The EMBO Journal Vol.17 No.8 pp.2436–2449, 1998

Devoted to the lagging strand—the χ subunit of DNA polymerase III holoenzyme contacts SSB to promote processive elongation and sliding clamp assembly

Zvi Kelman[1,2], Alexander Yuzhakov[1], Jelena Andjelkovic[1] and Mike O'Donnell[1,3]

[1]Rockefeller University, 1230 York Avenue and [2]Sloan-Kettering Institute, Department of Molecular Biology, 1275 York Avenue, New York, NY 10021, USA

[3]Corresponding author

Escherichia coli **DNA polymerase III holoenzyme contains 10 different subunits which assort into three functional components: a core catalytic unit containing DNA polymerase activity, the β sliding clamp that encircles DNA for processive replication, and a multisubunit clamp loader apparatus called γ complex that uses ATP to assemble the β clamp onto DNA. We examine here the function of the χ subunit of the γ complex clamp loader. Omission of χ from the holoenzyme prevents contact with single-stranded DNA-binding protein (SSB) and lowers the efficiency of clamp loading and chain elongation under conditions of elevated salt. We also show that the product of a classic point mutant of SSB, SSB-113, lacks strong affinity for χ and is defective in promoting clamp loading and processive replication at elevated ionic strength. SSB-113 carries a single amino acid replacement at the penultimate residue of the C-terminus, indicating the C-terminus as a site of interaction with χ. Indeed, a peptide of the 15 C-terminal residues of SSB is sufficient to bind to χ. These results establish a role for the χ subunit in contacting SSB, thus enhancing the clamp loading and processivity of synthesis of the holoenzyme, presumably by helping to localize the holoenzyme to sites of SSB-coated ssDNA.**

Keywords: clamp loader/DNA polymerase III holoenzyme/DNA replication/SSB/SSB-113

Introduction

DNA polymerase III holoenzyme (Pol III holoenzyme) is the multisubunit replicase of the *Escherichia coli* chromosome. Pol III holoenzyme is distinguished from other DNA polymerases in the cell by its high processivity (>50 kbp) and rapid rate of synthesis (750 nucleotides/s) (reviewed in Kornberg and Baker, 1992; Kelman and O'Donnell, 1995). The high processivity and speed is rooted in a ring-shaped subunit, called β, that encircles DNA and slides along it while tethering the Pol III holoenzyme to the template (Stukenberg *et al.*, 1991; Kong *et al.*, 1992). The ring-shaped β clamp cannot assemble around DNA by itself. For this, a multisubunit clamp loader is required which couples the energy of ATP hydrolysis to the assembly of the β clamp onto DNA. This clamp loader, called γ complex, is an integral component of the Pol III holoenzyme particle. A brief overview of the organization of subunits within the holoenzyme and their function follows.

Pol III holoenzyme consists of 10 different subunits, some of which are present in multiple copies for a total of 18 polypeptide chains (Onrust *et al.*, 1995b). The organization of these subunits in the holoenzyme particle is illustrated in Figure 1. As depicted in the middle column of the diagram, the subunits of the holoenzyme can be grouped functionally into three components: (i) the DNA polymerase III core is the catalytic unit and consists of the α (DNA polymerase), ε (3′–5′ exonuclease) and θ subunits (McHenry and Crow, 1979); (ii) the β sliding clamp is the ring-shaped protein that secures the core polymerase to DNA for processivity (Kong *et al.*, 1992); and (iii) the five protein γ complex (γδδ′χψ) is the 'clamp loader' that couples ATP hydrolysis to assembly of β clamps around DNA (O'Donnell, 1987; Maki and Kornberg, 1988). A dimer of the τ subunit acts as a 'macromolecular organizer' holding together two molecules of core and one molecule of γ complex forming the Pol III* subassembly (Onrust *et al.*, 1995b). This organizing role of τ in forming Pol III* is indicated at the right side of Figure 1. Two β dimers associate with the two cores within Pol III* to form the holoenzyme capable of replicating both strands of duplex DNA simultaneously (Maki *et al.*, 1998).

The Pol III holoenzyme assembles onto a primed template in two distinct steps. In the first step, the γ complex assembles the β clamp onto the DNA. The γ complex and the core polymerase utilize the same surface of the β ring and they cannot both utilize it at the same time (Naktinis *et al.*, 1996). Hence, in the second step, the γ complex moves away from β thus allowing access of the core polymerase to the β clamp for processive DNA synthesis. The γ complex and core remain attached to each other during this switching process by the τ subunit organizer.

