrocellulose filter binding assay. The binding of protein was sufficiently cooperative that an all-or-none mechanism governs and DNA was either free of or saturated with SSB. The stoichiometry of filter binding agrees with this model. It was found that the first-order kinetics of transfer of protein from donor DNA-protein complexes to recipient DNA of the same size was the same whether using ^{3}H-labelled donor or recipient DNA. The rate of transfer of protein from donor to recipient was found to be weakly temperature dependent, independent of salt concentration over a defined range, and inversely proportional to solvent viscosity. These results are consistent with a diffusion-controlled reaction mechanism. When much larger recipient DNA molecules were used, the rate of transfer of SSB was greatly reduced. Also the rate constant for transfer to intact circular single-stranded DNA molecules was no different than that for transfer to linear molecules of about the same length. This indicated that DNA ends are not required for the uptake of SSB. The magnitude and length dependence of the rate constants for protein transfer are incompatible with a mechanism involving uptake of free protein from solution. A model was proposed involving direct transfer of cooperative units of protein from donor to recipient strands.

SSB transfer studies analyzed by electron microscopic visualization were performed with 400 nucleotide single-stranded DNA molecules and circular single-stranded bacteriophage G4 DNA molecules. The transfer of protein from 400 nucleotide DNA donors to G4 recipients and vice-versa are presented in electron micrographs and a statistical compilation of the transfer products. They were found to be consistent with the model presented.

Finally, sucrose gradient sedimentation was used to separate and analyze the transfer of radiolabelled SSB from circular bacteriophage DNA molecules to 400 nucleotide long DNA molecules. The results of these experiments are also consistent with a model involving direct transfer of cooperative units of protein to recipient DNA strands.

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A special respect is reserved for my parents and family for their support and affection; they need not understand this dissertation in order to appreciate it.

There is no satisfactory way for me to express all of my gratitude to Patricia Doyle for her wisdom and kindness. It has helped construct a path out of turmoil and to dissipate the fog.

Finally, to Laura Coruzzi I would give a dozen roses if she only liked flowers. Her love has given the whole thing value and purpose, and has created some sanity out of an insane process.

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GENERAL INTRODUCTION

Many proteins have been purified which bind single-stranded DNA stoichiometrically, cooperatively and with little regard for nucleotide sequence. The first such protein to be extensively characterized was isolated from bacteriophage T4 infected Escherichia coli (E. coli), and was found to be the product of T4 gene 32 (Alberts and Frey, 1970). Subsequently, employing the technique of DNA-cellulose chromatography developed by Alberts et al. (1968; 1971), proteins qualitatively similar to T4 gene 32 protein have been isolated from a variety of prokaryotic sources, including E. coli (Sigal et al., 1972), as well as eukaryotic sources. The ability of some of these proteins to denature polydeoxyadenylic-thymidilic acid led to the designation "DNA-Unwinding Proteins" (Sigal et al., 1972). Many other names have been attached to these proteins, including helix destabilizing proteins, DNA melting proteins, DNA extending proteins and DNA binding proteins. The accepted name is now single strand binding protein, SSB, first suggested by Geider, (1978), and used as the designation for mutants of the E. coli protein (Meyer et al., 1980).

There is a large body of evidence indicating that E. coli SSB plays an important and vital role in the enzymology of DNA replication, recombination and repair. All three processes are inhibited at the non-permissive temperature with temperature sensitive (ts) SSB (Meyer et al., 1980). There is in vitro evidence for a significant role in the conversion of single-stranded circular bacteriophage DNA to the duplex replicative form (Weiner et al., 1975; Wickner and Hurwitz, 1974). There is also in vitro evidence for a vital role in the multiplication of the duplex replicative form of these viruses (Scott et al., 1977). Recent in vitro experiments on E. coli recA protein mediated recombination have suggested that SSB performs an important function in DNA recombination as well (Cassuto et al., 1980; Shibata et al., 1980; Cunningham et al., 1981). It appears, then, that the E. coli SSB is involved in all of the major aspects of DNA metabolism.

This dissertation presents work documenting the cooperativity of E. coli SSB binding to single-stranded DNA molecules, and the kinetics of transfer of E. coli SSB between single-stranded DNA molecules. The mechanism by which these extremely cooperative binding proteins are able to transfer between the single-stranded DNA substrates of DNA replication and recombination was unknown. Based on the in vitro transfer kinetics studies, a possible mechanism is presented which is unusual and may lead to a better understanding of many functions of SSB.

Because SSB is so integrally involved in the initiation, elongation and recombination of DNA, all three processes are discussed in this section. Where it is possible, the specific functions of SSB in each process are described in detail along with the known interactions between SSB and the DNA replication and recombination enzymes. For the sake of completeness, the basic enzymology of DNA replication and recombination is presented, even if certain areas do not directly concern a function of SSB. Following this discussion, the biochemical and physical properties of SSB are presented, along with some specific molecular- biological properties that did not lend themselves to the earlier discussion. Recent work concerning the ability of SSB to catalyze strand association is presented in this section, as well as work concerning the mutant temperature sensitive form of SSB. The last portion of the introduction contains a brief review of other SSBs and the functions in which they have been implicated.

1). OVERVIEW OF DNA REPLICATION

This section contains an overview of prokaryotic DNA replication. The enzymology of replication and the interactions of the proteins that comprise the machinery involved in each step are emphasized, including the role of SSB in each stage. The biosynthesis of DNA chain growth and of the nucleotide precursors are not included.

The E. coli chromosome is a covalently closed circular double-stranded DNA molecule of approximately four million base-pairs (Lewin, 1974). It is replicated bidirectionally from a unique origin. This mechanism occurs with all bacteria studied to date. The initiation of DNA replication at the origin involves a large number of initiation proteins, including dnaA (Wechsler and Gross, 1971; Kornberg, 1980), to recognize the appropriate start signals and establish a "replication apparatus". This replication apparatus proceeds along the leading strand synthesizing DNA in the 5' to 3' direction (elongation). Discontinuous synthesis occurs on the lagging strand also 5' to 3', with repeated reinitiation and elongation required. The fragments are primed by RNA and are called Okazaki fragments. The RNA primers are removed from small Okazaki fragments, DNA is synthesized within the gaps and then

the fragments are joined by DNA ligase. There may be multiple replication forks each of which was initiated at the unique origin of replication.

Some investigators have suggested that the bacterial membrane is actively involved in E. coli DNA replication (Sueaka et al., 1968; 1969; Ryter et al., 1969). There is still no good evidence, however, for a definitive role of the membrane in DNA replication, although the bacterial chromosome appears to be attached to the cell membrane at the origin of replication as well as at growing points (Tremblay et al., 1969; O'Sullivan and Sueoka, 1972).

The dissection of the components involved in DNA replication has been greatly aided by the use of the small E. coli bacteriophages G4, ØX174 and M13. The small size of these phage genomes (approximately 1/1000 the size of the E. coli genome), their dependence upon host enzymes for replication and the ease with which their genomes can be isolated have made them invaluable for the analysis of DNA replication enzymology. They have been especially important in elucidating the mechanisms by which DNA replication is initiated. Phage G4 DNA replication requires the fewest enzymes and proteins of any system studied. Replication begins with formation of an RNA primer which is synthesized by a special RNA polymerase called primase. Phage ØX174, although closely

related to phage G4, requires a number of additional host proteins called prepriming proteins to correctly initiate DNA replication. The replication of ØX174 DNA appears to resemble E. coli DNA replication more than any other bacteriophage studied. The entire prepriming plus priming machinery is necessary to reinitiate DNA synthesis in discontinuous replication of double-stranded DNA. Phage M13 initiates DNA replication by an entirely different mechanism from that used by G4 and ØX174. A large number of other E. coli bacteriophages have been investigated and all initiate DNA replication in a manner similar to either G4, ØX174 or M13. Once initiated, however, the elongation of DNA in these phages, as well as in E. coli, appears to proceed utilizing the same enzymes and mechanisms.

2). INITIATION OF DNA REPLICATION

The existence of a unique site or origin of DNA replication in E. coli has been established by a wide variety of techniques (Cairns, 1963; Masters and Broda, 1971; Bird et

al., 1972; Prescott and Kuempel, 1972; Louarn and Bird, 1974; Marsh and Worcell, 1977). The site located at 82.5 minutes on the circular E. coli map, approximately 20 megadaltons counterclockwise from the ilv locus (Bachman et al., 1976; von Meyenburg et al., 1977; 1978), has been designated oriC (Hiroga, 1976).

The phages G4, ØX174 and M13 also have unique origins for DNA replication. The formation of the (-) strand of these single-stranded bacteriophages might be considered to be models for leading strand DNA synthesis from any replication origin which is already open. ØX174 (-) strand synthesis is also a model for discontinuous DNA synthesis at a replication fork. The origin site in G4 has been identified by a number of investigators (Zechel et al., 1975; Bouche et al., 1975). The priming of DNA synthesis at a single site in ØX174 has recently been achieved in vitro, and the site has been located (Arai et al., 1980). Phage M13, which uses a different mechanism for the initiation of DNA synthesis, was the first bacteriophage exploited in DNA replication studies; the origin was located some years ago (Brutlag et al., 1971; Wickner et al., 1972; 1973).

Structural information concerning the origins for initiating DNA replication in G4, ØX174 and E. coli DNAs has been obtained from nucleotide sequences (Sanger et al., 1978; God-

son et al., 1978; Fiddes et al., 1978; Hirota et al., 1978; Messer et al., 1978). The initiation sites in G4 and ØX174 are similar, even at the level of nucleotide sequence homology (Hirota et al., 1978). All three initiation sites do not encode proteins (Messer et al., 1978), and there are a number of inverted repeat nucleotide sequences from which stable secondary structures may be drawn. There are two such sites in the E. coli initiation region, and one site in ØX174 and G4 DNAs (Godson et al., 1978). The stable stem-loop structures at initiation sites are probably recognised and bound by the initiation proteins (Kornberg, 1980). The evidence that the secondary structure is recognised specifically by initiation proteins will be presented later. The RNA polymerase promoter consensus sequence, TATAA (Pribnow, 1975), is also found within this region. All prokaryotic promoter sequences determined to date contain the consensus sequence 10 base-pairs 5' to the start of RNA transcription, and in most a stable secondary structure can be drawn (Gilbert, 1976). This consensus sequence for RNA transcription also seems to be an important part of the recognition signal for initiating DNA replication and may be related to the fact that DNA synthesis begins with the synthesis of an RNA primer.

The enzymology of DNA replication is very complex. Elucidation of the factors involved in phage DNA synthesis has largely been made possible through the use of in vitro comple-

mentation assays. Soluble protein lysates from E. coli mutants were complemented with wild-type E. coli fractions and examined for their ability to convert added purified viral single-stranded DNA circles to the double-stranded replicative intermediate (Weiner et al., 1974). A series of initiation proteins have been isolated.

The initiation of M13 DNA synthesis was found to be rifampicin sensitive (Brutlag et al., 1971). This suggested that the initiation occurred by RNA polymerase priming. Moreover, E. coli RNA polymerase mutants in which the enzyme no longer binds rifampicin were not only rifampicin resistant, but also permitted the replication of M13 DNA in the presence of rifampicin (Wickner et al., 1972; 1973). Priming takes place in the presence of SSB. Elongation then requires DNA polymerase III, ATP and the four dNTP's. It has since been shown that this mechanism for RNA priming in M13 is also used in leading strand plasmid replication in E. coli (Williams et al., 1973). Whether RNA polymerase functions at the origin of replication of E. coli DNA is not known.

The RNA polymerizing activity in E. coli that initiates nascent DNA chain growth for the bacterial chromosome reinitiation as well as for phages G4 and ØX174 is primase, the product of the dnaG gene (Rowen and Kornberg, 1978; Bouche et al., 1978). Primase appears to recognize a region of secon-

dary structure and bind (Wickner, 1977). In G4 there is a stable stem-loop structure near the promoter site (Fiddes et al., 1978; Sims and Dressler, 1978). It has been shown that primase reads through the stem-loop structure and synthesizes an RNA homologous to approximately 30 nucleotides at the origin of replication (Rowen and Kornberg, 1978; Hourcade and Dressler, 1978). The G4 promoter appears to be unusual in its simplicity, requiring only a few E. coli proteins for initiation. G4 requires addition of E. coli SSB, which is believed to coat the G4 circles except for the stable stem-loop structure at the origin of replication (Kornberg, 1977). Presumably, primase then binds or recognizes the only secondary structure remaining after melting-out of minor structures by SSB, and synthesizes an RNA primer from it. Elongation then requires DNA polymerase III, ATP and the four dNTP's. Completion of a (-) strand requires DNA polymerase 1 and DNA ligase.

The priming of ØX174 DNA synthesis is more complex than is the case of G4. A series of additional host proteins are required to promote initiation at the origin site in ØX174. The search for these other proteins disclosed an elaborate mechanism that may be used by E. coli to prime its own DNA replication (Zechel et al., 1975), but certainly is involved in discontinuous DNA replication in E. coli and in double-strand to double-strand DNA conversion in the various bacteriophage systems.

There is a prepriming stage in DNA synthesis (Kornberg, 1980). This is a stage in which the proper promoter is selected for initiation of DNA synthesis by an RNA primer. Selection of the correct initiation site seems to require several proteins. The E. coli SSB is required. Failure to include SSB lowers the selectivity of primase and reduces the subsequent levels of DNA synthesis. It should be noted that E. coli SSB is required for initiation of DNA synthesis in all three of the prototype bacteriophage systems. The dnaB protein appears to be absolutely required for the prepriming stage of DNA synthesis (Zyskind and Smith, 1977). The dnaB protein is probably a hexamer of 300,000 daltons, composed of identical subunits (Reha-Krantz et al., 1978). It binds single- stranded DNA and has an ATPase activity (McMacken et al., 1977). The dnaC protein is also required. This protein is poorly characterized. It is known that the dnaC protein associates with dnaB and dnaT proteins (Wechsler, 1978). The dnaT protein regulates termination of replication (Lark et al., 1978). There is evidence that the dnaT protein directly destabilizes the initiation complex (Lark and Lark, 1978). It has been suggested that dnaB protein is a major prepriming protein, since there is evidence that along with SSB, dnaB protein aids in the establishment of the promoter regions in ØX174 (-) strand and in E. coli or ØX174 discontinuous DNA synthesis. For discontinuous DNA synthesis, dnaB serves as a

"mobile promoter" that establishes primase on the DNA (McMacken et al., 1977; Kornberg, 1980).

A series of other E. coli proteins have been identified that are also necessary for the initiation of DNA replication in ØX174 at a unique site and for double strand DNA synthesis of ØX174. By analogy, they are presumed to be important in E. coli as well. The proteins have been designated n, n', n'' and i (Westergaard et al., 1973; Wickner and Hurwitz, 1974; Schekman et al., 1974; 1975; McMacken et al., 1977). These four proteins are also considered to be prepriming proteins that help prepare a priming site for primase. It appears that these proteins form a complex nucleoprotein intermediate with dnaB protein, primase, SSB and DNA (Weiner et al., 1976; McMacken et al., 1977).

The double-stranded helix of E. coli must be unwound in order for DNA replication to take place. The group of enzymes that are responsible for unwinding the helix are the helicases (Kornberg, 1980). These are enzymes that have an an ATPase activity upon binding DNA, and appear to be involved in unwinding the duplex for initiation and elongation at the replication fork. There are more than twelve DNA helicases already known (Kornberg, 1980).

The rep protein is a helicase from E. coli that unwinds duplex DNA using ATP (Scott and Kornberg, 1978). The rep pro-

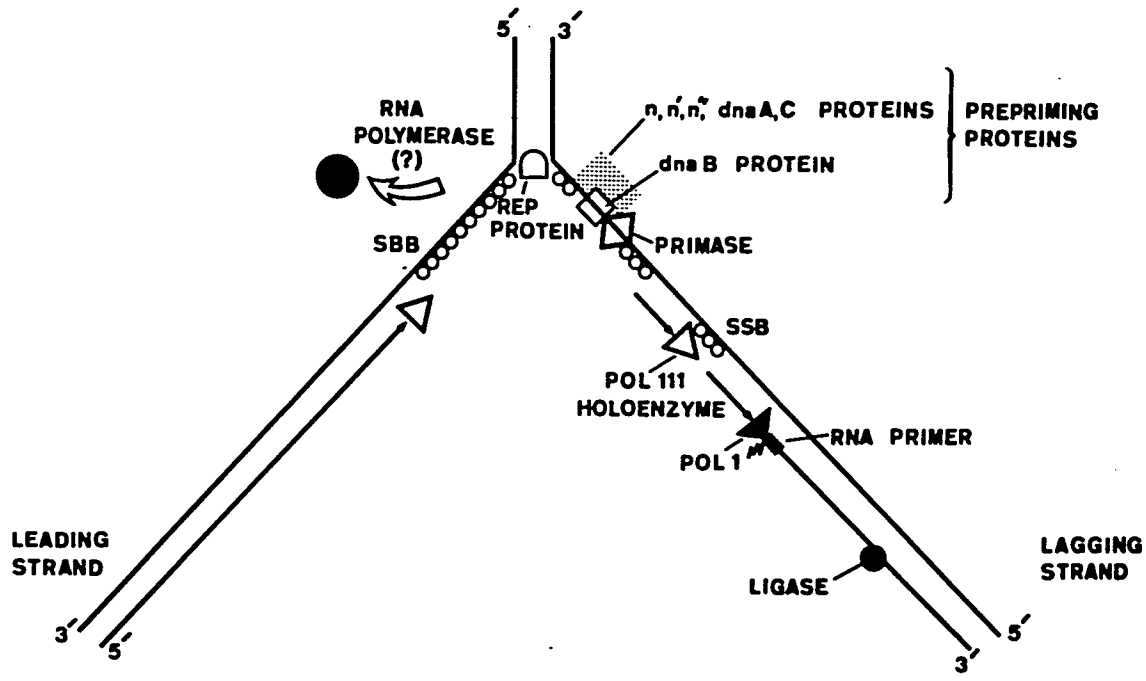
tein requires only four bases of single-stranded DNA in order to function (Kornberg et al., 1978). It may serve as the major ATP dependent unwinding enzyme that is involved in opening the replication fork. E. coli mutants in rep protein exist and are viable at restrictive temperatures (Denhardt et al., 1967), indicating that other helicases may function as well. The established properties of the enzyme and the inhibition of initiation at the replication origin in E. coli by rifampicin makes the suggestion attractive (Tomizawa and Selzer, 1979). The mechanism by which the DNA duplex is first opened for initiation is unknown. Because rep protein requires at least four single-stranded bases in order to bind to DNA, it is not likely that the rep protein is the initial strand melting protein. It has been suggested that RNA polymerase may actually fulfill the function of initial strand melting. Once unwound, the rep protein now operates at the replication fork and the single strands are bound by SSB which stabilizes the single-stranded state. SSB enhances the function of rep protein when rep protein is functioning at a replication fork (Kornberg, 1977). Analysis of E. coli mutants in rep protein indicates that the replication fork moves at a slower rate and that many more forks are generated (Lane and Denhardt, 1975).

Figure 1 is a hypothetical scheme for the initiation of E. coli DNA replication at the origin. There is a tremendous

FIGURE 1

Hypothetical scheme for initiation of DNA replication.

Hypothetical scheme for initiation of DNA replication. Proposed scheme for the enzymology of DNA chain initiation at the origin of replication.


5′
3′
RNA
POLYMERASE
(?)
n, n′, n″, dnaA,C PROTEINS
PREPRIMING
PROTEINS
dnaB PROTEIN
REP
PROTEIN
SBB
PRIMASE
SSB
POL 111
HOLOENZYME
RNA PRIMER
POL 1
LEADING
STRAND
LAGGING
STRAND
LIGASE
3′
5′
5′
3′

amount of confusion concerning the sequence of events, and other schemes can be drawn as well. The model presented here is a synthesis of models presented elsewhere (Wickner, 1978; Tomizawa and Selzer, 1979; Kornberg, 1980).

The duplex is unwound initially by RNA polymerase, which opens the first few bases. The rep protein, using ATP, binds and continues the unwinding proscess. Other helicases may be involved as well. As longer sections of DNA become unwound, SSB binds to them to effectively destabilize the helix. The dnaA protein is somehow involved. Prepriming protein (dnaB product) associates with dnaC protein and proteins n, n', n'' and i, as well as with Mg^{2+} and ATP. These associate with DNA to form a nucleoprotein complex. SSB binds single-stranded DNA as it becomes available, and presumably eliminates weak secondary structures from the DNA. In this manner, SSB helps to select the correct priming site for initiation, which will not become destabilized. Once the prepriming complex has been correctly positioned on the DNA, the dnaG protein, primase, binds the promoter sequence that has been selected. Primase synthesizes an RNA primer in the direction of chain growth, 5' to 3', using the DNA as a template at the origin of replication. The dnaB protein may remain bound to the DNA, functioning as a mobile promoter for further discontinuous replication of the other strand.