The γ complex consists of five different subunits (γ_{2-4} $\delta_1\delta'_1\chi_1\psi_1$). An overview of the mechanism of the clamp loading process has been fleshed out previously. The δ subunit is the major touch point to the β clamp and leads to ring opening, but δ is buried within the γ complex such that contact with β is prevented (Naktinis *et al.*, 1995). The γ subunit is the ATP-interactive protein but is not an ATPase by itself (Tsuchihashi and Kornberg, 1989). The δ' subunit bridges the δ and γ subunits, resulting in a γδδ' complex that exhibits DNA-dependent ATPase activity and is competent to assemble clamps on DNA (Onrust *et al.*, 1991). Upon binding of ATP to γ, a change in the conformation of the complex exposes δ for interaction with β (Naktinis *et al.*, 1995). The functions of the smaller subunits, χ and ψ, have remained elusive. These two

© Oxford University Press

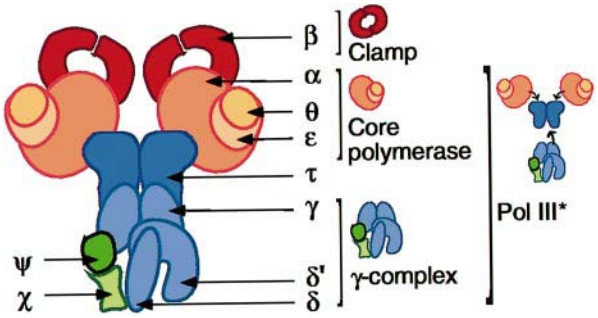


Fig. 1. Schematic of DNA polymerase III holoenzyme. DNA polymerase III holoenzyme contains 10 different subunits; arrows identify each of them. The three functional components of the holoenzyme are shown to the right of the diagram [clamp (β), core polymerase (αεθ), γ complex (γδδ'χψ)]. The τ subunit dimer acts as a glue to bind together two core polymerases and one γ complex to form the Pol III* subassembly (the holoenzyme lacking β), as shown in the diagram at the far right.

subunits form a 1:1 χψ complex (Xiao *et al.*, 1993b; Dallmann and McHenry, 1995) and the ψ subunit acts as a bridge between γ and χ in the γ complex (Xiao *et al.*, 1993b). In the absence of χ and ψ, Pol III activity becomes highly sensitive to ionic strength in the physiological range (40–150 mM) (O'Donnell and Studwell, 1990; Xiao *et al.*, 1993b). Presumably, either loading of β onto DNA, or polymerization itself, requires the χ and/or ψ subunits for full efficiency at elevated ionic strength.

This report identifies a function of the χ subunit, that of binding to single-stranded DNA (ssDNA)-binding protein (SSB). The χ subunit appears to be the only subunit of Pol III holoenzyme to form a major contact with SSB. This observation is consistent with an earlier study showing that the γ complex binds to ssDNA templates only in the presence of SSB (Fradkin and Kornberg, 1992), suggesting a direct interaction between SSB and one or more subunits of the γ complex.

SSB is an essential protein and it binds specifically and tightly to ssDNA, thereby melting hairpin barriers to polymerase progression (reviewed in Chase, 1984; Meyer and Laine, 1990; Lohman and Ferrari, 1994). The interaction of SSB with ssDNA increases the processivity of Pol III holoenzyme, resulting in a marked stimulation of its activity (Weiner *et al.*, 1975; Scott *et al.*, 1977; LaDuca *et al.*, 1983). Although a protein bound to ssDNA conceivably would inhibit chain extension by a DNA polymerase, its function in removing hairpin blocks would appear to be more beneficial to the polymerase than the detriment caused by the need to displace SSB during forward movement.

SSBs are ubiquitous in organisms and have been isolated from other bacteria and their bacteriophages (e.g. gene 2.5 protein of phage T7 and gene 32 protein of phage T4) and from eukaryotic cells [replication protein A (RP-A)] and their viruses. Generally, the SSBs in these systems interact with the corresponding DNA polymerase, thus explaining why the SSB of one system does not substitute efficiently for the SSB of another.