3). CHAIN ELONGATION IN DNA REPLICATION

The first enzymatic factors recognised for DNA replication were the DNA polymerases. DNA polymerase 1 (Pol 1) was the first to be purified and extensively studied (Lehman et al., 1958; Richardson et al., 1964; Jovin et al., 1969a; 1969b; Kornberg, 1969; Kornberg and Kornberg, 1974). It is unable to start chain growth de novo from a template, but requires a free 3'OH from a correctly base-paired nucleotide in order to elongate a pre-existing DNA or RNA primer (Bollum, 1964; 1967; Kornberg, 1969; 1974). Pol 1 was found to promote elongation from a nick without the use of any other proteins (Kornberg, 1974).

An associated 3' to 5' exonuclease activity of pol 1 was quickly appreciated as a proofreading function (Brutlag and Kornberg, 1972). It was found that the 3' to 5' exonuclease activity could remove incorrectly base-paired primer nucleotides or terminal mismatches, degrading the strand until it

was correctly base-paired. The correctly base-paired segment could then serve as a primer for DNA chain elongation. Mutants in E. coli pol 1 were used to examine the proofreading capacity of pol 1 (Muzyczka et al., 1972; Lo and Bessman, 1976).

The 5' to 3' exonuclease activity of pol 1 was found to degrade a duplex from the 5' end, removing "distorted" sections such as thymidine dimers or RNA from a RNA-DNA hybrid (Klett et al., 1969; Deutscher and Kornberg, 1969). The 5' to 3' exonuclease activity suggested that pol 1 could function in vivo to remove the RNA primers from DNA chains. The capacity of pol 1 to elongate DNA chains from a nick suggested a gap filling function which could be used to fill in between Okazaki fragments, as well as in the repair of DNA. This process is called nick translation (Kelly et al., 1970). E. coli mutants defective for pol 1 (DeLucia and Cairns, 1969) are not lethal but are defective in the gap filling function (Gross and Gross, 1969; Coukell and Yanofsky, 1970; Schekman et al., 1971).

DNA polymerase 11 of E. coli has not been as extensively studied as pol 1 or pol 111. The role of pol 11 in E. coli DNA metabolism is unknown, and despite research, the functions of pol 11 in DNA chain elongation remain a mystery (Kornberg and Gefter, 1970; Knippers, 1970; Kornberg and Gefter, 1971;

Kornberg, 1980). It is known that the optimal template-primer for pol 11 is a duplex DNA with short gaps (less than 100 nucleotides long), (Kornberg, 1980). The pol 11 enzyme lacks a 3' to 5' exonuclease activity. There is good evidence for a specific protein-protein interaction between pol 11 and E. coli SSB (Molineux and Gefter, 1975), in which they appear to bind each other. It was also shown that the activity of pol 11 on single-stranded DNA covered with SSB is increased approximately 100-fold, so that its activity on long stretches of single-stranded DNA is close to its activity on short gapped duplexes without SSB. Presumably, SSB functions with pol 11 to eliminate minor hairpin structures within the single-stranded DNA that would otherwise result in the inability of pol 11 to remain attached (Kornberg, 1980). E. coli mutants in pol 11 are viable and do not appear to be any more UV sensitive (Campbell et al., 1972). There is evidence that pol 11 is involved in the repair of UV lesions if pol 1 and pol 111 are rendered inactive (Masker et al., 1973; Tait et al., 1974).

The essential DNA polymerase which performs most of the chain elongation from a primer in DNA replication is pol 111 (Kornberg and Gefter, 1971; Kornberg, 1980). Temperature sensitive mutants in the E. coli dnaE (polC/111) gene become blocked in replication fork movement immediately upon shift to the restrictive temperature (Gefter et al., 1971; Kornberg,

1974; Wechsler, 1978). Pol 111 is involved in synthesizing DNA on both lagging and leading strands.

Pol 111 and pol 1 synthesize DNA at about the same rate (Kornberg, 1980). The optimal primer- template for pol 111 is different than that for pol 1. Double-stranded DNA templates containing long single- stranded gaps are optimal as primer-templates for pol 111, and the enzyme cannot use a nicked duplex or long single- stranded DNAs (Kornberg and Gefter, 1971; Kornberg, 1980). The primer-templates are analogous to the conditions at a replication fork in which a primer must be extended along an unfolding duplex DNA. Pol 111, like pol 1, has an associated 3' to 5' exonuclease activity (Livingston and Richardson, 1975), but unlike pol 1 the 3' to 5' exonuclease prefers single-stranded DNA (Kornberg, 1980). There is also an unusual 5' to 3' exonuclease activity associated with pol 111 that requires a free 5' end of a single-stranded DNA to start digestion, but can then progress into a double-stranded region (Livingston and Roberts, 1975). The exonuclease activities are probabaly important for editing and repair accompanying replication. Pol 111 has also been implicated as an important part of the excission-repair system (Wickner et al., 1973).

DNA polymerase 111 is an extremely complex enzyme, It actually appears to exist as a replication apparatus (Wickner,

1978), or as a holoenzyme (Kornberg, 1980), that consists of a basic core enzyme that associates with an extraordinary number of highly specific proteins. The holoenzyme has been purified with various associated subunits (Wickner and Kornberg, 1974), and also in a form that appears to be the fully intact holoenzyme containing all of the subunits (McHenry and Kornberg, 1977). The core enzyme and all of the subunits together comprise the complete enzyme that has the extended properties for chain elongation.

The core enzyme is normally referred to as pol 111. It is a single polypeptide of 140,000 daltons, the product of the E. coli dnaE gene, and forms the alpha subunit of the holoenzyme (Otto et al., 1973; Livingston et al., 1975; McHenry and Kornberg, 1977). Pol 111 appears to be released from pol 111 holoenzyme by dilution or aging (Kornberg, 1977). Another subunit, now designated as beta but formerly called copol 111* or DNA elongation factor 1 (EF1), has been purified and studied (McHenry and Kornberg, 1977). It is a 40,000 dalton polypeptide that enables the enzyme to use very long single-stranded DNA as a template, but only with the addition of SSB. Presumably, the function of SSB is the same in this case as it is with pol 11, but whether there is also an interaction between beta and SSB is not known. It is known that the activity of pol 111 holoenzyme is increased in the presence of SSB (Weiner et al., 1975).

There are a number of other proteins associated with pol III that comprise the holoenzyme. The gamma subunit is a 52,000 dalton polypeptide and the product of the E. coli dnaZ gene (Wickner and Hurwitz, 1976; Kornberg, 1980). The dnaZ gene product is an important component of the holoenzyme and is absolutely required for both in vivo and in vitro DNA replication (Wickner and Hurwitz, 1976). It appears to be integrally involved in the process of elongation from a primed single-stranded DNA (Wickner, 1978).

There are other subunits more transiently associated with the holoenzyme. The delta subunit is a 32,000 dalton polypeptide, also called DNA elongation factor III (EF III), that appears to interact specifically with the gamma subunit (Wickner and Hurwitz, 1976; McHenry and Kornberg, 1977; Kornberg, 1980). Three more polypeptides associate with the core enzyme, but very little is known about them. They are tau, an 83,000 dalton polypeptide, epsilon, a 25,000 dalton polypeptide and theta, a 10,000 dalton polypeptide. An extremely complex hypothetical mechanism has been presented for the interaction of the subunits, but there is very little substantive information to qualify any scheme at this point (Wickner, 1978).

In summary, elongation of DNA from a primer using a template can potentially occur by three mechanisms (Wickner,

1978). The first is extension of the primer by pol 1 alone (Kornberg, 1974). Pol 1 is certainly capable of such synthetic activity, but the optimal template-primer is a nick. Consequently, pol 1 is probably only involved in gap filling between Okazaki fragments. Pol 1 plus DNA ligase allow Okazaki fragments to be connected into a single DNA molecule. The second mechanism is extension by pol 11, with SSB covering the long single-stranded sections of DNA (Scherman and Gefter, 1976). However, little is known about pol 11 and deletion of pol 11 activity is not lethal. The third mechanism involves extension of a primer by pol 111 holoenzyme plus SSB (Wickner and Hurwitz, 1976; Wickner, 1978). This last mechanism is most likely the one which occurs *in vivo* (Wickner, 1978).

The requirement for 5' to 3' replication on both DNA strands indicates that there should be a leading strand from which DNA is synthesized continuously, and a lagging strand from which synthesis can take place only discontinuously at the single-stranded sections of the replication fork. On the lagging strand, Okazaki fragments are initiated with pppApC and two or three more nucleotides before extension by DNA polymerase 111 (Okazaki et al.,1978). Studies of Okazaki fragments in *E. coli* cells deficient in DNA ligase activity indicate that fragments are initiated at least every 1000 nucleotides (Sugimoto et al., 1968). Experiments using the

electron microscope to visualize the replication fork have resulted in the suggestion that there are single-stranded sections of DNA about 1000 nucleotides long, generally on only one parental strand (Inman and Schnoos, 1971; Wolfson and Dressler, 1972; Kriegstein and Hogness, 1974). Isolation of the DNA intermediates in E. coli replication led to the conclusion that discontinuous DNA replication could in fact occur from both strands.

The size of the Okazaki fragments isolated by different research groups varies considerably (Okazaki et al., 1978), as does the distribution of Okazaki fragments isolated on leading and lagging strands in E. coli (Louarn and Bird, 1974). Most of the experiments have been criticized for technical reasons, making analysis of the results difficult (Reichard et al., 1974; Uyemura et al., 1976).

It has been reported that incorporation of dUTP into DNA and subsequent excission of dUMP may result in pseudo- Okazaki fragments. It is known that dUTP incorporation results in chain scission (Lindahl, 1976; Tye and Lehman, 1977; Hochhauser and Weiss, 1978; Lehman et al., 1978). Presumably, the cell has an excission-repair system for removing dUMP from DNA (Alberts and Sternglantz, 1977), that could leave transient breaks in the DNA. Cells have an enzyme, dUTPase, to eliminate dUTP from the nucleotide pool (Shlomai and Kornberg,

1978), and E. coli DNA polymerases show no discrimination between the use of dUTP or dTTP (Kornberg, 1980). An argument that all of the DNA fragments made on the leading strand during DNA synthesis are a result of dUTP incorporation (Olivera et al., 1977), has yet to be refuted (Lehman et al., 1978).

4). DNA GYRASE

E. coli DNA gyrase appears to be absolutely required for DNA replication, since drugs that inhibit the enzyme stop DNA elongation almost immediately (Cozzarelli, 1977). Gyrase is a type 11 topoisomerase. It cuts both strands of DNA, and uses ATP to put in negative supertwists. In the absence of ATP, however, gyrase actually removes negative supertwists. There are two possible ways in which gyrase may function in DNA replication. One is that it untwists DNA, but this possibility seems unlikely (Wickner, 1978). Other topoisomerases, such as omega protein, a type 1 topoisomerase, could perform this

function. Another possible function makes use of the gyrase generated negative supertwisting (Wang and Hsieh, 1975). The increased negative supercoiling generated by gyrase and ATP may actually make it easier to open up the DNA helix. However, any mechanistic details at this point are purely speculative.

5). TERMINATION OF DNA REPLICATION

The control of DNA replication is effected at initiation, and consequently there has been much more work done in this area (Yoshikawa et al., 1964; Lark, 1979). Very little is known about how replication terminates and chromosomes separate.

After a chromosome has been fully replicated, the RNA primers have already been removed, the resulting gaps have been filled by DNA polymerase 1, and DNA ligase has functioned to seal the nicks. One major problem is gap filling of the first section of the chromosome initiated by RNA. This

section corresponds to the first RNA primer synthesized in initiation; it is opposite the extreme 3' end of the parental strand and comprises the extreme 5' end of the nascent strand. Removal of the RNA primer leaves only a 5' end free, and no DNA polymerase can extend a chain from the 5' end, nor can one elongate DNA in a 3' to 5' direction. Circular chromosomes, such as the E. coli chromosome, are a solution to the problem. The 3' end of the last DNA fragment synthesized can serve as a primer to fill in the gap created from the removal of the first RNA primer. All that is required is ligation of the remaining nick by DNA ligase.

In E. coli, termination of DNA replication takes place when the two replication forks meet at position 31 minutes on the circular map, at the rac locus (Kornberg, 1980). There is no evidence to indicate any site-specific nuclease events for termination of synthesis and separation of the double-stranded products.

6). GENETIC RECOMBINATION

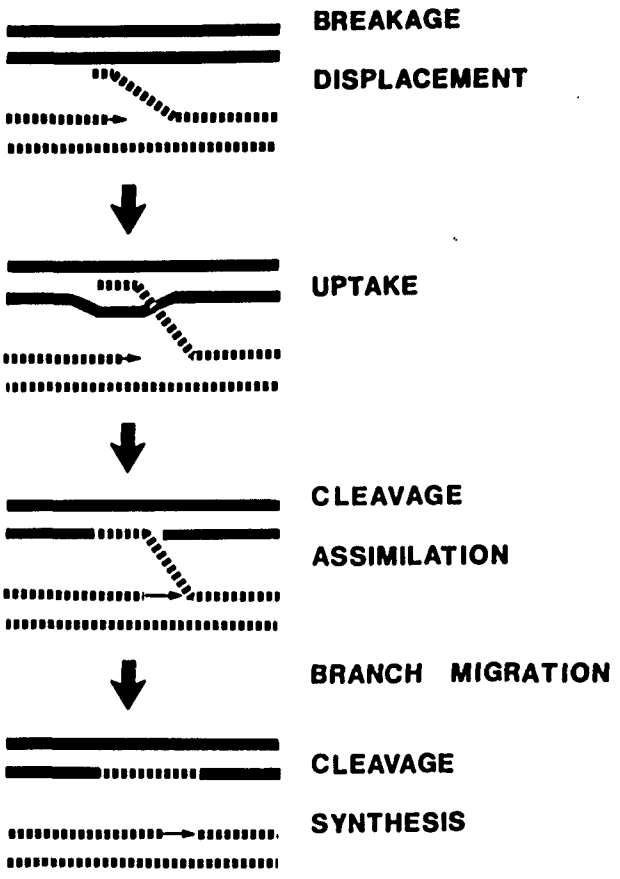
Classically, generalyzed recombination has been viewed as a complex process of strand breakage and reunion (Holloman et al., 1975). This scheme utilizes the machinery of DNA elongation (figure 2). First, there is a step of strand breakage and displacement in which the recombining strand is displaced by new DNA synthesis. The free strand is taken up, in some manner, by a homologous region on the recombining duplex, resulting in the formation of a D-loop. Removal of the loop permits assimilation. Symmetrical crossover may then occur but is not a prerequisite for the completion of the recombination event. Recombination is completed by strand cleavage and synthesis of DNA within the remaining gaps.

The involvement of the recA protein in DNA recombination in E. coli was assumed from genetic evidence. Mutants of E. coli in the recA gene were originally isolated as recombination deficient mutants (Clark and Margulies, 1965). RecA mutations were found to be pleiotropic and to be difficult to associate with one set of specific cellular functions (Clark, 1973). Mutants were found to be deficient in the repair of DNA after exposure to DNA-damaging agents (Witkin, 1976). The inducible system for repair of damaged DNA, the so called

FIGURE 2

Hypothetical scheme for strand transfer in DNA recombination.

Hypothetical scheme for strand transfer in DNA recombination. Mechanism of strand transfer involving replication and repair enzymes.


BREAKAGE
DISPLACEMENT
UPTAKE
CLEAVAGE
ASSIMILATION
BRANCH MIGRATION
CLEAVAGE
SYNTHESIS

"SOS" functions, was found to be severely affected in many ways (Gottesman, 1981). The production of recA protein itself was found to increase enormously after DNA damage by UV irradiation or by various mutagens (McEntee, 1977; Gudas and Mount, 1977). It was found that recA protein exhibited a specific protease activity for the phage lambda repressor (Roberts et al., 1978), as well as for the lexA protein, the repressor of the recA operon (Little et al., 1980). Thus the recA protein, besides its direct involvement in genetic recombination, also eliminates the control elements that restrain induction of the recombination and repair systems in E. coli (Gottesman, 1981). With the purification of the recA protein, however, (Roberts et al., 1978), its role in genetic recombination is becoming more clearly understood.

The recA protein is absolutely essential for generalyzed genetic recombination in E. coli (Clark, 1973; Kobayashi and Ikeda, 1978). Pure recA protein (Roberts et al., 1978), is now available. In fact, there is a recA clone in an E. coli plasmid which allows 20% of the protein in E. coli to be recA. In in vitro studies, recA protein has been shown to promote the pairing of complementary single- stranded DNAs (Weinstock et al., 1979). The protein promotes the pairing of homologous

DNAs in which the invading DNA is single-stranded and the invaded DNA is superhelical (Shibata et al., 1979a). Non-superhelical duplex DNAs also act as accepting species in the presence of recA protein (McEntee et al., 1979). A D-loop is formed under these conditions and, superficially at least, appears to resemble the generation of D-loops of recombining molecules seen in the electron microscope (Radding, 1978). D-loops appear to be necessary intermediates in generalized recombination (Cunningham et al., 1981). Recombination between gapped closed circular DNA and double-stranded closed circular DNA was also found to be catalyzed by recA (Cunningham et al., 1980). RecA mediated recombination can thus take place at gap junctions as well as between single and double stranded DNAs. Recently, recA has been shown to allow complete displacement of one strand of a double-stranded DNA by a homologous circular DNA strand (Cox and Lehman, 1981).

The addition of E. coli SSB to each of the systems just described results in a tremendous reduction in the amount of recA protein required for catalysis (Cassuto et al., 1980; Shibata et al., 1980). Hence SSB, in addition to its functions in DNA replication, also helps catalyze the strand transfer reactions mediated by recA protein, reactions that are probably analogous to recombination in vivo (Radding, 1978).

SSB may protect single-stranded DNA against deoxyribonucleases (Molineux and Gefter, 1975). The recBC nuclease is a nuclease in E. coli that appears to be involved in the inducible recA mediated recombination (MacKay and Linn, 1974; 1976; Tanaka and Sekiguchi, 1975). It functions as a double-stranded DNA exonuclease in either the 5'to 3' or 3' to 5' direction. It appears to unwind the duplex and digest the remaining single-stranded DNAs (Kornberg, 1980). Interestingly, however, if in the presence of saturating amounts of SSB, the recBC nuclease hydrolyzes ATP and functions in a manner analogous to the rep protein. The recBC nuclease unwinds the duplex like an ATP dependent helicase, and there is no DNA hydrolysis. Thus another function of SSB in recombination appears to be the protection of single- stranded DNAs by the conversion of the recBC nuclease into an ATP dependent helicase.

7). PHYSIOLOGICAL PROPERTIES OF E. COLI SSB

The physiological properties of SSB have been covered in the preceeding sections with respect to its functions in DNA replication and recombination. SSB was found to be required absolutely for the in vitro conversion of single- stranded circular bacteriophage DNA to the duplex replicative form (Wickner and Hurwitz, 1974; Weiner et al., 1975), as well as the in vitro synthesis of single- stranded DNA from the replicative form (Scott et al.,1977). It is also involved in protecting single-stranded DNA from nucleolytic enzyme degradation (Molineux and Gefter, 1975), including the recBC nuclease, a recombination/repair enzyme (MacKay and Linn, 1976). SSB complexes with E. coli DNA polymerase 11 and exonuclease 1 (Molineux and Gefter, 1975), and stimulates the activity of E. coli DNA polymerase 11 and 111 holoenzyme (Sigal et al., 1972; Molineux and Gefter, 1974; Weiner et al., 1975). The rep protein unwinding at replication forks was also found to be promoted by SSB (Kornberg, 1977), as well as recA mediated recombination in vitro (Cassuto et al., 1980; Shibata et al., 1980a). In addition, DNA strand reassociation without recA protein has also been found to be catalyzed some 5000-fold by SSB, under somewhat physiological conditions at 37 degrees C with 2mM spermidine (Christiansen and Baldwin, 1977).

Temperature sensitive mutants of the E. coli SSB have been isolated (Sevastopolous et al., 1977), and mapped at 91 minutes on the E. coli genome (Meyer et al., 1979). Mapping studies have placed the SSB gene, ssbA, very close and to the right of the uvrA gene in E. coli K12 (Johnson, 1977; Glassberg et al., 1979). A recombinant plasmid has been constructed that contains both the ssbA+ gene and the uvrA+ gene (Sancar and Rupp, 1979), as has a lambda transducing phage which carries both uvrA+ and ssbA+ genes. The lexC mutation (Johnson, 1977), occurs in the SSB gene. It also has a pleiotropic effect on DNA repair and replication, and is probably identical to ssbA (Meyer et al., 1979; Glassberg et al., 1979; Meyer et al., 1980).

The ts mutants in SSB do not grow well even at 30 degrees C, and exhibit a fast-stop phenotype at 42 degrees C (Meyer et al., 1979), indicating an essential function for SSB at the replication fork. The lower activity in vivo represents an actual decrease in specific activity and not merely a decreased production of protein. Interestingly, the purified mutant protein also displays inactivation at 42 degrees C in in vitro phage DNA replication systems, and has a four-fold lower specific activity at 30 degrees C (Meyer et al., 1979). Wild type SSB is known to withstand boiling for several minutes and regain its activity upon cooling (Weiner et al.,

1975); the mutant SSB also appears to withstand inactivation by boiling and to regain its activity at 30 degrees C (Meyer et al., 1979).

It has also been found that the ts SSB is far less effective than the wild type SSB in protecting single- stranded DNA against nucleolytic degradation (Meyer et al., 1980). DNA coated with ts SSB is more accessible to S1 nuclease. Assay of DNA dependent ATPases, such as the rep protein, also indicates that mutant SSB does not protect single-stranded DNA as well as wild type.

The E. coli SSB mutants exhibit a five-fold reduction in recombination activity at 37 degrees C (Glassberg et al., 1979). This is not a drastic decrease, considering that mutations in recA or recBC loci decrease recombination far more (Clark, 1973). However, the temperature sensitivity of one activity need not be the same as other activities of SSB. The ssbA- (lexC-) mutation is known to lead to extensive UV sensitivity (Johnson, 1977), indicating a role in repair. The UV sensitivity occurs at both permissive and non-permissive temperatures for DNA replication. Finally, there is evidence to suggest that the ssbA locus is a control element of the recA/lexA mediated inducible repair system in E. coli (Johnson, 1977), suggesting that SSB may function indirectly in recombination as well as directly.

8). PHYSICAL AND CHEMICAL PROPERTIES OF E. COLI SSB

The E. coli SSB is a double-stranded DNA "melting protein". It binds single-stranded DNA so strongly and cooperatively that it may destabilize the helical form of DNA, lowering the melting temperature (Tm). Sigal et al. (1972) found that addition of excess SSB could lower the melting temperature of DNA about 20 degrees C below Tm. However, the authors found it necessary to perform the experiments in extremely low salt, approximately 1 mM KCl. The low salt itself lowers the melting temperature. It is unclear to what extent SSB will lower the melting temperature of duplex DNAs in buffers containing high salt. The ability of SSB to lower the Tm has been used to obtain partial denaturation maps of lambda DNA that are similar to maps obtained using alkali (Sigal et al., 1972). As expected, the regions which melt first in alkali, those higher in AT, are the regions which are destabilized by SSB.

The cooperative binding exhibited by SSB probably results from protein-protein interactions (Alberts and Frey, 1970); the result is that proteins line up contiguously along a DNA strand. Consequently, a DNA strand becomes covered with SSB before the proteins initiate binding on another strand. This has been shown by EM visualization of single-stranded DNAs in the presence of sub-saturating amounts of SSB (Sigal et al., 1972; Ruyechan and Wetmur, 1975).

SSB holds DNA in a rigid conformation. DNA strands covered with SSB can be prepared for electron microscopy by spreading on an aqueous hypophase without the use of strong denaturing agents to maintain the single-stranded structures. The DNAs appear rigid, and do not collapse. If enough SSB is added to cover 50% of the DNA, then 50% of the DNA will be completely covered with SSB and 50% will be unbound.

The internucleotide spacing of DNA bound by SSB is reduced from 3.4 Angstroms (native form) to 1.8 Angstroms, a factor of 2.56 (Sigal et al., 1972). Presumably, the reduced internucleotide spacing is due to wrapping the DNA about the protein in some manner, possibly by coiling it into a broad helix (Kornberg, 1980). The suggestion has been made that the difference in base spacing represents a conformation of DNA which may more readily be utilized by recombination or replication proteins (Anderson and Coleman, 1975). However, there is no information to indicate that this is the case.

Presumably, SSB interacts with the phosphate backbone of DNA and not directly with the bases. Using equilibrium dialysis experiments to measure SSB binding to various oligonucleotides, Ruyechan and Wetmur (1976), found that there is little or no base specificity for binding. Even after removal of the purines from oligomers, the SSB-DNA interaction had the same apparent binding free energy. These results indicate that SSB binds DNA by the phosphate backbone with little regard for base sequence.

Binding of SSB to single-stranded DNA appears to take place without displacement of counterions from the phosphate backbone (Ruyechan and Wetmur, 1976). Analysis of the effects of sodium ion concentration upon the binding of SSB to oligomers indicated that the replacement of counterions by SSB does not occur upon binding. This indicates that the elimination of electrostatic repulsion between phosphate groups is probably not a function of SSB binding.

There has been much confusion concerning the native and reduced molecular weights of E. coli SSB, as well as the DNA binding form of the protein and the stoichiometry of binding. Sigal et al., (1972) found a reduced molecular weight of 22,000 daltons, as did Molineux et al., (1974), and Molineux and Gefter (1974), and a native molecular weight of approxi-

mately 90,000 daltons. Weiner et al., (1975) found a subunit molecular weight of 18,500 daltons and a native molecular weight of 76,000 daltons. Ruyechan and Wetmur found a subunit molecular weight of 19,000 daltons, which is in good agreement with the calculated molecular weight of 18,873 daltons from amino acid sequence analaysis (Sancar et al., 1981). The protein has a sedimentation coefficient of 4.9S, indicating a native molecular weight of 66,000 daltons assuming a spherical shape (Weiner et al., 1975). This indicates that the protein is a tetramer composed of four identical subunits of approximately 19,000 daltons each. No species of the protein larger than tetramers were found free in solution without DNA, unless the concentration of protein was increased to greater than 750 micrograms per ml (Molineux et al., 1974).

The single-stranded DNA binding species has been shown by a number of methods to be a tetramer. Sigal et al. (1972) obtained EM results suggesting SSB bound as a tetramer. Using sucrose gradient sedimentation analysis of SSB covered DNA they concluded that the protein bound DNA on a weight ratio of 8:1 (protein:DNA). This is one protein monomer for every 8 bases, or 32 bases per tetramer. Coleman and Anderson (1975) obtained a stoichiometry of 52-56 nucleotides per tetramer by circular dichroism analysis of DNA-protein complexes. Weiner et al. (1975), using gel filtration of SSB bound oligonucleotides, found a stoichiometry of 30-36 bases covered per

tetramer. SSB protection of single-stranded DNA against nucleolytic enzymes was used to determine binding stoichiometry of 30-36 nucleotides (Ruyechan and Wetmur, 1976).

The results of Bandyophadhyay and Wu (1978) suggest that tryptophan residues are near the SSB binding site for DNA. They also found that lysine residues are absolutely essential for binding, since chemical modification of them interferes with binding. Analysis of the SSB tryptophan fluorescence upon binding and dissociation led the authors to conclude that the tetramer does not dissociate into smaller subunits when not bound to DNA.

The binding of SSB to DNA can be broken into two parts (Ruyechan and Wetmur, 1975; 1976). First there is the initiation or non-cooperative interaction between one SSB tetramer with several nucleotides on a single-stranded DNA, followed by a very strong cooperative binding of other SSB tetramers. The binding constant for the association of one SSB with single-stranded DNA has been measured in a series of equilibrium dialysis experiments using oligomers of various lengths in various NaCl concentrations and pH conditions (Ruyechan and Wetmur, 1976). It has been found that the binding of SSB to four bases occurred optimally at physiological salt, temperature and pH. A non-cooperative binding constant on the order of 5×10^4 1/M per nucleotide site was calculated from a

Scatchard analysis. This corresponds to an association constant of about 1.6×10^6 1/M to a site size of 30-36 nucleotides. It was found that there were 4 nucleotides actually bound by each tetramer, although each tetramer covers 30-36 bases. Cooperative binding of an SSB next to an occupied site occurs with an association constant approximately 10^5 greater than the non-cooperative binding constant.

Equilibrium dialysis experiments of oligomers ranging from 8 nucleotides long to 12-18 long were used to draw a Scatchard plot to describe the number of sites available to react on SSB, as well as the number of nucleotides required for cooperative binding to occur (Ruyechan and Wetmur, 1976). It was found that the oligomers had two binding sites per tetramer, but that both could not be bridged at the same time. If they could, then oligomers up to 18 nucleotides long would have been bound cooperatively, and they were not. There appears to be, then, two identical binding sites per tetramer, which cannot be bound simultaneously, and only one of which is occupied during cooperative binding.

Very little is known about the kinetics and mechanism of exchange of SSB among single-stranded DNA molecules. Weiner et al., (1975) measured an exchange reaction of SSB between full length ØX174 viral DNAs and found an extremely slow rate of transfer. Bandyophadhyay and Wu (1978), have used

salt-jump kinetics for a detailed study of the DNA-T4 gene 32 protein complex, again with the same size DNA. The results are complex making their interpretation difficult.

9). A BRIEF SURVEY OF OTHER SINGLE STRAND BINDING PROTEINS

The first SSB purified was the T4 phage gene 32 protein from infected E. coli cells (Albert and Frey, 1970). Other bacteriophages from which SSBs have been purified and studied include the fd phage (Alberts et al., 1972; Oey and Knippers, 1972), and the T7 phage (Scherzinger et. al., 1973; Reuben and Gefter, 1973; 1974). Eukaryotic sources for SSB include the microsporocytes of the lily (Hotta and Stern, 1970; 1971a; 1971b) and calf-thymus tissue (Herrick and Alberts, 1976). Adenovirus infected cells have also been found to produce an adenovirus coded SSB (van der Vliet and Levine, 1973).

The T4 gene 32 protein remains the most extensively studied of the SSBs. The physiological role of gene 32 protein is accessible to genetic analysis since mutants in gene 32 exist.

Analysis of the gene 32 mutants has indicated that the protein is essential during the entire course of the T4 replication cycle (Kozinski and Felgenhauer, 1967; Alberts et al., 1968). Other experiments indicated that the protein is involved in T4 DNA replication (Epstein et al., 1963; Alberts and Frey, 1970; Huberman et al., 1971; Breschkin and Mosig, 1977), recombination (Tomizawa et al., 1966), repair (Wu and Yeh, 1973), and protection against nucleases (Mosig and Bock, 1976). The level of gene 32 protein within an infected cell has been found to be regulated by an elaborate autoregulatory response in which the excess protein binds and inhibits the translation of its own message (Gold et al., 1976; Russel et al., 1976; Krisch et al., 1977).

Biochemical characterization of the gene 32 protein showed that it is a 36,000 dalton protein (Alberts and Frey, 1970), and that it holds single-stranded DNA in an extended configuration in which the intranucleotide spacing is actually increased from 3.4 Angstroms to 4.6 Angstroms (Delius et al., 1972). The protein binds DNA cooperatively as a monomer (Alberts and Frey, 1970), and covers from 5-8 nucleotides (Delius et al., 1972; Jensen et al., 1976). Gene 32 protein will lower the melting temperature up to 40 degrees C for poly d(A-T) (Delius et al., 1972). However, the reduction in Tm is not large enough for gene 32 protein to melt native T4 DNA at 37 degrees C (Alberts and Frey, 1970). Under certain condi-

tions, gene 32 protein was also found to catalyze *in vitro* DNA strand reassociation (Alberts and Frey, 1970).

The gene 32 protein has been dissected into functional regions through the use of genetic mutations at various parts of the protein (Breschkin and Mosig, 1977a; 1977b; Mosig et al., 1978), as well as by proteolytic modification of the protein (Hosoda et al., 1974; Anderson and Coleman, 1975; Moise and Hosoda, 1976; Williams and Konigsberg, 1978; Tsugita and Hosoda, 1978; Spicer et al., 1979). Together, the results of these studies have established that the N-terminal portion of the protein is responsible for its cooperative binding (its protein- protein interactions), and the C-terminal portion is involved in DNA binding. There have been many suggestions that specific proteolysis may play an important *in vivo* role in the functioning of gene 32 protein. It is possible that enzymatic modification occurs *in vivo*, and it has been shown that the removal of 50 C-terminal amino acids (Coleman and Anderson, 1975) results in a more cooperatively binding protein; modified gene 32 protein can melt T4 duplex DNA at 37 degrees C (Hososda, et al., 1974).

The physical and chemical properties of T4 gene 32 protein have been quantitatively studied extensively with respect to its binding parameters and cooperativity (Jensen and von Hippel, 1976; Jensen et al., 1976; Kelly and von Hippel, 1976;

Kelly et al., 1976; Peterman and Wu, 1978; Kowalczykowski et al., 1981; Newport et al., 1981). There is now a good quantitative understanding of the details of the molecular interactions between gene 32 protein and single-stranded DNA and RNA. There has also been some research on the molecular interactions of enzymatically modified gene 32 protein with single-stranded DNA and RNA (Burke et al., 1980; Lonberg et al., 1981).

Adenovirus infected cells have a SSB that appears to have a molecular weight of 72,000 daltons (Levine et al., 1976; van der Vliet and Levine, 1973). Genetic analysis and biochemical studies of the adenovirus SSB have shown that the protein is involved in the initiation and elongation of DNA for virus replication, and that it is produced early in virus infection (Arens et al., 1977; Ginsberg et al., 1977; Horwitz, 1978; van der Vliet et al., 1975; 1978). The protein binds single-stranded DNA without regard to nucleotide sequence (van der Vliet and Levine, 1973; Sugawara et al., 1977). In addition, it has been found that at low protein concentrations the adenovirus SSB shows a greater specificity for the ends of double-stranded DNA, both blunt and staggered ends, than for single-stranded DNA (Fowlkes et al., 1979). It had been suggested that the adenovirus SSB may be analogous to the T4 SSB in its function, such as DNA melting and strand displacement, since it does saturate single-stranded DNA (Shanmugam et

al., 1975). However, research into the DNA interactions of the adenovirus SSB has shown that the protein does not significantly lower the Tm of duplex DNA (Fowlkes et al., 1979; Schechter et al., 1980). Moreover, genetic analysis of adenovirus SSB ts mutants has indicated that the protein may play a regulatory role. Under restrictive conditions the amount of early mRNA increases, indicating a regulatory function in early gene expression (Carter and Blanton, 1978a; 1978b). Also, adenovirus SSB ts mutants under restrictive conditions may actually replicate in normally non-permissive monkey cells (Klessig and Grodzicker, 1979), indicating that the adenovirus SSB role in virus replication is extremely complex.

MATERIALS AND METHODS

PREPARATION OF E COLI SINGLE STRAND DNA BINDING PROTEIN

E. coli K12 frozen cell paste was purchased from Grain Processing Corporation, Muscatine, Iowa. SSB was isolated using a modification of the procedure of Sigal et al., (1972). All operations were carried out at 4 degrees C unless otherwise specified. 250 grams of cell paste were added to 1200 ml of 0.05M NaCl, 0.01M $MgCl_2$, 0.002M EDTA, 0.001M 2-mercaptoethanol, 0.0001M dithiothreitol, 0.05M TrisHCl pH 7.6, 130 ug/ml lysozyme (Worthington), 23 ug/ml phenylmethylsulfonylfluoride, 20 ug/ml DNAse 1 (Sigma), 0.4 percent sodium deoxycholate. After blending in a Waring blender, the suspension was allowed to stand at least 15 minutes and was sonicated for 3 minutes in 100 ml aliquots with a Bronson Power Sonifier. The extract was incubated for 4 hours at 10 degrees C before clarification by low speed centrifugation followed by centrifugation at 54,000g for 45 minutes. The supernatant was dialyzed against buffer A of Sigal et al., (1972): 0.05M NaCl, 0.001M EDTA, 0.001M 2-mercaptoethanol, 0.05M TrisHCl pH 7.6.

A 100 ml single-strand DNA cellulose column (Alberts and Herrick, 1971) with 1.5 mg/ml DNA was prepared using denatured calf-thymus DNA and highly purified cellulose powder (Whatman) and was poured in buffer A plus 10% glycerol. The dialyzed extract was made 10% in glycerol and pumped through the DNA-cellulose column at 30 ml/hr. The column was eluted with buffer A containing 0.46 mg/ml dextran sulfate (Pharmacia) followed by buffer A containing 0.15M NaCl until the effluent contained no detectable absorbance at 280nm. The SSB and other contaminating proteins were obtained by elution with buffer A containing 2M NaCl and were dialyzed into 0.2M NaCl, 0.02M Tris HCl pH 7.6, 0.001M EDTA, 0.001M 2-mercaptoethanol, 20 percent glycerol. The SSB containing sample was pumped through a 20 ml Blue -sepharose column at 20 ml/hr. A blue dextran column has been used by Meyer et al., (1979) for SSB purification. The elution properties differ from those found using Blue -sepharose CL6B. The column was washed with the same buffer containing 2M NaCl instead of 0.2M NaCl to remove all of the bound protein except SSB. The SSB was eluted with 4M guanidine HCl (Schwartz Mann, ultrapure) in 0.02M sodium phosphate buffer pH 6.86, 0.001M EDTA, 0.001M 2-mercaptoethanol, 20 percent glycerol, and dialyzed into 0.05M NaCl, 0.02M TrisHCl pH 7.6, 0.001M EDTA, 10% glycerol to remove the guanidine and renature the protein. The protein may be stored at 4 degrees C over $CHCl_3$, or frozen once at greater than 100 ug/ml protein and stored at -20 degrees C.

LOWRY PROTEIN CONCENTRATION DETERMINATIONS

The SSB concentration was determined by the method of Lowry et al. (1951). All reagents including the Phenol (Foln) Reagent were purchased from Fisher Scientific and used without further purification. The protein standard was electrophoretically pure bovine serum albumen, BSA, (Sigma). A plot of the absorbance at 750 nm versus ug, between 0 and 70 ug, was drawn for the BSA standards. The absorbance at 750 nm for SSB at various dilutions was determined from the BSA standard curve.

SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

REDUCING SDS-PAGE

The polypeptide molecular weight of SSB was determined by SDS-PAGE (Weber and Osborn, 1969), using a 7% to 15% linear acrylamide gradient gel. Protein samples were prepared by precipitating with eight volumes of acetone at -70 degrees C and centrifugation in an Eppendorf mini-centrifuge to pellet. Pellets were dried, then resuspended in 20 ul of a 1% 2-mercaptoethanol + 2% SDS solution and 5 ul of a 50% glycerol + 0.25% bromophenol blue solution, and submerged in boiling water for 2 minutes to denature the proteins. Electrophoresis was carried out overnight at 100 volts. Gels were stained with 0.1% Coomassie Brilliant Blue and destained with a mixture of 7% glacial acetic acid and 10% methanol in water. Molecular weight was determined relative to a set of standard protein markers by plotting the logarthm of the distance migrated versus molecular weight.

NATIVE SDS-PAGE

Non-reducing SDS-polyacrylamide gel electrophoresis was carried out as described above, except for a few modifications in sample preparation. Samples were prepared by making 2% in SDS, but the addition of 2-mercaptoethanol and the boiling step were eliminated.

IODINATION OF SSB

The iodination of SSB was performed with modifications from Greenwood , Hunter and Glover (1963). 15 ug of SSB in 250 ul was dialyzed against 0.1M sodium phosphate buffer pH 7.3. This buffer was also used for the preparation of chloramine T and sodium meta-bisulfite. The protein was transferred to a small siliconized stoppered glass vial, 50 uCi of ^{125}I was added and stirred at 4 degrees C for two minutes. 100 ul of buffer containing 0.0044M chloramine T was added and stirred at 4 degrees C for 5 minutes. 100 ul of buffer containing 0.0058M sodium meta-bisulfite was added and stirred at 4 degrees C for 5 minutes. The protein was dialyzed exhaus-

tively against 0.05M NaCl, 0.01M TrisHCl pH 7.7, 0.0002M EDTA. Specific activity was determined by Lowry protein determination and gamma -counting.

SEPHADEX GEL FILTRATION

Sephadex G-100 was prepared in 0.15M NaCl, 0.01M TrisHCl pH 7.7, 0.0002M EDTA, according to the recommendations of the manufacturer. A column 1.5 cm x 18 cm was prepared. The void volume was determined by elution of Blue dextran T-2000. Protein markers consisted of cytochrome C, myoblobin, ovalbumin and BSA. Elution of protein markers was followed by absorbance at 280 nm. The elution of iodinated SSB was followed by gamma-counting. The native molecular weight for SSB was estimated from a plot of the elution voulume versus the logarithm of the molecular weight of the known protein markers.

SINGLE-STRANDED PHAGE DNA CIRCLES

Single-stranded DNA circles from bacteriophage G4, isolated by Dr. N. Godson, were a gift of Dr. J. Young. The ØX174 single-stranded DNA circles were purchased from Bethesda Research Labs. The molecules were found to be almost entirely circles when visualized in the electron microscope. DNA circles were visualized by two techniques. (1) Single-stranded DNA circles at 1 ug/ml were incubated with excess SSB in 0.20M ammonium acetate and made between 20-100 ug/ml in cytochrome c prior to spreading on a 0.25M ammonium acetate aqueous hypophase as described by Davis et al. (1971). DNA was picked up on parlodion covered copper grids and stained for 30 seconds in 90% ethanol containing 2 x 10^{-5}M uranylacetate and dried for 10 seconds in petroleum ether. (2) DNA circles at 1 ug/ml in 40% formamide containing between 20-100 ug/ml cytochrome c, 0.1M TrisHCl pH 7.7, 0.01M EDTA were spread on a formamide hypophase containing 15% formamide, 0.01M TrisHCl pH 7.7, 0.001M EDTA as described by Davis et al. (1971). DNA was picked up on parlodian covered grids as before and stained. Grids were usually also shadowed with platinum from a platinum-tungsten basket at an 8 degree angle to the grid in a Varian evaporator.