SSB-113 is a conditional lethal mutant with a pleiotropic phenotype having defects in replication and recombination (Chase, 1984; Chase and Williams, 1986; Meyer and Laine, 1990; Lohman and Ferrari, 1994). SSB-113 contains a single amino acid substitution at the penultimate residue to the C-terminus whereby Pro176 is replaced by Ser (Chase *et al.*, 1984). Study of SSB-113 showed that it binds ssDNA with affinity comparable with the wild-type SSB (Chase *et al.*, 1984), leading to the suggestion that the C-terminus of SSB is involved in contact with DNA metabolic proteins involved in replication and recombination (Chase *et al.*, 1983). Here we confirm this hypothesis by demonstrating that SSB-113 binds weakly to the χ subunit of DNA polymerase III holoenzyme. Consistent with this conclusion, a peptide corresponding to the C-terminal 15 amino acids of SSB is sufficient to interact with χ, but a similar peptide corresponding to SSB-113 is not.

The functional consequences of the χ–SSB contact for holoenzyme action have been explored in this report in two ways: (i) Pol III* and γ complex have been reconstituted in the absence of the χ subunit for comparison of their properties; and (ii) the effects of substituting SSB-113 for SSB in replication reactions have been examined. The results of both systems show that the χ–SSB contact provides resistance in elevated salt to the efficiency of loading the β clamp onto DNA and the processivity of chain elongation. Presumably, in high salt, the χ–SSB contact helps keep the enzyme localized to sites containing SSB for these clamp loading and chain elongation functions.

Results

The ability to reconstitute Pol III* from pure isolated subunits makes it possible to reassemble holoenzyme lacking one or more subunits by reconstituting it in the absence of a particular subunit(s). This report focuses on a role for χ by reconstituting Pol III* and γ complex either in the presence or absence of χ, purifying the resulting complexes from unbound subunits, and then comparing their DNA synthetic and β clamp loading activities. The comparison provides insight into the contribution of χ to clamp loading and processivity. As the study unfolded, we discovered that χ binds to SSB. Therefore, one may presume that results using Pol III holoenzyme in the absence of SSB would be similar to use of Pol III holoenzyme lacking χ in the presence of SSB. However, very little synthesis is observed in the absence of SSB whether χ is present or not. This is due to the fact that SSB not only interacts with χ, but it is also needed to melt hairpin barriers in ssDNA. This dual role of SSB precluded our ability to obtain information about the role of χ by comparisons of Pol III holoenzyme in the presence and absence of SSB. Hence, the experiments presented in this report are perfomed on primed templates in the presence of SSB (or SSB-113).

Omission of χ from Pol III holoenzyme leads to salt-sensitive DNA synthesis

First, we examined the role of χ in elongation by Pol III holoenzyme. For these experiments, the nine subunit Pol III*, and Pol III* – χ (i.e. eight subunits), were reconstituted from pure subunits. In the experiment of Figure 2, Pol III* and β were pre-incubated with SSB-coated primed M13mp18 ssDNA in the absence of NaCl to form the initiation complex of the holoenzyme on the primed

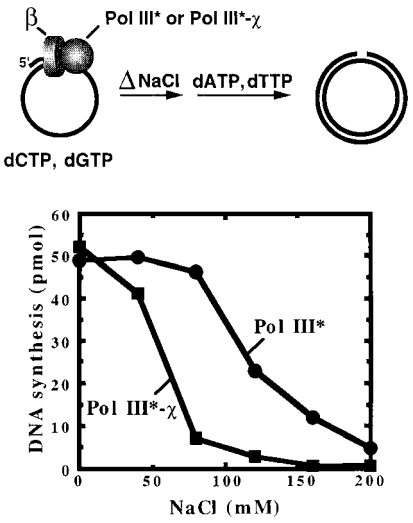


Fig. 2. χ confers resistance to added NaCl on chain elongation by Pol III holoenzyme. Pol III* (or Pol III* – χ) and β were used to assemble Pol III holoenzyme onto singly primed M13mp18 ssDNA coated with SSB. Then different concentrations of NaCl were added and a 20 s burst of DNA synthesis was initiated. ●, Pol III*; ■, Pol III* – χ.