PREPARATION OF ^{3}H-(T)-DNA OF PLASMID PBR322

A thymidine requiring mutant (CR34) of E. coli K12 transformed with pBR 322 was a gift of Dr. J. Young. These bacteria were grown in M9 minimal media plus 0.4% casein hydrolysate, 0.4% glucose, 2 ug/ml thymidine, 25 ug/ml each of ampicillin and tetracycline. The plasmid was selectively replicated and labelled using 4uCi/ml 6-^{3}H-thymidine (New England Nuclear) and 200ug/ml chloramphenicol (Sigma) when the culture reached an absorbance of 0.8 at 590 nm, according to the procedure of Clewell (1972). 3-H-thymidine was added 2 hours after chloramphenicol addition. ^{3}H-labelled DNA was isolated by the method of Birnboim and Doly (1979) scaled up for large preparative isolation. The method involved alkaline denaturation of DNA, renaturation of plasmid DNA, centrifugation to selectively precipitate the bacterial DNA and repeated phenol/chloroform extractions to remove protein. Plasmid DNA was purified by CsCl density gradient sedimentation. Specific activities of 10^{5} cpm/ug were routinely obtained. Purified plasmid was judged free from contaminating DNAs by agarose gel electrophoresis of 2 ug of DNA, followed by staining with a 2 ug/ml solution of ethidium bromide. Plasmid was stored frozen at -70 degrees C in 0.015M NaCl, 0.01M TrisHCl pH 7.7, 0.0002M EDTA.

SONICATION OF DNAS

A solution of double-stranded calf-thymus DNA (Sigma) at 150 ug/ml in 0.015M NaCl, 0.01M TrisHCl pH 7.7, 0.0002M EDTA was sonicated using a Bronson Power Sonifier. Sonication was performed on ice for 8 x 30 seconds at the maximum setting, with intermittent cooling of the solution.

DETERMINATION OF DNA SIZE, INTEGRITY AND SEDIMENTATION COEFFICIENT

DNA sedimentation coefficients, integrity and size were determined by sedimentation in 3M CsCl at 0.1 N NaOH pH 13 in a Beckman Model E Analytical Ultracentrifuge equipped with ultraviolet optics. Approximately 50 ul of a solution containing between 0.5 ug to 1 ug of DNA was loaded into a band-forming centerpiece of a 30mm cell. Photographic negatives were taken during sedimentation and later scanned on a Canalco Model J Densitometer. Sedimentation coefficients and molecular weights were calculated according to Studier (1965).

RESTRICTION ENZYME DIGESTS

Restriction endonuclease digestions were performed using buffers recommended by the manufacturer (New England Biolabs).

AGAROSE GEL ELECTROPHORESIS

Agaraose (SeaKem or BRL, ultrpure) was prepared in either TBE buffer (0.089M Tris-borate pH 7.7, 0.001M EDTA) or Tris Acetate buffer (0.04M Tris Acetate pH 7.9, 0.005M Sodium Acetate, 0.001M EDTA). Gels between 0.7% to 1% agarose were run according to McDonell et al. (1977), using ethidium bromide at 0.5 ug/ml in a horizontal gel apparatus. Before loading, DNA samples were made 10% in glycerin and 0.025% in bromophenol blue. All DNA molecular weights were determined relative to the known molecular weights of various published restriction digests of either lambda DNA or pBR322.

PURIFICATION OF RESTRICTION FRAGMENTS FROM AGAROSE GELS

DNA restriction fragments were isolated on 1% agarose gels containing ethidium bromide and removed from the gels by a variety of methods (Yang et al., 1979). The methods were either (1) electroelution from gel slices into dialysis sacs (2) repeated freeze-thaw extraction of DNA from gel slices (3) phenol extraction from low melting point agarose (BRL). All restriction fragments were extracted with phenol several times after removal from gel slices, ethanol precipitated several times and stored in 0.015M NaCl, 0.01M TrisHCl pH 7.7, 0.0002M EDTA at 4 degrees C. DNA concentrations were determined by molar absorbtivity at 260 nm and also specific activities when the fragments were radiolabelled.

IODINATION OF DNAS

All DNAs were radio-iodinated according to Orosz and Wetmur (1974). Iodinations were performed in sodium acetate buffer at pH 4.3. Iodinated DNAs were dialyzed exhaustively against 0.015M NaCl, 0.01M TrisHCl pH 7.7, 0.0002M EDTA and stored at 4 degrees C.

SSB NUCLEASE PROTECTION ASSAY

The amount of active SSB was determined by the nuclease protection assay of Ruyechan and Wetmur (1976), using radioiodinated single-stranded DNA. Digestions were performed in 0.006M $MgCl_2$, 0.01M TrisHCl pH 7.4, 0.05M NaCl at 37 degrees C for 20 minutes in small siliconized glass tubes using an equal volume of a stock solution containing 100 ug/ml DNase 1 and 10 ug/ml snake venom phosphodiesterase (Sigma). Reactions were quenched by addition of EDTA to 0.02M. Aliquots were spotted onto PEI-cellulose strips (Brinkman Industries) and separation of SSB protected ^{125}I-labelled single-stranded DNA was achieved by thin layer chromatography using 1M HCl. The strips were cut in half, and bottom and top counted separately by gamma-counting to determine the fraction of DNA protected.

PREPARATION OF HIGHLY ACCURATE CAPILLARY TUBES

Siliconized glass micropipetting tubes (Clay Adams) were individually calibrated and scored for the precise volumes delivered in the transfer experiments. The micropipetting tubes were outfitted to a Pipettman automatic pipetting device (Gilson). The pipetting accuracy was within 1%.

SSB TRANSFER EXPERIMENTS VISUALIZED BY ELECTRON MICROSCOPY

Donor DNA was either highly sonicated and denatured calf-thymus DNA with an average length of 400 bases or intact G4 single- stranded DNA circles. Donor DNA was incubated with SSB at 80% site saturation in 0.15M NaCl, 0.01M TrisHCl pH 7.6, 0.001M EDTA for 20 minutes at 25 degrees C. An equal concentration of single-stranded DNA recipient (either sonicated calf thymus DNA or G4 DNA) was added and incubated for 30 minutes at 25 degrees C. The final nucleotide concentration was 9×10^{-6}M. As a control, SSB was incubated only with G4 circles at 40% site saturation using the same conditions. Cytochrome C was added to both reaction mixtures to a

final concentration of 0.1mg/ml. Each mixture was spread on a 0.2M ammonium acetate aqueous hypophase using the aqueous technique described by Davis et al., (1971). Photographs of uranylacetate stained preparations were taken with an AEI EM801 at a magnification of 6300x, and further enlarged during printing.

PROTEIN TRANSFER EXPERIMENTS ASSAYED BY NITROCELLULOSE FILTER BINDING

A 375 base pair fragment of ^{3}H-labelled pBR 322 DNA resulting from digestion with both EcoR1 and BamH1 restriction endonucleases (New England Biolabs) was isolated and used for the donor strands for all transfer experiments unless otherwise specified. SSB was added to the denatured donor DNA to produce 80% coverage corresponding to a protein to DNA weight ratio of almost 8:1. All buffers and concentrations are given in Results. Each donor DNA-SSB mixture was incubated at 20 degrees C for 15 minutes and then equilibrated in a 37 degree C water bath. A donor DNA-SSB sample was mixed by vortexing with an equal volume and concentration of a recipient single-

stranded DNA sample at 37 degrees C. Aliquots were removed with very accurate siliconized glass capillary tubes as a function of time to detect transfer of SSB from the ^{3}H-labelled donor strands. Nitrocellulose filtration was sufficiently rapid compared to the transfer reaction to serve to terminate the SSB protein transfer reaction.

Millipore nitrocellulose filters (type HA, 0.45uM, HAWP 024-00) were soaked in 0.01M TrisHCl pH 7.6, 0.001M EDTA, and rinsed with the same buffer in a Hoefer Scientific stainless steel filtration assembly equipped with a vacuum regulator to assure a flow rate of 2 ml/min. After application of a sample, filters were again rinsed with 2 ml of the same buffer before drying. The dried filters were counted in a high efficiency toluene based scintillation mixture (Fisher Scientific) in a Beckman LS9000 scintillation counter.

PROTEIN TRANSFER EXPERIMENTS ASSAYED BY SUCROSE GRADIENT SEDIMENTATION

Linear sucrose gradients between 10% to 45% sucrose were prepared in 0.015M NaCl, 0.01M TrisHCl pH 7.7, 0.0002M EDTA,

and layered over a 0.5 ml 60% sucrose cushion in 5-ml SW-50 polyallomar centrifuge tubes.

Radioiodinated SSB was added to single-stranded DNA circles of either bacteriophage ØX174 or G4 and incubated at 25 degrees C for 20 minutes. All buffers, concentrations and the extents of SSB coverage of DNA circles are given in Results. A donor DNA-SSB sample was mixed with highly sonicated, denatured calf-thymus DNAs of approximately 400 nucleotides long. Mixtures were incubated at 25 degrees C for one hour. Transfer experiments were then made 5% in sucrose and layered onto sucrose gradients. Centrifugation was for 14 hours at 39,000 rpm at 4 degrees C in a SW-50 rotor. Fractions of 0.4 ml were collected by dripping the tubes into gamma-counting vials, and counted in a Beckman Biogamma gamma-counter.

ALKALINE AGAROSE GEL ELECTROPHORESIS

Alkaline agarose gels were prepared and used according to the method of McDonnel et al. (1977). Agarose (SeaKem or BRL, ultrapure) was prepared in 0.03M NaCl, 0.002M EDTA. The standard alkaline buffer was 0.03N NaOH, 0.002M EDTA. All gel

electrophoresis experiments were performed at 4 degrees C. All gels were pre-run for at least 30 minutes before loading DNA samples, in order to convert the gels to alkaline (pH 12.5) conditions. The buffer was recirculated at approximately 15 ml/hr. Prior to loading, all DNA samples were ethanol precipitated, dried and resuspended in 0.1N NaOH plus 0.002M EDTA. This procedure served to denature all DNA samples not previously denatured, and was found to be superior to simply making the existing sample volume alkaline by the addition of NaOH. Samples were made 10% in glycerin and 0.0025% in bromocresol green. Bromocresol green has the same relative electrophoretic mobility as bromophenol blue under these conditions and does not bleach in alkaline pH. Electrophoresis was carried out overnight at 25 volts.

AGAROSE GEL FLUOROGRAPHY

Alkaline agarose gels were soaked in Enhance (New England Nuclear) with gentle shaking for 3-4 hours so that the gels were cleared of any white precipitate for at least one hour. The Enhance was removed and enough distilled water added to

completely cover the gel. The gel was soaked for 2-3 hours with gentle shaking, ensuring that an even white precipitate had been formed for at least one hour. The gel was then removed to a piece of filter paper, covered with plastic film wrap and dried at a moderate heat setting in a commercial gel dryer (BioRad Labs). The plastic wrap was removed before film exposure. Fluorograms were made using Kodak XAR-5 high speed X-ray film. All films were exposed for 24-36 hours at -70 degrees C. All films were developed for a full 5 minutes as recommended by the manufacturer.

DNA-LOOP CUTTING EXPERIMENTS USING PBR322 PLASMID DNA

^{3}H-(T) labelled pBR322 was prepared as described, to a specific activity of approximately 100,000 cpm/ug. Plasmid DNAs were linearized by EcoR1 restriction digestion, isolated by agarose gel elctrophoresis with ethidium bromide staining, and purified by a variety of techniques as previously described.

DNAs were denatured by the addition of NaOH to 0.1N and incubated at 25 degrees C for 10 minutes, followed by chilling

on ice and the addition of cold TrizmaHCl to 0.3M. DNAs were then made 0.3M in sodium acetate, ethanol precipitated, washed, dried and resuspended in either S1 or DNase 1 buffer and incubated for at least 30 minutes before the addition of SSB. The salt and buffer conditions are given in Results. SSB was added to the DNA and incubated for at least 30 minutes before addition of either S1 or DNase 1. The site coverage, temperature conditions and nuclease concentrations are given in Results. Reactions were stopped by addition of EDTA to 0.01M. Samples were made 0.3M in sodium acetate, ethanol precipitated, washed and resuspended. In general, 20,000 to 50,000 cpm per slot were loaded onto 1% agarose gels. This was sufficient to expose a film quite strongly in a 24 hour period at -70 degrees C. Marker DNAs consisted of ^{3}H-pBR322 and RsaI or HincII restriction digests of plasmid DNA.

5' END LABELLING OF DNA FRAGMENTS

All reactions were performed in 1.5 ml polypropylene tubes. DNA fragments were ethanol precipitated, dried, and resuspended in 40 ml of 0.05M Tris HCl pH 8.2. Approximately

2 units of calf-alkaline phosphatase (Boehringer-Mannheim) were added and incubated for 30 minutes at 45 degrees C (Efstradiadis et al., 1977). Then 0.03M sodium acetate was added to a final volume of 200 ul, and the DNA was extracted twice with phenol:chloroform (1:1 v/v) using distilled phenol buffered with 0.05M sodium borate. The DNA was ethanol precipitated, dried, and resuspended in 35 ul water plus 1 ul of 0.001M EDTA and submerged in a boiling water bath for 1 minute followed by ice quenching. The DNA was then transferred to a tube containing lyophilized gamma-^{32}P-ATP, plus 1 ul of 0.01M spermidine, 5ul of 10x kinase buffer (10x= 0.1M $MgCl_2$, 0.5M Tris HCl pH 8.8, 0.05M DTT), 6 ul water, and 10 units of polynucleotide kinase (New England Biolabs) and incubated at 37 degrees C for 1 hour. This is a modification of the technique of Chaconas and van de Sande (1980). In general, the amount of label used for end-labelling was approximately 20uCi per picomole of ends available to react.

Unincorporated label was eliminated by column chromatography. Disposable plastic columns (0.5cm x 10cm, Kontes) were poured with Sephadex G-100 (Pharmacia) in 0.1M NaCl, 0.01M Tris HCl pH 7.6, 0.001M EDTA. Columns were permitted to flow by gravity and 0.5ml fractions were collected. DNA fragments were usually several fractions ahead of the label in the elution profile. Cerenkov counting was used to approximate the specific activity. Samples prepared for gels were often etha-

nol precipitated with small carrier DNA, dried, and resuspended before loading onto alkaline agarose gels.

RESULTS

1). PURIFICATION OF SSB

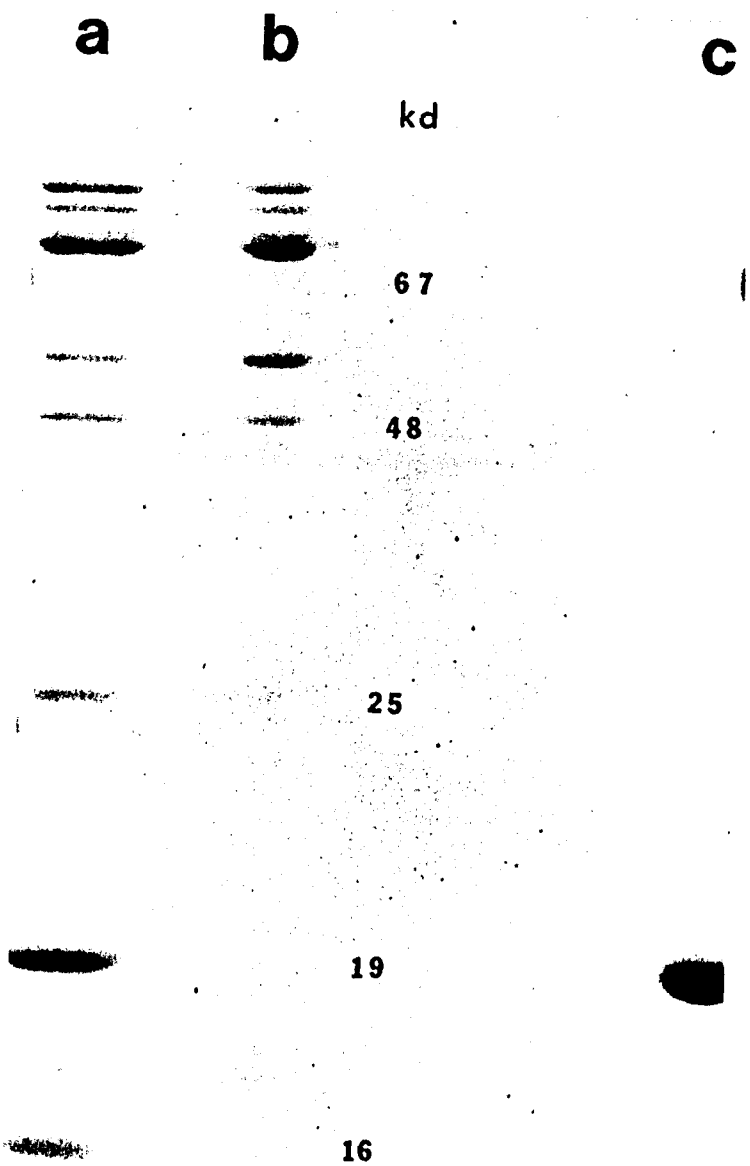
The SSB was purified by chromotography on single-stranded DNA cellulose and Blue-sepharose CL6B. The SSB protein was removed from the DNA-cellulose with 2M NaCl, along with a number of other contaminating proteins. The ability of the purified SSB to remain tightly bound to ssDNA-cellulose at NaCl concentrations in excess of 0.5M is in keeping with the known physical properties of previously isolated *E. coli* SSB (Sigal et al., 1972).

Figure 3 is a 7% to 13% gradient denaturing SDS- polyacrylamide gel stained with Coomasie brilliant blue and destained with a mixture of 7% glacial acetic acid and 10% methanol in water. Lane A contains all of the proteins removed from DNA-cellulose in the 2M NaCl wash. The SSB polypeptide is visible as an enriched band at about 19,000 daltons. The SSB was purified to homogeneity by the use of Blue-sepharose. The 2M NaCl wash from DNA-cellulose (lane A) was dialyzed into 0.2M NaCl buffer and pumped through a Blue-sepharose column.

FIGURE 3

Purification and polypeptide molecular weight of SSB.

Purification and polypeptide molecular weight of SSB. Denaturing SDS-polyacrylamide gel. DNA-cellulose 2M NaCl wash (lane A). Blue-sepharose CL6B 2M NaCl wash (lane B). 50 ug of purified SSB (lane C). Standard protein markers (not shown) were used to determine molecular weight.


a
b
c
kd
67
48
25
19
16

A 2M NaCl wash of the column removed all of the proteins that bound the matrix except SSB (lane B). A few other bands not present in lane B, the 16,000 and 32,000 dalton bands, probably did not bind the Blue-sepharose at the loading conditions used.

The SSB was eluted from the column with 4M guanidine HCl, which denatures the protein. The ability to withstand treatment with guanidine HCl is consistent with the ability of this protein to withstand denaturation by boiling (Weiner et al., 1975). The protein could not be removed by elution with 0.5M phosphate buffer pH 7.5, or 0.05M sodium acetate buffer pH 4.2. A Blue dextran column has been used by Meyer et al. (1979) for SSB purification. The elution properties differ from those found using Blue-sepharose CL6B; apparently it is more difficult to remove SSB from Blue-sepharose than from blue dextran. The guanidine HCl was removed by exhaustive dialysis at 4 degrees C. Lane C contains approximately 50 ug of the purified protein, which has an apparent polypeptide molecular weight of 19,000 daltons. This polypeptide molecular weight is in good agreement with the molecular weight values reported from other isolations (Sigal et al., 1972; Molineux et al., 1974; Weiner et al., 1974; Ruyechan and Wetmur,

1975), and with the calculated molecular weight from sequence data of E. coli K12 SSB (Sancar et al., 1981). It was judged that the SSB was greater than 95% pure. From 250 grams of cell paste, approximately 3 mg of homogeneous SSB was purified.