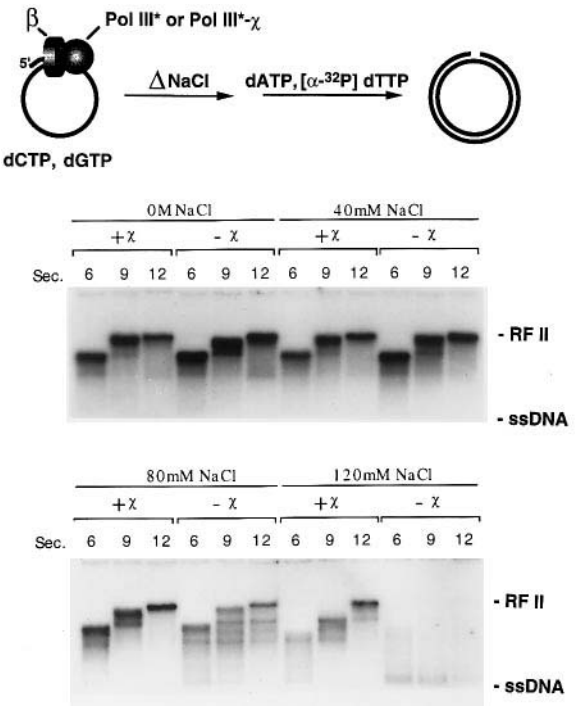


Fig. 3. Time course of chain extension by Pol III holoenzyme in the presence or absence of χ. The initiation complex of β and either Pol III* or Pol III* – χ was pre-formed on primed M13mp18 ssDNA followed by addition of NaCl and then the remaining dNTPs to initiate synthesis. Reactions were quenched at either 6, 9 or 12 s, followed by analysis in a native agarose gel and autoradiography. The positions of completed circular products (RF II) and starting primed template (ssDNA) are indicated on the right.

template. This pre-incubation is performed in the presence of only two dNTPs, thus preventing extension of the DNA primer for more than only a few nucleotides. After assembly of Pol III* and β on DNA, NaCl was added to the reaction to different concentrations, and dATP and [α-^{32}P]dTTP were added to initiate synthesis (see scheme in Figure 2). The result, in Figure 2, shows that at low ionic strength, both forms of polymerase have equal chain elongation activity. However, as NaCl is added, Pol III* − χ becomes much less efficient than Pol III* containing χ. This result indicates that χ serves a role in elongation.

The defect in elongation by holoenzyme lacking χ in high salt can be explained by either decreased processivity of Pol III holoenzyme lacking χ or a slower speed of elongation. To distinguish between these, the experiment of Figure 2 was repeated, but rapid time points from each reaction were withdrawn and the products were analyzed on a neutral agarose gel. The results of these time courses are shown in Figure 3.

At the 6 s time point, the enzyme should have proceeded for ~4200 of the 7200 nucleotides of the template. The results show that the leading edge of synthesis at the 6 s time point in 0, 40 and 80 mM NaCl is approximately the same for Pol III holoenzyme with or without χ. However, for holoenzyme lacking χ, the DNA products appear as a smear at 80 mM added NaCl, whereas products of reactions containing χ do not change significantly in going from 0 to 40 or 80 mM NaCl. The template is mostly completed within the 9 and 12 s time points in reactions containing either 0, 40 or 80 mM NaCl. However, at 80 mM NaCl, reactions lacking χ show that the smear continues to be evident at both 9 and 12 s. At 120 mM NaCl, reactions containing χ are diminished, appear slower at 6 s compared with lower salt and start to smear, but the reactions lacking χ at 120 mM NaCl are barely visible at all. Presumably, products that appear as a smear are the result of premature dissociation of the holoenzyme from the template. Thus, these results indicate that the defect in Pol III holoenzyme lacking χ lies mainly in a decreased processivity of elongation rather than a decreased speed of synthesis.

Next we examined the effect of χ on the clamp loading activity of the γ complex (Figure 4). To assay γ complex ± χ for assembly of β onto DNA, [^{3}H]β subunit was incubated with SSB-coated singly primed M13mp18 ssDNA with either γ complex or γδδ'ψ. The reaction was then gel filtered over BioGel A15m. The A15m matrix has such large pores that free [^{3}H]β elutes in the included fractions. However, large [^{3}H]β–DNA complexes elute in the excluded volume (as does DNA) and resolve from free [^{3}H]β. First the reaction was performed in the absence of added NaCl (Figure 4A and B). The [^{3}H]β assembled onto DNA elutes in fractions 9–13, and resolves from free [^{3}H]β in the included volume (fractions 18–30). The results, in Figure 4A and B, show that γ complex and γδδ'ψ assemble approximately equal amounts of [^{3}H]β onto primed DNA under these low salt conditions.