DETERMINATION OF SSB NATIVE MOLECULAR WEIGHT

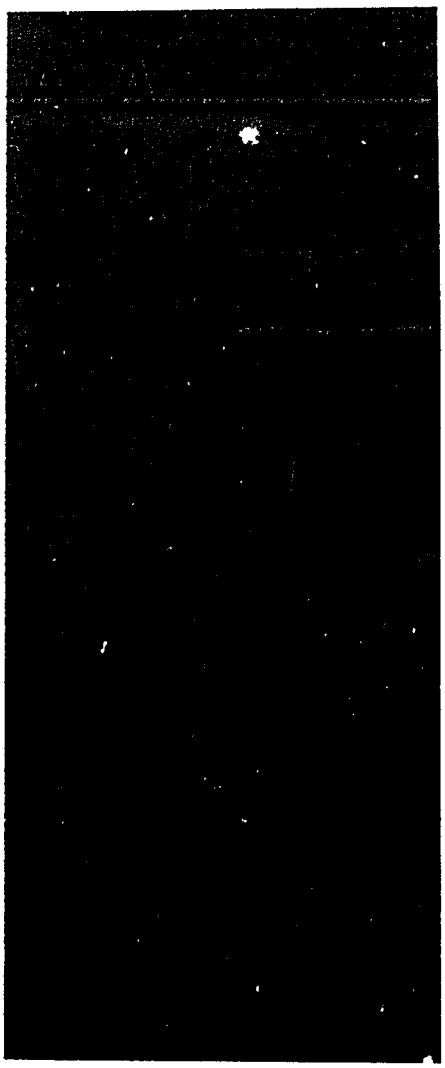
The native molecular weight of the purified SSB was estimated in two ways. One way was by the use of gel filtration chromotography of ^{125}I-labelled SSB on Sephadex G-100. The elution profile of SSB was compared with the elution profiles of standard proteins. A native molecular weight between 70,000 to 80,000 daltons was estimated by this technique.

The native molecular weight was also estimated by polyacrylamide gel electrophoresis at non-reducing conditions in which the addition of 2-mercaptoethanol and the boiling step to prepare the samples were eliminated (figure 4). SSB does not contain any cysteine residues, hence the addition of 2-mercaptoethanol is not necessary for denaturation. The molecular weight was determined from a standard set of protein

FIGURE 4

Native molecular weight of SSB.

Native molecular weight of SSB. Non-denaturing SDS- polyacrylamide gel containing native (78,000 d) and some polypeptide (19,000 d) SSB. Protein markers (not shown) were used to estimate molecular weight.



markers. There is a predominant band at about 75,000 to 80,000 daltons molecular weight, and a fainter minor band at 19,000 to 20,000 daltons. The 19,000 dalton band probably represents partial denaturation of SSB tetramers under the loading conditions used, and the 75,000 to 80,000 dalton band is in good agreement with the previously published native molecular weight determination (Weiner et al., 1975).

DETERMINATION OF SSB CONCENTRATION AND ACTIVITY

The concentration of purified SSB was determined in several ways. The previously determined molar extinction coefficient of the protein at 280 nm may be used (Ruyechan and Wetmur, 1976). An absorbance at 280 nm equal to 1 represents 660 ug/ml of SSB protein. Another method used was the protein concentration determination of Lowry et al. (1951), in which BSA was used for generating the standard curve.

In general, the most reliable method for determination of both SSB concentration and activity was the nuclease protection assay first developed by Ruyechan and Wetmur (1975). The assay when performed as described previously is extremely accurate and reproducible. Only active SSB is measured in this assay. Consequently, loss of activity or the extent of purity can be easily determined by comparison with the Lowry protein determination. Comparison of the nuclease protection assay with the Lowry assay indicated that all of the protein isolated was fully active in the nuclease protection assay. It has been shown previously that the nuclease protection assay responds linearly to increasing SSB concentration up to approximately 98% saturation by SSB of available DNA sites (Ruyechan and Wetmur, 1975). A binding stoichiometry of 8:1 weight/weight (protein:DNA) was found to completely protect ^{125}I labelled DNA from nucleolytic degradation. This agrees with the published binding stoichiometry determinations for SSB (Sigal et al., 1972; Weiner et al., 1975; Ruyechan and Wetmur, 1976).

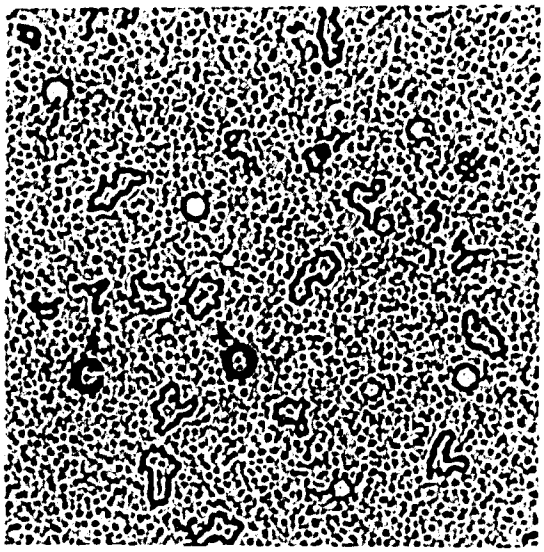
PURIFIED SSB EXTENDS SINGLE-STRANDED DNA CIRCLES

The purified SSB was shown to be able to extend G4 single-stranded DNA circles to the extent previously published (Sigal et al., 1972). G4 viral DNA circles may be prepared for visualization in the electron microscope by aqueous procedures only if complexed to SSB. Uncomplexed single-stranded molecules collapse at identical conditions. G4 DNA circles were complexed with SSB at approximately 40% saturation (figure 5). Circles appear mostly as fully open or closed structures with only a small proportion of molecules partially open. This is evidence for the cooperative binding of SSB; circles are generally fully covered and extended or not covered and collapsed. ØX174 double-stranded replicative form (RF) DNA was linearized by a single restriction cut and added to the reactions to serve as a reference for the determination of the contour length of G4-SSB complexes. The DNA molecules were photographed, the negatives projected and the molecules traced on a blackboard. The published values of 5577 nucleotides for G4 DNA (Godson et al., 1978) and 5762 nucleotides for ØX174 DNA (Sanger et al., 1978) were used to determine the reduction in contour length of G4-SSB complexes. A reduction in contour length of approximately 40% was found, which is in good agreement with the value of 35% published by Sigal et al., 1972). This corresponds to a reduction in base-spacing from 3.4 Angstroms to approximately 1.8 Angstroms.

FIGURE 5

Electron Micrographs of complexes of SSB and G4 single-stranded DNA circles.

Electron Micrographs of complexes of SSB and G4 single-stranded DNA circles. G4 DNA circles complexed with SSB at 40% site saturation, spread on 0.2M ammonium acetate aqueous hypophase. Open and closed circles are indicated. Magnification x16,000.



2). DETECTION OF SSB-SINGLE STRANDED DNA COMPLEXES BY NITROCELLULOSE FILTRATION

A nitrocellulose filter binding assay was used to detect single-stranded DNA-SSB complexes to determine SSB transfer rates between single-stranded DNA molecules. In this assay, single-stranded DNA only binds to the filters when complexed with SSB. Uncomplexed single- stranded DNA is not retained by the filters and is washed through. Similar assays have been used by Oey and Knippers (1972) to study the binding of bacteriophage fd gene 5 SSB to single-stranded DNA, by Weiner et al. (1975) to determine the stoichiometry of *E. coli* SSB binding to single-stranded DNA, and by Fowlkes et al. (1979) to study the binding of adenovirus type 2 DNA binding protein (DBP) to adenovirus DNA. Fowlkes et al. (1979) found that full length adenovirus DNA (3.6×10^4 base- pairs) was retained on the nitrocellulose filters when complexed with as

little as one adenovirus DBP of 72,000 daltons molecular weight.

The assay was calibrated using a denatured 375 nucleotide long EcoR1 plus BamH1 restriction fragment of pBR322 ^{3}H-labelled DNA. When SSB was added to the DNA, the amount of ^{3}H-DNA fragment retained on the filters was proportional to the saturation of sites on the DNA as determined by the nuclease protection assay. This is shown in table I. Addition of enough SSB to cover 80% of the ^{3}H-DNA fragment sites as determined by nuclease protection resulted in retention of 690 cpm. Reduction in SSB to 60% site saturation by nuclease protection resulted in the retention of 550 cpm, or 64% of the ^{3}H-DNA fragments. Uncomplexed single-stranded ^{3}H-DNA does not bind the filter at any ionic strength tested between 0.002M to 0.4M NaCl.

The amount of ^{3}H-DNA retained on nitrocellulose filters is proportional to the extent to which it is covered by SSB. The linear relationship between the nuclease protection assay and the filter binding assay provides additional evidence for cooperativity and is a prerequisite for the use of nitrocellulose filters to accurately detect SSB-^{3}H-DNA complexes.

The low background, linearity and stoichiometry of the assay were confirmed in 0.003, 0.15 and 0.3M NaCl plus 0.01M Tris HCl pH 7.8, 0.001M EDTA, and in buffers containing gly-

TABLE 1

Calibration of Nitrocellulose Filters.

Ionic Strength Dependence for Filter Retention of SSB-^{3}HDNA Complexes

NaCl Concentration (M)	cpm Retained on Filters
0.003 M	600 cpm ± 20
0.03 M	620 cpm ± 20
0.15 M	620 cpm ± 20
0.3 M	615 cpm ± 20

cerol. Table II contains representative data for the retention on nitrocellulose filters of ^{3}H-DNA fragment-SSB complexes at 80% site saturation in 50% glycerol buffer at various ionic strengths. The amount of ^{3}H-DNA retained on filters was constant within the range of ionic strengths used in the experiments presented. The assay was also found to be independent of DNA-SSB complex concentration over a 4-fold range around the concentration of 4×10^{-7}M nucleotides, which was used for the experiments presented.

TABLE 2

Effect of NaCl on Filter Retention of SSB-DNA Complexes.

Comparison of Nuclease Protection Assay with Filter Binding Assay

Site Saturation by Nuclease Protection Assay	cpm Retained on Filters[@]
80%	690 ± 20
60%	550 ± 20
+ 0%	25 ± 20

@ At 25°C in 0.15 M NaCl, 0.01 M TrisHClpH 7.6, 0.001 M EDTA

+ Tested between 0.003 M to 0.3 M NaCl

3). REVERSIBLE SSB TRANSFER REACTIONS: AN ALL-OR-NONE MECHANISM

REACTION KINETICS

When a labelled DNA-SSB complex is mixed with an equal amount of unlabelled DNA of the same length, SSB proteins may exchange until the SSB is equally partitioned between the labelled and unlabelled DNAs. The progress of the reaction may be followed by detecting labelled DNA-SSB complexes by filter binding at various times after mixing. The reverse reaction may be followed by first mixing the SSB with unlabelled DNA. If the only significant species in solution at all times are free DNA and fully complexed DNA, these reactions may be said to follow an all-or-none mechanism.

The exchange of SSB may be represented by the following reaction

$$\text{D-SSB} + \text{R} \underset{k_2}{\overset{k_2}{\rightleftharpoons}} \text{D} + \text{R-SSB} \qquad (1)$$

where D and R represent donor and recipient strands and k_2 is the rate constant for transfer of all of the SSB molecules from one strand to another.

If the DNA lengths are the same, then

k_2 (forward) = k_2 (reverse) = k_2

The rate equation for this reaction is then

$$\frac{-d}{dt}\,\text{D-SSB} = k_2\,(\text{D-SSB})\,(\text{R}) - k_2\,(\text{D})\,(\text{R-SSB}) \qquad (2)$$

Let Δ be the absolute value of the difference between the labelled DNA-SSB complex bound to a filter at time t and the labelled DNA-SSB complex bound at infinite time (equilibrium).

Then

$$\frac{-d}{dt} \ln \Delta = k_1 = 2k_2 \, Co \qquad (3)$$

where k_1 is the apparent first order rate constant.
A first order reaction is one in which the reaction rate is proportional to the concentration of only one reacting species. When the rate of the forward and reverse reactions is the same, and each species is present in stoichiometric concentrations (that is, the concentration of reactants and products is the same), then the reaction between two species may appear first order and may be described by an apparent first order rate constant.

In the reaction described above, the reverse reaction will give the same rate constant as the forward reaction. If an all-or-none mechanism is not followed, the prediction of identical first order reactions in both directions would not be expected to be fulfilled. Partially complexed DNA with SSB would bind to a nitrocellulose filter, which would result in an apparent increase in the reverse reaction rate and the loss of a significant forward reaction rate.
Δ is easily represented as change in cpm, (the amount of ^{3}H-DNA retained on a filter at any time t minus the final value at equilibrium). The slope of the rate plot gives k_1, the apparent first order rate constant for the reaction. The

half-life of the reaction, $t_{1/2}$, is a useful term that describes the time required for Δ to drop to one-half its initial value. It is defined by

$$t_{1/2} = \ln 2/k_1 \qquad (4)$$

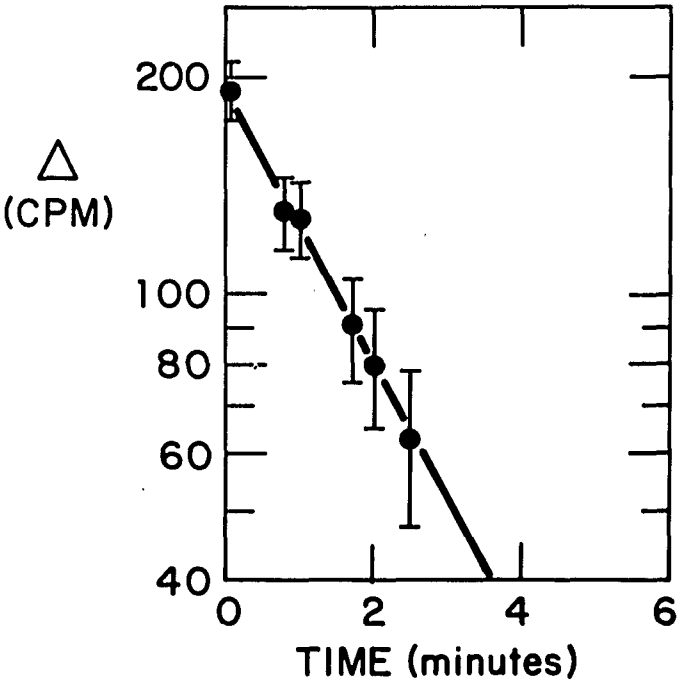
PROTEIN TRANSFER EXPERIMENTS

A 375 base-pair fragment of ^{3}H-labelled pBR322 DNA resulting from digestion with both EcoR1 and BamH1 restriction endonucleases was isolated and used as the donor strands for all transfer experiments unless otherwise specified. This DNA was used to test the all-or-none hypothesis. SSB was added to the denatured donor DNA to produce 80% coverage corresponding to a protein to DNA weight ratio of almost 8:1. This DNA can bind 11-12 SSB proteins at about 32 nucleotides per tetramer. The competing unlabelled DNA was extensively sonicated, denatured calf-thymus DNA which had an average length of 400 nucleotides as determined by gel electrophoresis. Each donor DNA-SSB mixture was incubated at 20 degrees C for 15 minutes and then equilibrated in a 37 degree C water bath. A donor

FIGURE 6

A first-order,all-or-none rate plot for the forward reaction.

A first-order, all-or-none rate plot for the forward reaction. Donor DNA is denatured 375 nucleotide ^{3}H-labelled DNA, 80% site saturated with SSB. Recipient DNA is sonicated, denatured 400 nucleotide (average) calf thymus DNA. Final nucleotide concentration is 4×10^{-7}M. Reactions were at 37 degrees C in 0.15M NaCl, 0.01M Tris HCl pH 7.6, 0.001M EDTA, 50% glycerol.


200
100
80
60
40
Δ
(CPM)
0
2
4
6
TIME (minutes)

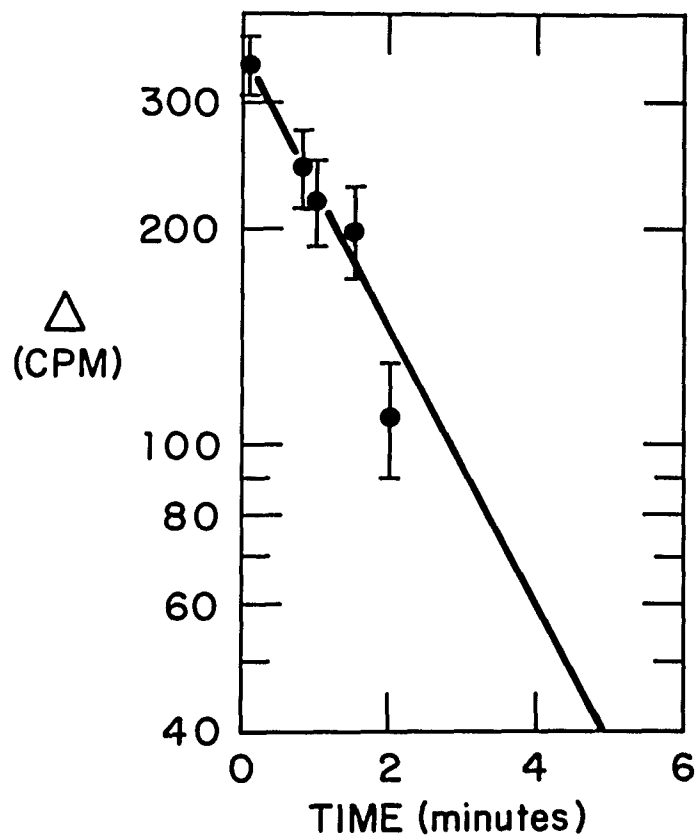
DNA-SSB sample was mixed by vortexing with an equal volume and concentration of a recipient single-stranded DNA sample at 37 degrees C. For the reverse experiment, SSB was complexed to single-stranded calf-thymus DNA at 80% site saturation prior to addition of an equal concentration of competing ^{3}H-labelled DNA. The solvents were 0.15M NaCl, 50% glycerol, 0.01M Tris HCl pH 7.6, 0.001M EDTA at 37 degrees C. The DNA fragments were present at $10^{-9}M$ (about $4x\ 10^{-7}M$ nucleotide) prior to mixing, in both sets of experiments described. Aliquots were removed as a function of time to detect transfer of SSB from ^{3}H-labelled donor strands. Nitrocellulose filtration was sufficiently rapid compared to the transfer reaction to serve to terminate the SSB protein transfer reaction. The reaction was followed until ^{3}H-binding results were constant at several widely spaced time points. First order rate profiles were observed for both reactions, as shown in figure 6 and in figure 7.

The reactions not only appear to be first order, but the amount of ^{3}H-DNA retained on the nitrocellulose filters at equilibrium accounts for approximately half of the ^{3}H-DNA retained at the start of the reaction. As given in table III, the forward and reverse rate constants were the same within

FIGURE 7

A first-order, all-or-none rate plot for the reverse reaction.

A first-order, all-or-none rate plot for the reverse reaction. Donor DNA is sonicated, denatured 400 nucleotide (average) calf thymus DNA, 80% site saturated with SSB. Recipient DNA is denatured 375 nucleotide DNA. Final nucleotide concentration is 4×10^{-7}M. Reactions were at 37 degrees C in 0.15M NaCl, 0.01M Tris HCl pH 7.6, 0.001M EDTA, 50% glycerol.


300
200
100
80
60
40
Δ
(CPM)
0
2
4
6
TIME (minutes)

experimental error. Consequently, the kinetics of the exchange reaction for SSB between short DNA molecules may be treated using an all-or-none model. Furthermore, the 375 nucleotide donor strands behave like random sequence calf thymus DNA.

4). VISCOSITY AND TEMPERATURE DEPENDENCE OF THE RATE OF SSB TRANSFER

The viscosity of the solvent may affect chemical reaction rates. Reactions that are affected by solvent viscosity, diffusion-controlled (limited) reactions, are ones in which a reaction occurs at each correct collision of reactants. These reactions generally have low activation energies and fast reaction rates. Common examples are free-radical reactions and ionic reactions. There are many reactions in which solvent viscosity has little or no effect on reaction rate. Examples include all chemical reactions that rearrange chemical bonds with a concommitantly large activation energy; the

TABLE 3

Reversibility of SSB Transfer Reactions.

REVERSIBILITY OF ssb TRANSFER REACTIONS

Donor DNA	Recipient DNA	$k_1(s^{-1})$	$t_{\frac{1}{2}}=\ln 2/k_1(s)$	$k_2(M^{-1}s^{-1})$
375 nucleotide ^3H DNA	400 nucleotide sheared DNA	$7.0\pm0.3\times10^{-3}$	98	7×10^6
400 nucleotide sheared DNA	375 nucleotide ^3H DNA	$8.0\pm0.3\times10^{-3}$	87	8×10^6

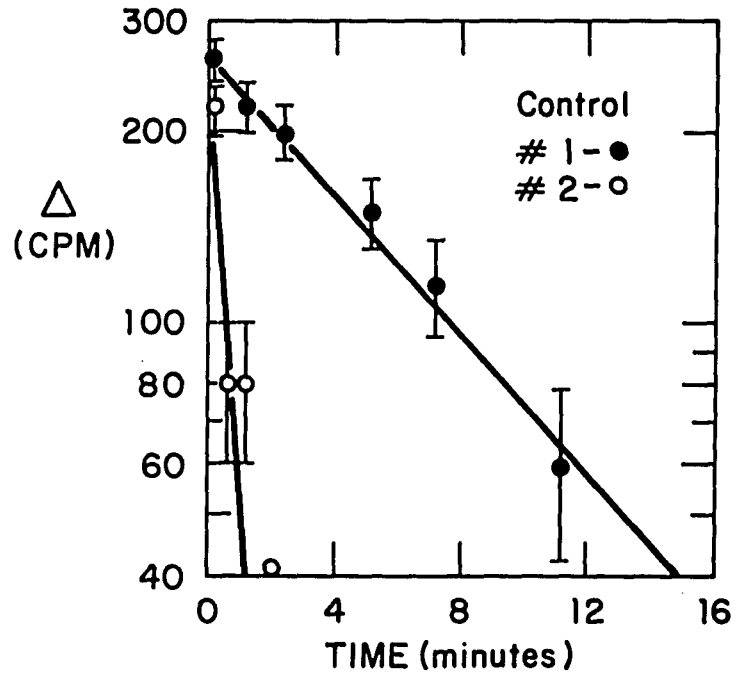
rate-controlling step is the capacity to rearrange chemical bonds and not the frequency of encounters of the reactants. Most enzymatic reactions are not affected by solvent viscosity. The turnover rate in enzymatic processes is generally so low that the rate-limiting step is not the frequency of encounters of the reactants.