This experiment was then repeated in the presence of added 160 mM potassium glutamate (Figure 4C and D). The results show that the γ complex was nearly as active in high salt as in low salt, but the γδδ'ψ complex was only 20% as efficient in high salt compared with low salt.

Next, the clamp loading activity of γ complex and γδδ'ψ was compared on a plasmid DNA containing a single nick (Figure 4E–H). Nicked DNA contains a 3′ terminus, but no ssDNA. In contrast to use of singly primed M13mp18 ssDNA, most of the [^{3}H]β is assembled onto the nicked template. The underlying reason for accumulation of β on nicked DNA is the ability of β to slide on duplex DNA (Stukenberg *et al.*, 1991). Therefore,

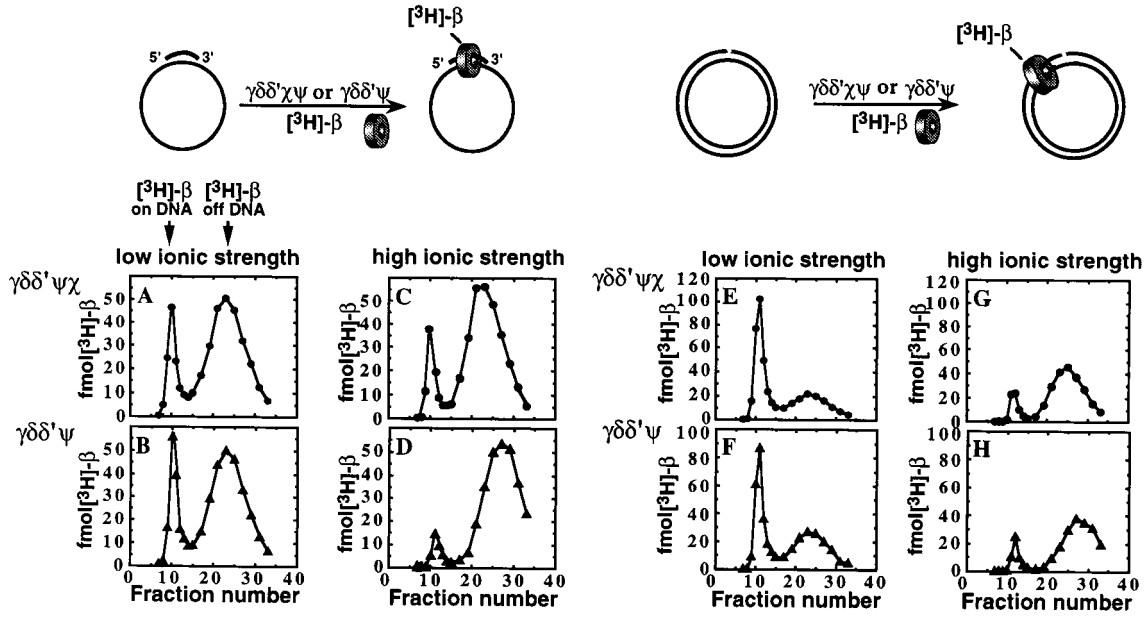


Fig. 4. The χ subunit aids γ complex in assembly of [^{3}H]β onto SSB-coated primed ssDNA at physiological ionic strength. (**A–D**) [^{3}H]β was assembled onto circular singly primed ssDNA by either the γ complex (●, A and C) or γδδ'ψ (▲, B and D) in the presence or absence of 160 mM potassium glutamate as described in Materials and methods. Reactions were applied to BioGel A15m gel filtration columns and the amount of [^{3}H]β in each fraction was quantitated by scintillation counting. (**E–H**) χ does not assist γ complex in assembly of β onto nicked DNA. [^{3}H]β was assembled onto a singly nicked circular duplex plasmid by either the γ complex (E and G) or γδδ'ψ (F and H) in the presence or absence of 160 mM potassium glutamate as described in Materials and methods. Reactions were applied to BioGel A15m gel filtration columns and the amount of [^{3}H]β in each fraction was quantitated by scintillation counting.

upon assembly of β onto a nicked site, the [^{3}H]β clamp slides away from the 3′ terminus allowing the γ complex repeatedly to load multiple [^{3}H]β clamps onto the nicked template. In contrast, primed DNA contains only a short duplex region that can support occupancy of only one or two [^{3}H]β clamps. At low ionic strength, both γ complex and γδδ'ψ are highly active in loading [^{3}H]β onto nicked DNA (Figure 4E and F, respectively). However, at elevated ionic strength, neither γ complex nor γδδ'ψ are efficient in clamp loading (Figure 4G and H). Nicked DNA contains no ssDNA, and thus is incapable of binding SSB. Therefore, the lack of salt-resistant clamp loading activity on nicked DNA implies that either ssDNA, or SSB bound to ssDNA, is needed for χ to stimulate clamp loading at elevated ionic strength.