Viscosity and temperature effects on SSB transfer were studied using as donor, ^{3}H-labelled 375 nucleotide DNA plus SSB at 80% site saturation. The recipient DNA was unlabelled sonicated calf-thymus DNA. Equal single-stranded concentrations ($10^{-9}M$ fragments) were employed. The solvent contained 0.15M NaCl, 0.01M Tris HCl pH 7.6, 0.001M EDTA plus various amounts of glycerol. The glycerol was added weight/volume and viscosities (η) are stated in centipoise (cp, from Wolf et al., 1976). Reactions were performed at 37 degrees except where noted. Data were obtained from first order rate profiles, as shown in figure 8. Increasing the viscosity had a marked effect on reaction rate. The reaction in 75.6% glycerol, 29 fold more viscous than water, was extremely slow. The effect of viscosity on the second order rate constant for SSB transfer is depicted in figure 9. Studies were performed in solutions with 2.7, 5.3, 11 and 29 times the viscosity of

FIGURE 8

A first-order rate plot for SSB transfer in 0% and 75.6% glycerol.

A first-order rate plot for SSB transfer in 0% and 75.6% glycerol. DNA concentrations and reaction conditions, except for glycerol concentrations, were the same as in figure 6. 0% glycerol (open circles); 75.6% glycerol (filled circles).


300
200
100
80
60
40
Δ
(CPM)
Control
1-●
2-o
0
4
8
12
16
TIME (minutes)

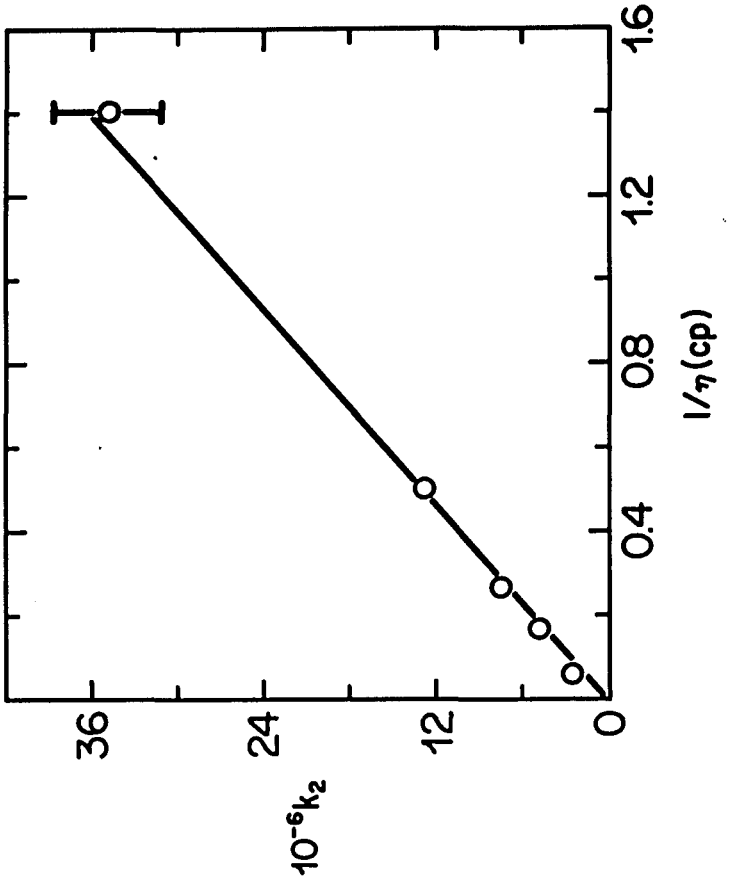
the solvent without glycerol. There is a reciprocal relationship between solution viscosity and the rate constant for SSB transfer. The inverse relationship is observed over the extremes of solution viscosity from 0 to 75.6% w/v glycerol.

The dependence of SSB transfer rates on temperature was tested comparing reaction rates at 37 degrees C and 15 degrees C. The rate constant was reduced about 1.4 fold at 15 degrees C. The decrease in solution viscosity from 37 degrees C to 15 degrees C is 1.6 fold. Thus, within experimental error, there appears to be no temperature dependence of the SSB transfer rate except that which is accounted for by the viscosity dependence of the reaction. The inverse linear relationship between solution viscosity and SSB transfer rate is consistent with the hypothesis that SSB exchange is a diffusion controlled reaction.

FIGURE 9

The effect of viscosity on the rate constant for transfer between 375 nucleotide donor and 400 nucleotide recipient strands.

The effect of viscosity on the rate constant for SSB transfer between ^{3}H-375 nucleotide donor and 400 nucleotide recipient strands. Donor DNA 80% site saturated with SSB. Final concentrations were 4 x 10^{-7}M nucleotides. Reactions were at 37 degrees C in 0.15M NaCl, 0.01M Tris HCl pH 7.6, 0.001M EDTA, and glycerol concentrations as indicated.


36
24
12
0
$10^{-6}k_2$
0.4
0.8
1.2
1.6
$1/\eta$(cp)

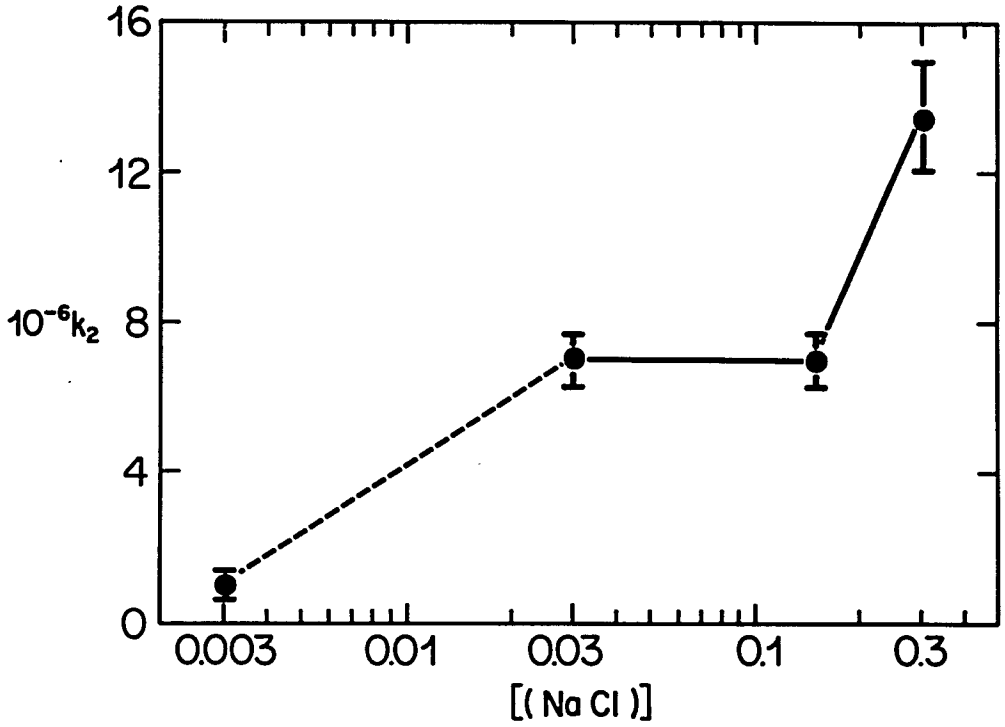
5). THE EFFECT OF IONIC STRENGTH ON THE RATE OF SSB TRANSFER

The effect of salt concentration on SSB transfer rates was studied with the same DNAs and SSB site saturation as in the viscosity experiments, and at 37 degrees C. The solvent contained 0.01M Tris HCl pH 7.6, 0.001M EDTA, 50% glycerol, and various concentrations of NaCl. Figure 10 shows the effect of NaCl concentration on the second order rate constant for SSB transfer. There is a broad plateau from 0.03 to 0.15M NaCl where the rate of SSB transfer is constant. The rate of SSB transfer increases 2-fold upon increasing the NaCl concentration from 0.15 to 0.3M. The plateau range for SSB transfer rates occurs in the same ionic strength region where the association constants for SSB binding to DNA are independent of NaCl concentration (Ruyechan and Wetmur, 1975; 1976). The 2-fold increased rate of transfer at 0.3M NaCl occurs in the same range in which the association constant for SSB binding to DNA was found to fall by half. It is not clear why the transfer rate in 0.003M NaCl is 7-fold lower than it is above 0.03M. The ^{3}H-DNA is still retained on the filters, which provides evidence that at 0.003M NaCl SSB still binds DNA. Further evidence for this can be found in the ability of SSB to protect DNA in the nuclease protection assay at very low

FIGURE 10

The effect of NaCl concentration on the rate constant for SSB transfer between strands.

The effect of NaCl concentration on the rate constant for SSB transfer between strands. The reaction conditions were the same as in figure 6. NaCl concentrations are indicated.


16
12
8
4
0
$10^{-6}k_2$
0.003
0.01
0.03
0.1
0.3
[(Na Cl)]

ionic strengths. Whether the protein binds DNA more strongly at very low salt concentrations is unknown. The dotted line in figure 10 is used to indicate the uncertainty concerning the actual lower salt limit of the plateau region.

6). THE EFFECT OF RECIPIENT DNA STRAND LENGTH OR CIRCULARITY ON THE RATE OF SSB TRANSFER

All of the studies of DNA strand length effects on SSB transfer rates were carried out at 37 degrees C in 0.15M NaCl, 0.01M Tris HCl pH 7.6, 0.001M EDTA, 50% glycerol. The donor DNA was ^{3}H-labelled 375 nucleotide DNA complexed with SSB at 80% site saturation. The DNA fragment concentration was 10^{-9}M before 2-fold dilution with the recipient DNA. The recipient DNA was present at the same site (nucleotide) concentration as the donor DNA.

The recipient single-stranded DNA molecules were either 400 nucleotides (average) calf thymus DNA, 2180 nucleotide AvaI plus PstI digest of pBR322, 4361 nucleotide EcoRI digest of pBR322, or circular 5577 nucleotide bacteriophage G4 DNA (Godson et al., 1978). All linear double-stranded restriction fragments of pBR322 were purified by agarose gel electrophoresis and elution before denaturation and use. The single-stranded circular DNA was purified by CsCl density gradient sedimentation. The various sizes were confirmed by observation of SSB-DNA complexes in the electron microscope. The G4 DNA was more than 90% circular.

First order, as well as second order rate constants based on the all-or-none model for SSB transfer reactions involving DNAs of different length or shape are given in table IV. These reactions proceed approximately 50% to completion, an equilibrium concentration required for the all-or-none model. Figure 11 is a first order rate profile for the 2180 nucleotide competing DNA fragment. Some small downward curvature was observed at the start of the rate plots with 4361 and 5577 nucleotide recipient DNAs, as shown in figure 12, a first order rate profile for transfer to the 4361 nucleotide DNA. This indicates that incomplete long DNA-SSB complexes might be better recipients than long DNA alone. The rate constants were calculated from the entire rate plot. The reported values include the range of systematic error which could have been introduced by fitting a first order reaction.

TABLE 4

Effect of DNA Length of SSB Transfer Reactions.

EFFECT OF DNA LENGTH OF ssb TRANSFER REACTIONS

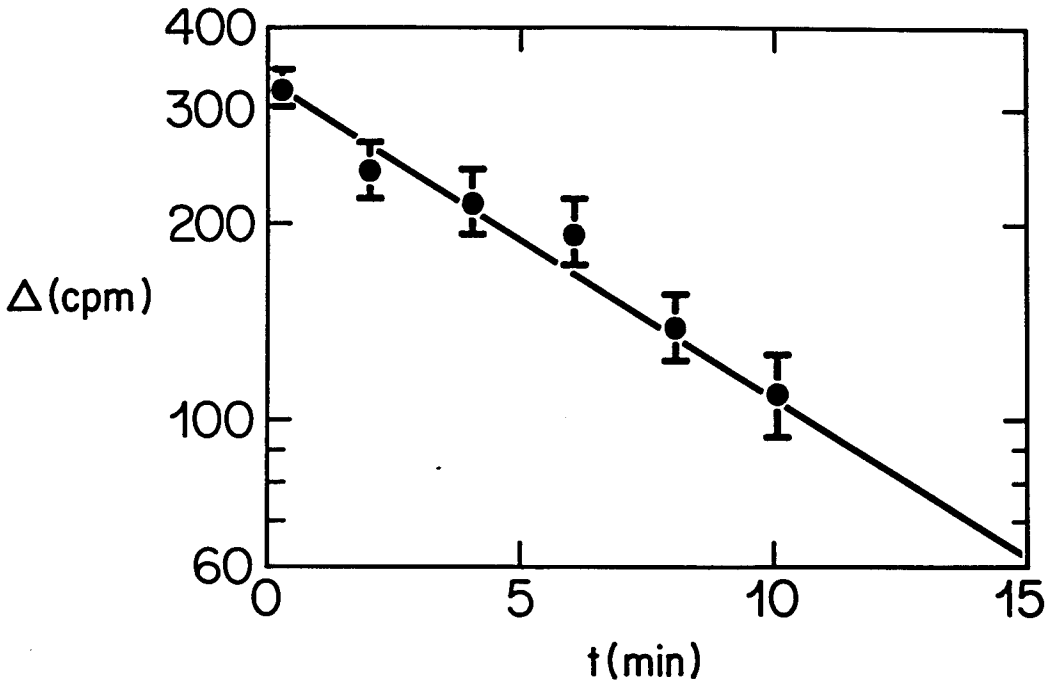
Length of Recipient	Recipient/Donor Length Ratio	NaCl (M) Concentration	$k_1(s^{-1})$	$k_2(M^{-1}s^{-1}$
400	1	0.15	$7.0\pm0.3\times10^{-3}$	7×10^{6}
		0.03	$7.0\pm0.3\times10^{-3}$	7×10^{6}
2180	6	0.15	$1.9\pm0.2\times10^{-3}$	1.9×10^{6}
4361	12	0.15	$1.0\pm0.15\times10^{-3}$	1.0×10^{6}
		0.03	$5.9\pm0.9\times10^{-4}$	5.9×10^{5}
5577 Circle	15	0.15	$1.3\pm0.2\times10^{-3}$	1.3×10^{6}

The first order rate constant for 400 nucleotide DNA is 7 x 10^{-3} sec^{-1}. It has already been observed that this result is independent of NaCl concentration in the range of 0.03 to 0.15M. When the recipient DNA is increased in length by 6-fold to 2180 nucleotides, the rate constant drops 3.5-fold. Further increase of the recipient DNA length to 12-fold (4361 nucleotides) leads to a total rate constant decrease of 7-fold, down to 1 x 10^6 M^{-1} s^{-1}. When this 4361 nucleotide DNA was used as a recipient in 0.03M NaCl, the total rate constant decrease was 12-fold. The rate of SSB exchange becomes somewhat dependent upon ionic strength in the 0.03 to 0.15M NaCl range when the recipient DNA becomes long. Finally, it was found that recipient circular DNA that is 5577 nucleotides long reacts as rapidly as linear DNA of 4361 nucleotides. This result eliminates any mechanism requiring nucleation of SSB-DNA complexes at the end of DNA molecules.

FIGURE 11

A first-order rate plot for SSB transfer from 375 nucleotide donor to 2180 nucleotide recipient.

A first-order rate plot for SSB transfer from 375 nucleotide donor to 2180 nucleotide recipient DNA. Reaction conditions were the same as in figure 6.


400
300
200
100
60
Δ(cpm)
0
5
10
15
t(min)

7). VISUALIZATION OF SSB TRANSFER BY ELECTRON MICROSCOPY

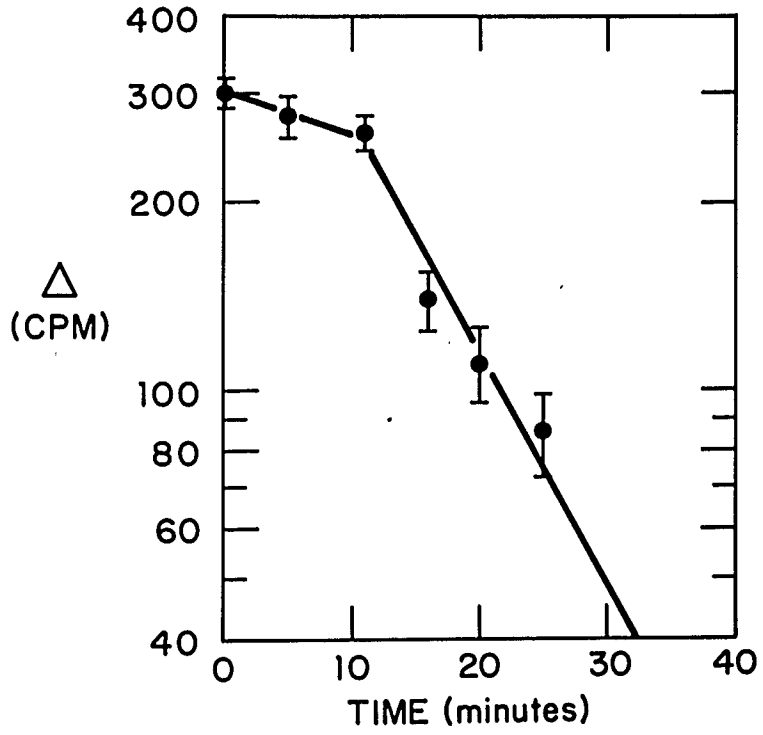
Circular single-stranded viral DNA complexed fully with SSB is easily distinguished from uncomplexed or partially complexed circles. Consequently, SSB transfer was studied using electron microscopy in addition to nitrocellulose filter binding. Electron microscopy offers additional information about the mechanism of SSB exchange, as will be described in the Discussion.

Complexes of single-stranded G4 circles incubated at 40% site saturation are shown in plate A of figure 13. Uncomplexed circles appear as folded and compact structures. Fully complexed circles appear round and large, whereas partially complexed circles appear folded or twisted with one or more extended loops. Uptake of SSB from solution by G4 DNA results predominantly in structures that are fully extended circles (plate A) indicating cooperative binding of protein throughout the entire length of the DNA. A statistical analysis of the types of molecules observed appears in Table V ,line 1. Collapsed circles are present in roughly an equal concentration, and only occasionally are partially complexed circles visible. When this solution is treated with endonuclease S1 or endonu-

FIGURE 12

A first-order rate plot for SSB transfer from 375 nucleotide donor to 4361 nucleotide recipient DNA.

A first-order rate plot for SSB transfer from 375 nucleotide donor to 4361 nucleotide recipient DNA. Reaction conditions were the same as in figure 6. There is downward curvature at the start of the rate plot.


400
300
200
Δ
(CPM)
100
80
60
40
0
10
20
30
40
TIME (minutes)

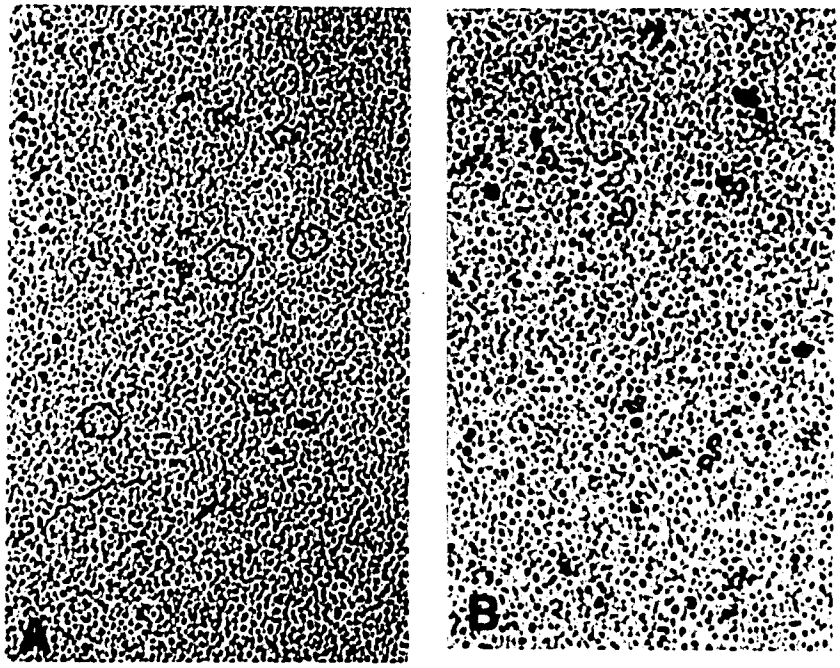
clease 1 before mounting, the extended DNA molecules remain full length.