Pol III interacts with SSB-coated ssDNA via the γ complex*

The inability of χ to confer salt resistance to γ complex on nicked DNA suggests that ssDNA or SSB is required for χ to manifest its activity, possibly through direct χ–SSB contact. To initiate the study of which subunit of Pol III*, if any, interacts directly with SSB, we reconstituted several forms of Pol III* using a ^{3}H-labeled subunit to follow it. Then the [^{3}H]subassembly was mixed with M13mp18 ssDNA coated with SSB and gel filtered over BioGel A15m. The results are shown in Figure 5. Use of [^{3}H]Pol III* (reconstituted from pure subunits using [^{3}H]θ) showed co-migration of [^{3}H]Pol III* with the SSB–ssDNA complex (Figure 5A). Pol III' is a subassembly of Pol III* lacking the γ complex (i.e. core$_2\tau_2$) (McHenry, 1982; Studwell-Vaughan and O'Donnell, 1991). As shown in Figure 5B, [^{3}H]Pol III' (reconstituted using [^{3}H]θ) did not co-migrate with the SSB–ssDNA, indicating that α, ε, θ and τ are not major actors in binding this template. In Figure 5C, [^{3}H]γ complex (reconstituted using [^{3}H]δ') was found to co-migrate with the SSB–ssDNA, consistent with earlier reports indicating an interaction between SSB–ssDNA and γ complex (Fradkin and Kornberg, 1992). To help distinguish which subunit of γ complex underlies its interaction with the SSB–ssDNA complex, two [^{3}H]sub-assemblies of γ complex, [^{3}H]γδδ'ψ (Figure 5D) and [^{3}H]γδδ' (Figure 5E) were prepared (each of them were constituted using [^{3}H]δ'). The results in Figure 5D and E show that neither of these subassemblies interact with the ssDNA–SSB complex as is evident by their absence in the excluded fractions and presence in the included volume (fractions 16–33). The γδδ'ψ complex lacks only the χ subunit, suggesting that χ is essential for interaction of the γ complex with SSB-coated ssDNA.

To determine whether SSB is needed for the interaction between γ complex and ssDNA, the experiment was repeated in the presence or absence of SSB (Figure 6). Figure 6A shows the expected co-migration of [^{3}H]γ complex and SSB-coated ssDNA. In the absence of SSB (Figure 6B), [^{3}H]γ complex does not bind the ssDNA template. Hence, SSB appears essential to the interaction between χ and SSB–ssDNA complex. Figure 6C shows that [^{3}H]γ complex does not interact with double-stranded DNA. In order to have detected a weak interaction between χ and either double-stranded DNA or ssDNA, these experiments were performed with a larger quantity of [^{3}H]γ complex than those of the experiments of Figure 5.

The χ subunit interacts with SSB–ssDNA

Direct interaction between χ and SSB–ssDNA complex is demonstrated in Figure 7 using an FPLC Superose-12 gel filtration column. This column resolves SSB tetramers

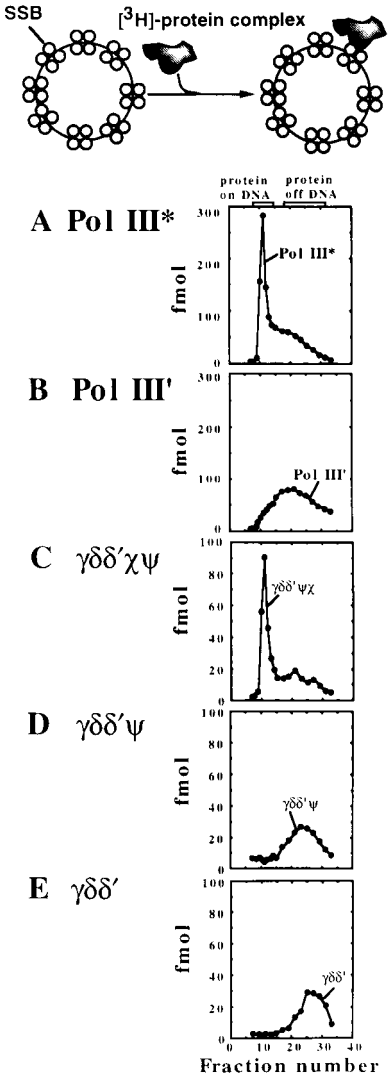


Fig. 5. The γ complex needs χ to interact with SSB-coated ssDNA. Protein–DNA interactions were analyzed by gel filtration of mixtures of SSB-coated ssDNA and tritiated subassemblies of Pol III* as described in Materials and methods. (**A**) [^{3}H]Pol III*(1 pmol) was incubated with 125 fmol (as circles) of M13mp18 ssDNA coated with SSB, then gel filtered. (**B**) [^{3}H]Pol III' (1 pmol) was incubated with 125 fmol of M13mp18 ssDNA coated with SSB, then gel filtered. (**C**) [^{3}H]γ complex (250 fmol) was incubated with 300 fmol of M13mp18 ssDNA coated with SSB, then gel filtered. (**D**) [^{3}H]γδδ'ψ (250 fmol) was incubated with 300 fmol of M13mp18 ssDNA coated with SSB, then gel filtered. (**E**) [^{3}H]γδδ' (250 fmol) was incubated with 300 fmol of M13mp18 ssDNA coated with SSB, then gel filtered. The presence of protein complex in column fractions was analyzed by liquid scintillation as described in Materials and methods.

(75 kDa) from χ (17 kDa), and thus co-migration of χ and SSB would reflect formation of a complex between them. χ and SSB were incubated in the presence or absence of an 87mer DNA oligonucleotide, and then applied to the column. Fractions were collected and analyzed by Coomassie Blue staining of an SDS–polyacrylamide gel. In the absence of ssDNA (Figure 7A), the SSB tetramer and χ monomer resolve, indicating that they do not interact strongly in the absence of ssDNA. However, in the presence of the ssDNA oligonucleotide (Figure 7B), the SSB and χ co-eluted (fractions 10–19), indicating formation of a complex that depends on ssDNA. This experiment was repeated with oligo(dT_{65}) and oligo(dT_{35})

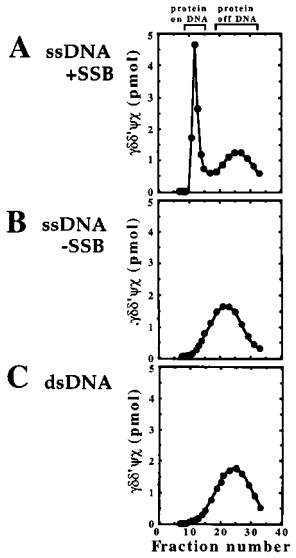


Fig. 6. SSB is required for γ complex to bind ssDNA. [^{3}H]γ complex (10 pmol) was incubated with the following components and then gel filtered as described in Materials and methods: (**A**) 440 fmol of M13mp18 ssDNA and 8 μg of SSB; (**B**) 440 fmol of M13mp18 ssDNA; and (**C**) 440 fmol of M13mp18 plasmid DNA and 8 μg of SSB.

with similar results (data not shown). No interaction was observed between χ and the 87mer oligonucleotide in the absence of SSB (data not shown). Further, use of the T4 gene 32 protein (the T4 SSB) with oligo(dT_{65}) did not suffice to bind χ, suggesting that χ does not bind ssDNA coated with just any SSB, but requires *E.coli* SSB (data not shown). Finally, these experiments needed to be performed in column buffer containing only 40 mM NaCl (the χ–SSB–ssDNA complex was not observed using 100 mM NaCl) and were carried out in the cold room.

To estimate the K_d value for the interaction between χ and SSB–oligo(dT_{65}), the equilibrium gel filtration technique was performed (Figure 7C). In this experiment, the smaller protein, [^{3}H]χ, was included in the column buffer to equilibrate it throughout the column, and the larger SSB–ssDNA complex was loaded onto the column. Due to the continual presence of [^{3}H]χ in the column buffer, the [^{3}H]χ–SSB–ssDNA complex remains at equilibrium as it flows through the column. The [^{3}H]χ that is bound to SSB–ssDNA elutes as a peak of [^{3}H]χ early, and the segment of column buffer that lost [^{3}H]χ to the SSB–ssDNA complex elutes later and appears as a dip, or trough, in the radioactive profile of column fractions. From the area under the peak of [^{3}H]χ–SSB–ssDNA complex, a K_d value of 53 nM was calculated (see Materials and methods). Further, this experiment showed a total of 807 pmol of χ bound to 212.5 pmol of SSB (as tetramer). Hence, ~4 molecules of χ bind one SSB tetramer (i.e. one χ per SSB monomer).