Transfer experiments were performed in which a short 400 base DNA was incubated with SSB at 80% site saturation and then an equal concentration of G4 DNA circles was added. The final nucleotide concentration was 9 x 10^{-6}M. This is roughly 20 times more concentrated than the reactions assayed by nitrocellulose filter binding, ensuring that only equilibrium products would be visualized. The results of the transfer reactions are given in Table V, line 2. Most DNA structures found in transfer reactions were partially collapsed with extended loops as in figure 13 part B, or collapsed uncomplexed circles. Well extended circles were extremely rare, being present in only a few cases out of many hundreds of molecules photographed. Transfer reactions were also performed in 0.01M and 0.2M NaCl, as previously dscribed, and the same results were obtained. Finally, the reverse reaction with 80% site saturated G4 DNA circles plus 400 base recipient DNA was observed. The extended G4 DNA circles remained for the most part extended (Table V line 3). The lack of fully extended DNA circles in transfer reactions onto circles suggests that a mechanism other than uptake of free SSB from solution might be involved.

FIGURE 13

Electron micrographs of single-stranded G4 DNA circles at 40% coverage by SSB.

Electron micrographs of single-stranded G4 DNA circles at 40% coverage by SSB. Plate A: DNA-SSB complexes prepared by mixing SSB and G4 DNA. Plate B: DNA-SSB complexes prepared by transfer of SSB from 400 nucleotide DNA-SSB complexes to G4 DNA.


A
B

8). DETECTION OF SSB TRANSFER BY SUCROSE GRADIENT SEDIMENTATION

Sedimentation through sucrose gradients was used to study the transfer of radioiodinated SSB from bacteriophage single-stranded circular DNAs to small (approximately 400 nucleotide long) single-stranded calf thymus DNA fragments. All experiments were performed at 25 degrees C. Control experiments were performed in which only ^{125}I labelled SSB covered G4 circles, SSB covered 400 nucleotide DNA fragments, or SSB alone were centrifuged.

Curve 1 in figure 14 shows the position of SSB saturated G4 viral DNA circles in a 10% to 45% linear sucrose gradient. The fully complexed circles sediment fully to the bottom of the centrifuge tube. For this reason, a 60% sucrose cushion was later employed in the experiments. This explains the slight shift in the position of SSB-G4 complexes in curves 2 and 3, experiments in which a sucrose cushion was used.

TABLE 5

Electron Microscopic Analysis of SSB Transfer Reaction.

ELECTRON MICROSCOPIC ANALYSIS OF SSB TRANSFER REACTION

	FULLY OPEN CIRCLES	FULLY CLOSED CIRCLES	PARTIALLY* OPEN CIRCLES
CONTROL[a] (405 molecules scored)	45%	44%	11%
SSB TRANSFER TO G4 DNA[b] (260 molecules scored)	3%	61%	36%
SSB TRANSFER FROM G4 DNA[c] (350 molecules scored)	70%	24%	6%

[a] G4 ssDNA circles + SSB at 40% saturation in 0.15M NaCl.

[b] Sonicated DNA -SSB complex at 80% saturation + G4 DNA in 0.15M NaCl.

* Defined as less than one-half the contour length of fully open circles.

[c] G4 DNA - SSB complex at 80% saturation + sonicated DNA in 0.15M NaCl.

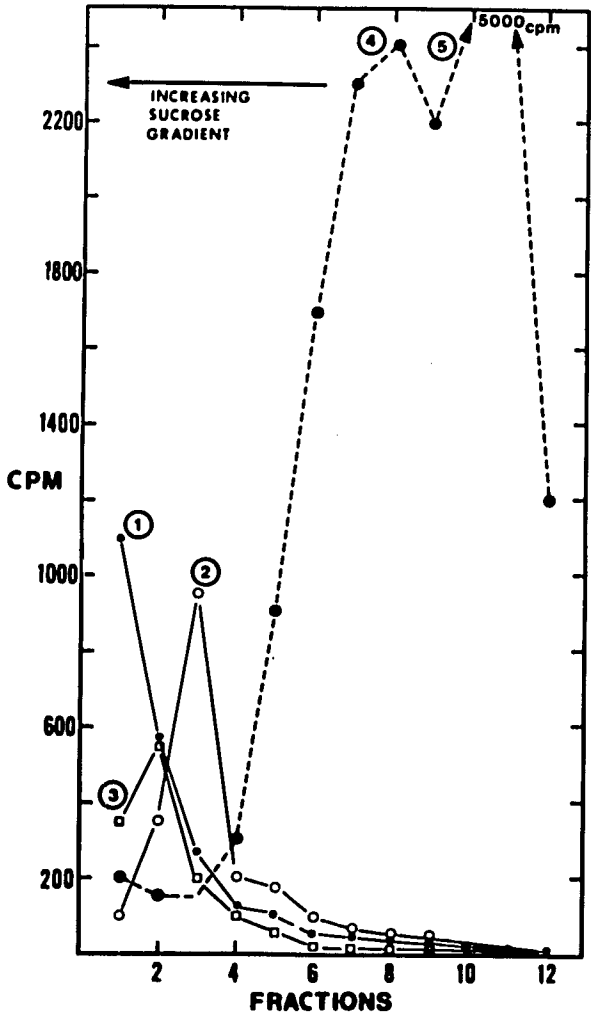
G4 viral DNA circles were complexed with SSB at approximately 75% saturation, in 0.015M NaCl, 0.01M Tris HCl pH 7.6, 0.002M EDTA at 25 degrees C for 30 minutes. The final viral DNA concentration was 4.6 x 10^{-9}M nucleotides. A small volume of short (400-long) sonicated, denatured calf thymus DNA fragments was added. The final concentration of short fragments was 6 x 10^{-8}M nucleotides (1.5 x 10^{-10}M fragments), approximately a 13-fold excess over the G4 DNA. The mixture was incubated at 25 degrees C for one hour, then centrifuged through a sucrose gradient. Curve 2 shows the results of the experiment. It is apparent that there is no exchange of ^{125}I-SSB from G4-SSB complexes to the short competing DNAs under these conditions, despite the long incubation time and excess competing DNA.

ØX174 viral DNA circles were complexed with ^{125}I-SSB (at a somewhat lower specific activity) to a level of 75% saturation in 0.2M NaCl, 0.01M Tris HCl pH 7.6, 0.002M EDTA at 25 degrees C for 30 minutes. The final viral DNA concentration was 5.5 x 10^{-8} M nucleotides, roughly 12-fold more concentrated than before. A small volume of short, sonicated, denatured calf thymus DNA fragments was added. The final concentration of short DNAs was 1 x 10^{-6}M nucleotides (2.5 x 10^{-9}M fragments), roughly a 20-fold excess over the viral DNA con-

FIGURE 14

Sedimentation analysis of SSB transfer from bacteriophage DNA circles to 400 nucleotide recipient strands.

Sedimentation analysis of SSB transfer from bacteriophage DNA circles to 400 nucleotide recipient strands. Sedimentation of ^{125}I-SSB bound DNA. Curve 1: DNA circles complexed with ^{125}I-SSB at 100% saturation and no competing DNA. Curve 2: DNA circles complexed with ^{125}I-SSB at 75% saturation plus 400 nucleotide recipient DNA. Reactions were at 25 degrees C in 0.015M NaCl, 0.01M Tris HCl pH 7.6, 0.002M EDTA for 30 minutes. Donor DNA circles were at 4.6 x 10^{-9}M nucleotides. Recipient fragments were at 6 x 10^{-8}M nucleotides. Curve 3: DNA circles complexed with ^{125}I-SSB at 75% saturation plus 400 nucleotide recipient DNA. Reactions were at 25 degrees C in 0.2M NaCl, 0.01M Tris HCl pH 7.6, 0.002M EDTA for 30 minutes. Donor DNA circles were at 5.5 x 10^{-8}M nucleotides. Recipient fragments were at 1 x 10^{-6}M nucleotides. Curve 4: 400-long DNA-SSB. Curve 5: SSB.


5000cpm
INCREASING
SUCROSE
GRADIENT
2200
1800
1400
CPM
1000
600
200
1
2
3
4
5
2
4
6
8
10
12
FRACTIONS

centration. This represents a mixture about 15-fold more concentrated than before. The mixture was incubated at 25 degrees C for one hour before centrifugation through a sucrose gradient. Curve 3 shows the results of this experiment. It is obvious that even under these higher salt conditions there was no detectable transfer of ^{125}I-labelled SSB from the SSB covered viral DNA circles to the competing short fragments. These results agree with those obtained using electron microscopy where transfer of SSB from preformed G4 DNA-SSB complexes to 400 base-pair oligomers failed to occur.

The position of ^{125}I-SSB covered 400-long fragments is shown by curve 4 in figure 14. SSB alone is shown in curve 5. Transfer of ^{125}I-SSB from the viral DNA circles would have been detected in these experiments, due to the large separation between the relative positions of SSB covered viral and short DNAs, and the low degree of background contamination. The slight shift in the peaks of curves 2 and 3 are taken to be normal variation resulting from experimental error inherent in the production and dripping of sucrose gradients.

9). SSB INTERACTION WITH STEM-LOOP STRUCTURES

Supercoiled pBR322 contains a region which serves as a substrate for single-strand specific endonuclease S1 (Lilly, 1980; Panayotatos and Wells, 1981). Precise restriction enzyme mapping has been used to locate the site to position 3061 +/- 5 base pairs on the pBR322 map. Analysis of the DNA sequence in this region reveals the presence of an inverted repeat which can be drawn into a stable stem-loop configuration consisting of an 11 base pair stem and a 3 nucleotide loop (Panayotatos and Wells, 1981). There is an absolute requirement for superhelicity in the recognition of this structure by S1. Linearized pBR322 no longer contains the S1 sensitive site. It is believed that the decrease in superhelix free energy upon forming a stem-loop structure more than compensates for the increase in free energy due to the formation of the stem-loop structure itself.

SSB, because of its highly cooperative binding, may melt-out minor stem-loop structures. There is not enough known about the interaction between SSB and DNA for an accurate determination to be made of the stability of larger stem-loop structures in the presence of SSB. The stem-loop structure present in supercoiled pBR322 DNA should also be present in the linearized and denatured plasmid. For this

reason, pBR322 DNA was chosen to study the interaction of SSB with DNA molecules containing a known stable stem-loop structure.

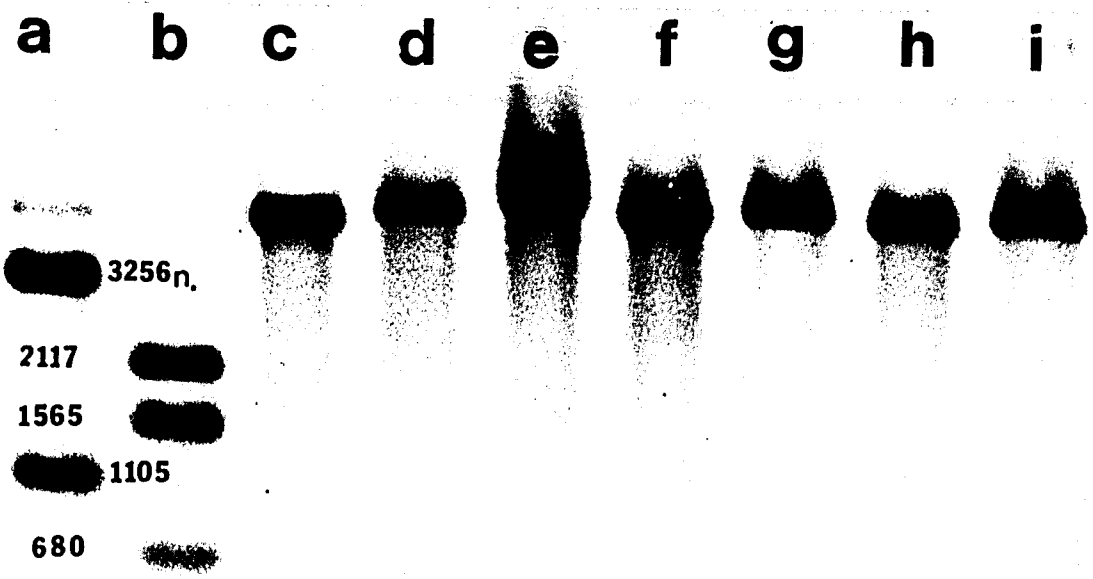
^{3}H-DNA was prepared, purified, linearized with EcoRl, and denatured as previously described. Single-cut single-stranded molecules were complexed with SSB at various site saturations in many different buffer conditions and subjected to digestion with either Sl or DNasel endonucleases. The solution was made 0.1N in NaOH to remove the protein and inactivate the enzymes, ethanol precipitated, resuspended and subjected to electrophoresis in 1% alkaline agarose gels. Gels were prepared for fluorography as previously described and fluorograms were made.

Figure 15 is a fluorogram of ^{3}H-pBR322 DNA complexed with SSB at 85% saturation and subjected to either Sl or DNasel degradation at either 4 degrees C, 20 degrees C or 37 degrees C, in 0.03M sodium acetate buffer pH 4.8 - 5.0 plus 0.008M $ZnCl_2$ (Sl) or $MgCl_2$ (DNasel). Digestions were carried out using 5 units of enzyme for each 0.1 ug of DNA for 30 minutes. In every case digestion products were identical to full length pBR322 DNA. Control experiments in which no SSB was added indicated that complete digestion took place. These results indicate that the proposed stem-loop structure, if present, was not cut by either enzyme with these reaction conditions.

FIGURE 15

Fluorogram of 1% alkaline agarose gel electrophoresis of S1 or DNaseI digested 85% SSB saturated pBR322 DNA.

Fluorogram of 1% alkaline agarose gel electrophoresis of S1 or DNaseI digested 85% SSB saturated pBR322 DNA. All digestions were performed with 5 units of enzyme per 0.1 ug DNA for 30 minutes in 0.03M sodium acetate buffer pH 4.8-5.0 plus either 0.008M $MgCl_2$ (DNaseI) or 0.008M $ZnCl_2$ (S1). Markers are HincII digest (lane A), RsaI digest (lane B), and EcoRI digest (lane C) of pBR322 DNA. S1 digest at 37 degrees C (lane D), 20 degrees C (lane F), and 4 degrees C (lane H). DNaseI digest at 37 degrees C (lane E), 20 degrees C (lane G), and 4 degrees C (lane I).


a
b
c
d
e
f
g
h
i
3256n.
2117
1565
1105
680

Other experiments were performed with S1 endonuclease in sodium acetate buffer pH 4.8 - 5.0 containing 0.1M or 0.2M NaCl, at 4 degrees C, 20 degrees C or 37 degrees C and at 65% SSB saturation. Digest products were again identical to full length pBR322 DNA. Lowering the pH in steps from 5.0 to 4.2 resulted in a gradual loss of material from the pBR322 DNA band, but without concommitant production of any other bands. Lowering the pH from 5.0 to 4.2 reduces the ability of SSB to bind DNA.

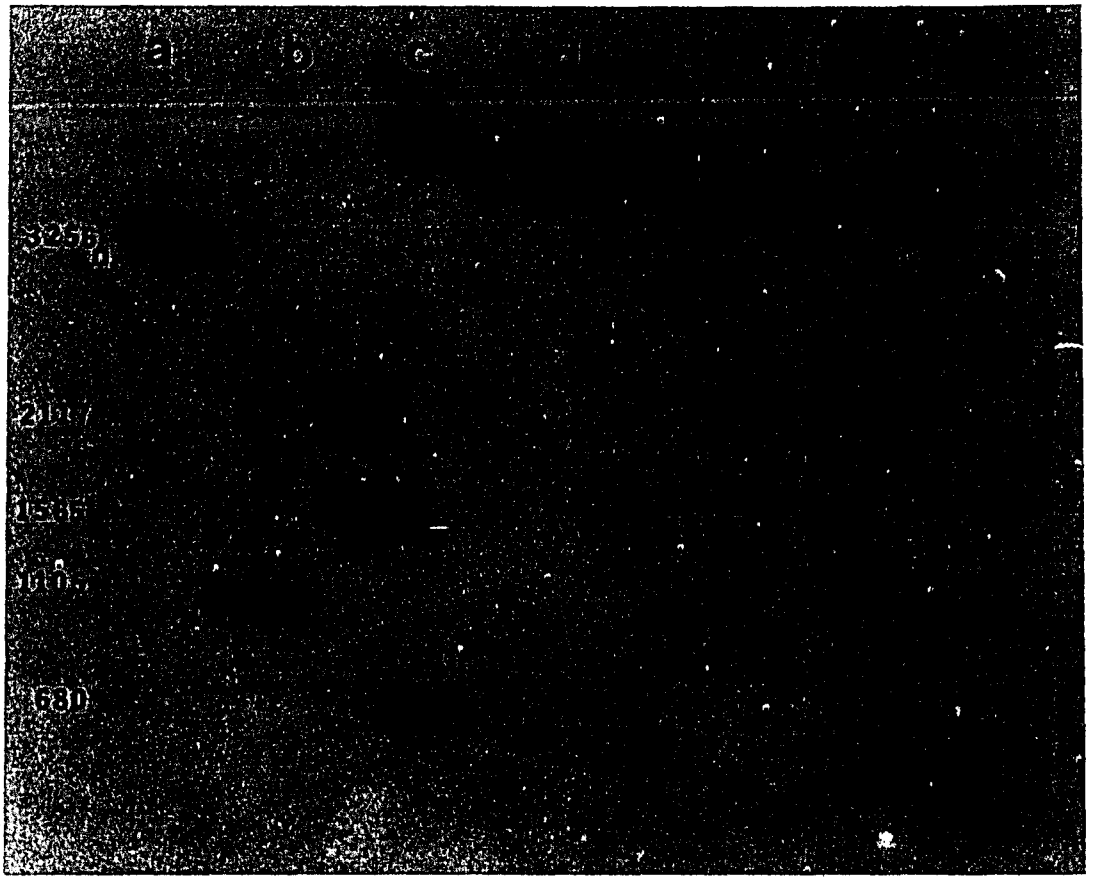
Complexes were prepared of pBR322 DNA with SSB at 75% saturation and subjected to DNaseI digestion in 0.01M Tris HCl pH 7.6 buffer containing 0.2M NaCl plus 0.008M $MgCl_2$ at 20 degrees C (figure 16). S1 endonuclease only functions at or below pH 5, whereas DNaseI is active at both low and physiological pH ranges. The digestion products were identical to full length pBR322 DNA. Control experiments in which SSB was not added resulted in complete digestion.

G4 single-stranded DNA circles (greater than 95% intact) were incubated with SSB at 75% site saturation in 0.15M NaCl, 0.01M Tris HCl pH 7.6, 0.0125M $MgCl_2$ at 37 degrees C for 30 minutes. The final DNA concentration was 2.5 ug/ml. Then 3ug

FIGURE 16

Fluorogram of 1% alkaline agarose gel electrophoresis of DNasel digested 75% SSB saturated pBR322 DNA.

Fluorogram of 1% alkaline agarose gel electrophoresis of DNasel digested 75% SSB saturated pBR322 DNA. Digestions were performed at 20 degrees C for 30 minutes with 5 units of enzyme per 0.1 ug DNA in 0.01M Tris HCl pH 7.6, 0.008M $MgCl_2$, and 0.2M NaCl. Markers are the same as in figure 15.DNasel digest of SSB-^{3}H-DNA (lane D).


a
b
c
d
3255
2807
1538
1010
680

of electrophoretically pure DNasel (Sigma) was added and the mixture incubated for 30 minutes at 37 degrees C. Digestion was halted by addition of EDTA to 0.03M and the mixture chilled on ice. A 25 ul aliquot was removed at 30 minutes, made 50 ug/ml in cytochrome c and prepared for visualization in the elctron microscope as previously described. The remaining DNA was end-labelled as described, and subjected to electrophoresis in 1% alkaline agarose gels. The gels were dried and autoradiograms were made.

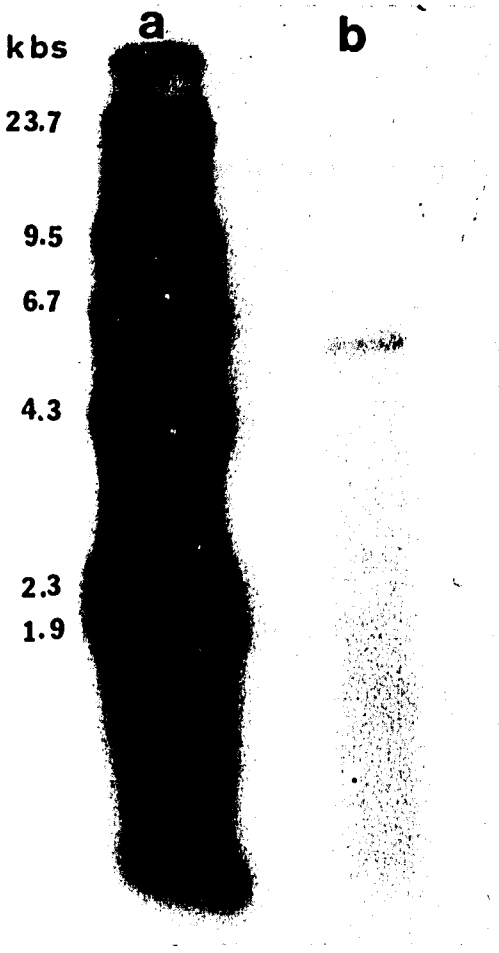
With the conditions used above, approximately 75% of the circular molecules were converted to linears, as determined by visualization in the electron microscope. The autoradiogram (figure 17) contains end-labelled Hindlll cut lambda DNA markers (lane A) and the end- labelled G4 DNA digestion products (lane B).

Although these results are preliminary, it can be seen that there is a single band present in the SSB protected G4 DNA experiment (lane B), roughly 6 kb in size. It remains to be seen whether circularity of the G4 genome is required for cleavge.

FIGURE 17

Autoradiogram of 1% alkaline agarose gel electrophoresis of DNasel digested 75% SSB saturated G4 DNA.

Autoradiogram of 1% alkaline agarose gel electrophoresis of DNasel digested 75% SSB saturated G4 DNA. Labelled markers consist of Hindlll cut lambda DNA (lane A). End labelled G4 digestion products (lane B). Reaction conditions are given in the text.


kbs
a
b
23.7
9.5
6.7
4.3
2.3
1.9

G4 DNA has been shown by sequence data to be 5577 nucleotides (Godson et al., 1978). These results would indicate that cutting occurred at a single site in the majority of the molecules. It remains to be demonstrated , however, whether the single species is a product of a unique cutting event or random nicking. The DNA 5' ends must be mapped to determine whether they are unique, and if so, the region must be identified. G4 complementary DNA synthesis commences at position 3997 nucleotides, within the 5a fragment of a Haelll digest. G4 viral strand DNA synthesis commences at position 507 nucleotides, within the 2b fragment of a Haelll digest. In both cases, a stable stem-loop structure may be drawn at the origin of DNA replication. It remains to be determined whether the cutting demonstrated in this experiment is unique, and if so, whether it may be mapped to either of the two DNA replication origins.

DISCUSSION

1). QUALIFICATIONS AND PREDICTIONS OF A DIRECT TRANSFER MECHANISM

There are two classes of DNA binding proteins. There are those that recognize specific sites and bind to a small number of DNA sequences. These are generally the regulatory proteins, such as repressor proteins, which have large association constants for DNA and bind quite strongly. The second class of proteins bind to DNA non-specifically, apparently without regard to nucleotide sequence, and exhibit a specificity only with respect to their ability to bind single-stranded or double-stranded DNAs. The single-strand DNA binding proteins (SSBs) exhibit an enormous affinity for single-stranded DNA. The enormous affinities that these proteins have for their respective substrates create special problems for their transfer from incorrect to correct sites, or from one set of correct sites to another.

There is no reason to believe that the mechanism by which initially free protein becomes bound must be, a priori, the

same mechanism by which it is transferred between sites. Direct transfer of binding proteins, should it exist, offers a faster mechanism than dissociation for these proteins to select new or correct sites. The lac repressor protein binds non-specific sites quite strongly as evidenced by its large association constant for them, and it binds specific sites only one order of magnitude better (Riggs et al., 1972; Lin and Riggs, 1972). Hence selection of the correct sites can take an enormously long period of time if the repressor protein- DNA complex must dissociate each time. In the case of the E. coli SSB proteins, direct and cooperative transfer between single-stranded DNAs would be an effective way of quickly providing contiguous units of protein required for DNA replication, recombination and repair.

The direct transfer of proteins requires that they be essentially bifunctional, that is, they must have two potential binding sites. The construction of a protein about a two-fold axis of symmetry could provide two binding sites. Construction of a tetrameric protein composed of four subunits with two sides a mirror image of each other fulfills this requirement. The lac repressor exists as a tetramer with a two-fold axis of symmetry (Steitz et al., 1974), and the E. coli SSB appears to as well (Weiner et al., 1975). Ruyechan and Wetmur (1976) found that the E. coli SSB probably posseses two identical binding sites which cannot be occupied simulta-

neously. A prediction of a direct transfer mechanism is the appearance of a transition state in which both sites must be occupied simultaneously for a short period of time. The binding sites of the proteins during this intermediate step would then undergo a switching from strong affinity to weak affinity and vice-versa until all of the SSB remains on either the donor or recipient DNA strands.

2). EQUILIBRIUM CONSIDERATIONS OF A DIRECT TRANSFER MECHANISM

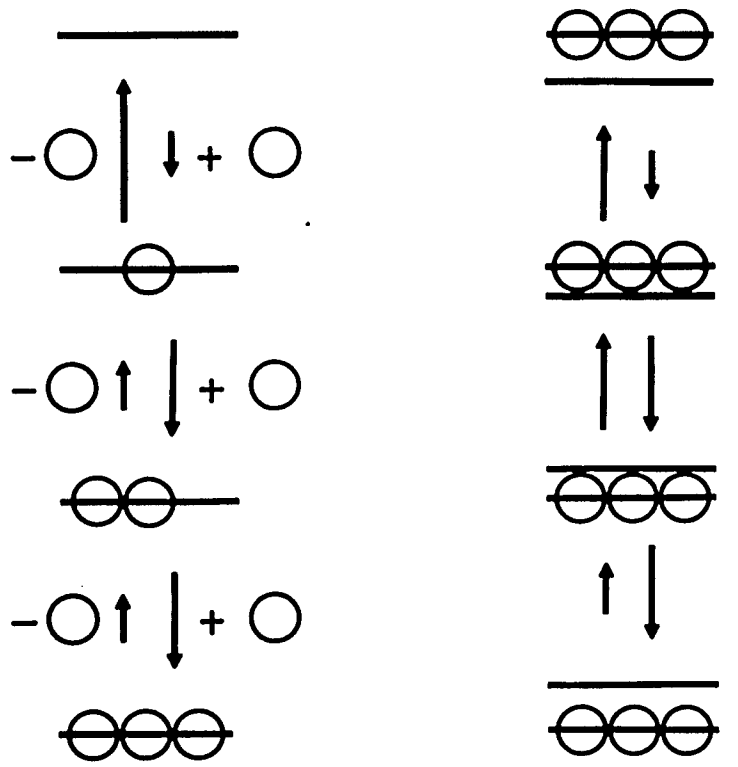
The two ways by which SSB could be transferred from one DNA to another are illustrated in figure 18.SSB could dissociate from the first DNA to join a pool of free molecules. SSB from this pool could then bind to another DNA strand. This mechanism is illustrated in part A. The second possibility would involve direct and cooperative transfer from one strand of DNA to another. This mechanism is illustrated in part B.

When free SSB and free single-stranded DNA are mixed together, a DNA-SSB complex will be formed. This uptake of free SSB by DNA, illustrated in part A, will involve a nucleation reaction and a propagation reaction analogous to those

FIGURE 18

Models for SSB transfer between DNA molecules.

Models for SSB transfer between DNA molecules.


A.
FREE
PROTEIN
B.
DIRECT
TRANSFER

occurring in DNA renaturation (reassociation) kinetics. First, a noncooperative, reversible binding may occur between one SSB and a DNA molecule. This step, like the formation of the first base pair in DNA reassociation, rarely results in complex formation. The pre-equilibrium is described by the weak non-cooperative binding constant,

$$K_1o = k_n/k_{-n}$$

where k_n and k_{-n} are the rates of formation and dissociation of a single SSB-DNA complex. Using equilibrium dialysis of oligonucleotides, Ruyechan and Wetmur (1976) determined K_1o to be about 5×10^4 per specific nucleotide. If the specific four nucleotide - SSB interaction may occur at any position along an S'=32 nucleotide binding site for an SSB tetramer, an observed association constant to such a site would be $K_1 = S' K_1o = 1.6 \times 10^6$.

The next step in a nucleation reaction involves binding of an SSB molecule next to a non-cooperatively bound SSB. The cooperative binding constant is $K_2 = k_c/k_{-c}$ where k_c and k_{-c} are rate constants for association of SSB from the end of a group of bound SSB molecules. This cooperative binding step is effectively irreversible and stabilizes the formation of an SSB-DNA complex. The cooperative binding of an SSB, following the reversible non-cooperative binding, is the rate determin-

ing step for nucleation. Propagation involves addition of more SSB molecules to fill the remaining sites.

3). MAGNITUDE OF THE COOPERATIVE BINDING CONSTANT

The cooperative binding constant for the association of SSB to single-stranded DNA is quite large. As a result there is relatively little free SSB when single-stranded DNA is present. To obtain a clearer understanding of the difficulties inherent in a transfer mechanism that requires a free pool of SSB, it is first necessary to discuss more thoroughly the magnitude of the cooperative binding constant of SSB for single-stranded DNA.

The cooperative binding of SSB to single-stranded DNA may be shown in many ways. Single-stranded pBR322 DNA, when complexed with SSB at sub-saturating amounts, remains resistant to nucleases as shown (figures 15,16). Even a 1500-fold excess of either S1 or DNaseI endonucleases could not produce a significant population of DNA molecules smaller than unit size.

When SSB is mixed with bacteriophage G4 DNA, even at 40% coverage, highly extended molecules are seen in the electron microscope (figure 5). When these molecules are subjected to extensive digestion with DNasel or endonuclease S1, these molecules remain full length. Ruyechan and Wetmur (1975) showed that the cooperativity,

$$w=K_2/K_1, = K_2/S'K_1o,$$

at 50% site saturation, must be equal to the square of the average number of sites covered by SSB on an infinitely long DNA strand. The nuclease data with bacteriophage G4 DNA means that

$$w>3 \times 10^4 \quad \text{or} \quad K_2> 5 \times 10^{10} M^{-1}.$$

Using longer DNA molecules, Ruyechan and Wetmur (1975) also obtained

$K_2> 5 \times 10^{10} M^{-1}$. Sigal et al. (1972) showed that SSB could lower the melting temperature of T4 DNA from T = 328 degrees K to below Tm' = 310 degrees K when present at a concentration less than S = 1.7×10^{-6}M. A minimum association constant may be calculated to be, (Crothers, 1971),

$$K_2 = (1/S)\ e^{-(Tm-Tm')\Delta H/B_c RTmTm'} \qquad (5)$$

where ΔH for DNA melting is about -8000cal/mole of base pairs, R is the gas constant and $B_c = 1/16$ is the density of sites

for SSB (1 per 16 base pairs). The melting temperature depression data also implies that $K_2 > 5 \times 10^{10} M^{-1}$.

4). KINETICS OF FREE PROTEIN TRANSFER MECHANISM

This section contains a discussion of the possibility that the mechanism involved in formation of DNA-SSB complexes from DNA and SSB is the same as the mechanism involved in the transfer of SSB from one DNA molecule to another. For the transfer between 375 and 400 nucleotide DNA strands the all-or-none reaction observed is consistent with this mechanism involving uptake of free SSB. However, it will be shown below that the rate of transfer is too great to be explained by uptake of the exceedingly low concentration of free SSB. The dependence of the rate of SSB transfer on inverse viscosity suggests the calculation of a maximum possible rate of transfer based on a diffusion limited reaction. Consider the reaction

$$R + S \underset{k_{-n}}{\overset{k_n}{\rightleftharpoons}} RS \qquad (6)$$

$$RS + S \xrightarrow{k_c} RS2 \qquad (7)$$

where R is recipient DNA, S is free SSB, RS is a non-cooperative DNA-SSB complex and RS2 is the first cooperative DNA-SSB complex.

A rate equation may be derived from this using the kinetics of opposing reactions. The rate equation with respect to R is

$$\frac{dR}{dt} = k_n(R)(S) - k_{-n}(RS) \qquad (8)$$

RS, the non-cooperative DNA-SSB complex, never accumulates, and it can be said to exist in a steady state. The rate equation with respect to RS may be written.

$$\frac{d(RS)}{dt} = k_n(R)(S) - k_{-n}(RS) - k_c(RS)(S) = 0 \qquad (9)$$

Isolating (RS),

$$(RS) = \frac{k_n(R)(S)}{k_{-n} + k_c(S)} \qquad (10)$$

and substituting into (8) we obtain the rate equation for this reaction.

$$-\frac{d(R)}{dt} = \frac{2N\, k_n\, k_c\, (R)\, (S)^2}{k_{-n} + 2\, k_c\, (S)} \qquad (11)$$

where N is the number of nucleotides minus three (372 for a 375-nucleotide oligomer) and the 2 comes from the two ways of obtaining a cooperative binding interaction. We know $k_n/k_{-n} = 6 \times 10^4$ and we may assume $k_n = k_c$ =rate constant for diffusion of SSB to a DNA site (nucleotide). For the 375-nucleotide molecules

$$-\frac{d\ln(R)}{dt} = \frac{744\, k_n\, (S)^2}{1/6\times10^{-4} + 2(S)} = 4.5 \times 10^7 k_n (S)^2 \qquad (12)$$

for the small values of (S) used in the experiments. Relating this rate equation to the all-or-none reactions (equations 1 and 3), there is an upper limit for k_1 of

$$k_1 < 4.5 \times 10^7 \, k_n (S)^2 \qquad (13)$$

if the free protein mechanism is correct. The total protein concentration in the experiments is 4.5×10^{-9}M and most of it is bound. In fact, it may be shown that (S) must be of the order of $3/k_2$ or 6×10^{-11}M. k_n for a diffusion controlled reaction involving SSB and single-stranded 375 nucleotide DNA may be estimated by the Smoluchowski equation to be

$$k_n \leq 4\pi D_{1,2} \frac{No}{1000} e \qquad (14)$$

where No is Avogadro's number, $D_{1,2}$ is the mutual diffusion coefficient and e is the required approach distance. Letting e be 3 Angstroms and using sedimentation data to obtain $D_{1,2}$,

$$k_n < 2.3 \times 10^8 M^{-1} sec^{-1} \qquad (15)$$

in water. This leads to a prediction of

$$k_1 \text{ (Theory)} < 3.7 \times 10^{-5} \text{ sec}^{-1} \quad (16)$$

whereas

$$k_1 \text{ (Experiment)} = 3.7 \times 10^{-2} \text{ sec}^{-1} \quad (17)$$

The theory is unable to account for the rapid observed reaction rates even after taking all statistical factors into account. The mechanism involving uptake of free SSB from solution also fails to account for the observed DNA length dependence of SSB uptake. In fact, the yield of SSB molecules per nucleation event should rise with increasing recipient DNA length leading to a length dependence of the transfer rates opposite to that observed. Furthermore, this mechanism also predicts that the products of the transfer reactions involving DNA circles and fragments should be identical, regardless of which DNA acts as SSB donor or recipient. Comparison of lines 1 and 2 in table V , the electron micrographic data, shows that this is not the case. The sucrose gradient sedimentation studies involving DNA circles and fragments also indicate that the transfer products are different.

5). KINETICS OF DIRECT TRANSFER MECHANISM

To account for the relatively rapid rate of transfer of SSB from one strand to another and to satisfactorily account for the data obtained, one needs to invoke a mechanism where the formation of a cooperative complex takes place in a single-step and is diffusion controlled. One such mechanism would involve uptake of polymers (dimers or higher) of SSB by a recipient strand. Sedimentation studies of SSB (Ruyechan and Wetmur, 1976) failed to indicate such aggregates even at 100,000 times greater concentration than the free SSB in the experiments. Furthermore, invoking a mechanism involving association and dissociation of SSB-SSB complexes does not help to explain the DNA length dependence of SSB transfer rates or the dependence of the reaction products on the order of addition of the reactants.

The most likely explanation of the kinetics is that transfer of SSB between strands, unlike addition of SSB to DNA, where the free SSB concentration is much greater, follows the mechanism outlined in figure 18, part B. In this case,

the reaction involves the mutual diffusion of donor DNA-SSB complex and recipient DNA into the proper orientation followed by direct and cooperative transfer of bound SSB from one strand to the other. It is not known whether the two binding sites per SSB tetramer (Ruyechan and Wetmur, 1976) are involved in this process. The rate constant for this process, the same as k_2 for the all-or-none mechanism, would be limited by diffusion to

$$k_2 \leq \frac{4\pi D_{1,2} No}{1000} e N \qquad (18)$$

where $D_{1,2}$ is now the mutual diffusion coefficient of a DNA-SSB complex and a recipient DNA, and N is the number of ways the bound SSBs could interact with the recipient strand to produce a productive transfer. For all reasonable calculations of $D_{1,2}$, the theoretical k_2 is much larger than the experimental k_2. Thus, there is no a priori reason to exclude a direct transfer mechanism.

The direct transfer mechanism also helps to explain the length effect of recipient DNAs. The yield per transfer reaction is limited to the number of SSB molecules complexed to a donor strand. Thus, unlike the rate of uptake of SSB from solution, the rate of uptake of SSB by direct transfer will

not be expected to increase with increasing recipient DNA strand length when the recipient DNA is longer than the donor DNA. In fact, the rates of transfer are found to decrease with increasing recipient DNA strand length.

The decrease could be the result of some combination of two effects. First, the mutual diffusion coefficient will decrease when the recipient strand becomes longer. This would result in a slower rate of transfer of SSB. Second, the availability of sites on the recipient DNA may decrease when the recipient length increases. The longer DNA molecules could have a smaller concentration of sites available since single-stranded DNA at 37 degrees C in 0.03 - 0.15M NaCl is known to have a significant amount of secondary structure. Also, the denatured long strands of DNA assume a random coil configuration, making the interior portions of the molecule more inaccessable. Consequently, the effective concentration of competing DNA sites decreases with increasing DNA length. At the concentrations of SSB used in these experiments, if transfer required dissociation of SSB to a free pool it would not be affected by the change in the concentration of competing sites. Direct transfer, however, is much more affected by the concentration of competing sites.

The products of SSB transfer from short DNA-SSB complexes to recipient G4 DNA, seen in part B of figure 13, and compiled

in Table V, line 2, appear to be the result of all of the G4 DNA molecules acquiring clusters of SSB rather than the highly cooperative product distribution seen in part A of figure 13, and compiled in Table V, line 1. This product of G4 DNA-SSB clusters explains the continued adherence of the kinetics to reversible all-or-none behavior even with long DNA recipients. The observed product distribution is not an equilibrium distribution. If G4 DNA-SSB complexes are formed first and the recipient 400-nucleotide DNA added later, the open circular G4 DNA molecules retain that conformation (Table V, line3, and figure 13). Eventually, the observed product distribution (Table V, line2) will convert to the expected cooperative distribution (Table V, line 1) by intramolecular and intermolecular redistribution of the SSB molecules. Weiner et al. (1975) and I (unpublished observations) have found, however, that SSB transfer between such long DNA molecules is an extremely slow process, even in the presence of facilitating short single-stranded DNA.

6). CONCLUSION

It may be concluded that there are two possible mechanisms which may facilitate the transfer of SSB from a single-stranded DNA-SSB mixture onto newly added single-stranded DNA. If there is excess SSB, then free SSB from solution will simply add on to the newly added single-stranded DNA. If there is more single- stranded DNA than SSB in the mixture, then direct transfer may occur of SSB clusters from complexes of DNA-SSB onto added DNA, according to mechanism B in figure 18.

The relative importance of these two mechanisms for governing the redistribution of SSB during such processes as DNA replication and recombination is unknown. However, several points should be noted. (1) The redistribution of SSB in single-strand DNA excess by dissociation is exceedingly slow and cannot provide the rapid redistribution required during DNA replication. (2) The redistribution of SSB by an all-or-none direct transfer mechanism could conceivably provide for SSB redistribution rapidly during DNA replication. (3) The efficient and catalytically mediated recA protein DNA recombination also requires substantial amounts of SSB. This process could also require the rapid redistribution of SSB. (4) A purported role of SSB in bacterial and bacteriophage DNA replication is the selection of the origin of replication by its

capacity to melt-out all other stem-loop structures. My attempts to cut the purported stable stem-loop structure at the origin of DNA replication in pBR322 DNA in the presence of SSB failed to confirm this hypothesis. However, it is conceivable that pBR322 DNA is not the proper model system for the analysis of this role of SSB. Possibly some other DNA, such as viral G4 or ØX174 DNA, would prove to be a better system for study. The results of the G4 loop-cutting experiments are potentially exciting, but they are preliminary. It must be shown rigorously whether there is specific cleavage in the G4 system, and if so, the cleavage site must be determined accurately.

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