χ binds SSB in the absence of ssDNA

Perhaps χ interacts weakly with SSB or ssDNA alone. To detect a possible weak interaction of χ with SSB, or χ with ssDNA, we analyzed these components by surface plasmon resonance (SPR). In the SPR technique, one macromolecule is immobilized to a sensor chip and another macromolecule is passed over the chip. Interaction between

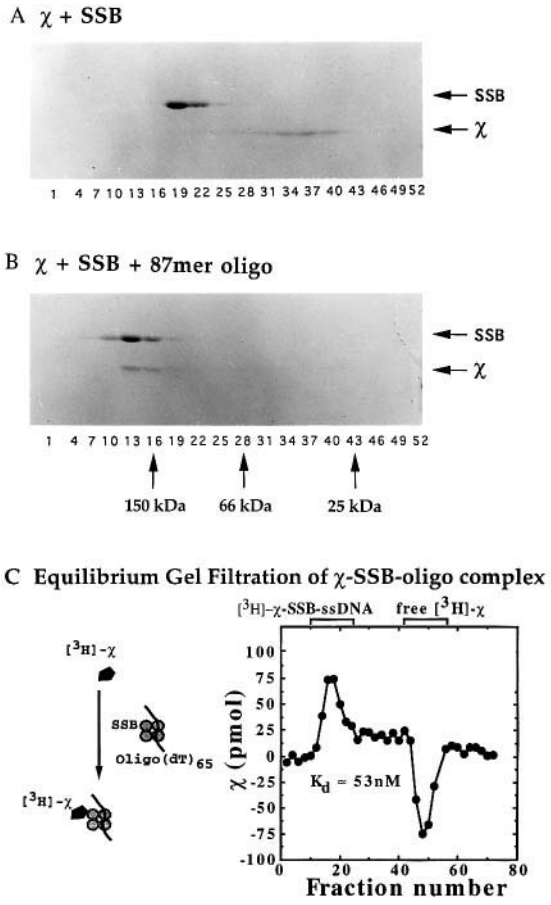


Fig. 7. The χ subunit interacts with SSB in the presence of ssDNA. (**A** and **B**) The interaction between χ and SSB was analyzed using a Superose-12 gel filtration column in the presence or absence of an 87mer DNA oligonucleotide as described in Materials and methods. (A) χ and SSB alone; (B) χ and SSB in the presence of the 87mer DNA oligonucleotide. The position of molecular weight markers, analyzed in a parallel analysis, is indicated. The standards used were: 150 kDa (alchohol dehydrogenase from yeast); 66 kDa (BSA), 25 kDa (bovine pancreas ribonuclease A). (**C**) The K_d of the χ–ssDNA–SSB complex was measured by equilibrium gel filtration analysis as described in Materials and methods. The baseline amount of χ was 1 μM, but has been set to zero in the figure. The column buffer contained [^{3}H]χ, and then a mixture of [^{3}H]χ–SSB–oligo(dT_{65}) was injected over the column. A K_d value of 53 nM was obtained from the data.

the two macromolecules leads to an accumulation of mass on the sensor chip surface leading to SPR [measured as response units (RU)] (Granzow and Reade, 1992).

As a control, we first examined interaction of χ with an immobilized SSB–ssDNA complex. In Figure 8A, oligo(dT_{65}), 5′ end labeled with biotin, was passed over a sensor chip to which streptavidin was covalently attached. Then SSB was passed over the immobilized oligonucleotide. Different concentrations of χ were serially passed over the SSB–ssDNA complex. The increase in RU observed with each injection indicates interaction between χ and the immobilized SSB–ssDNA complex. At the end of each injection, buffer is passed over the surface, with a resulting drop in RU, indicating rapid dissociation of χ. The rise and fall of RU in these experiments was too rapid for calculation of k_{on} and k_{off} values under these typical conditions for the SPR technique (22°C and 150 mM NaCl).

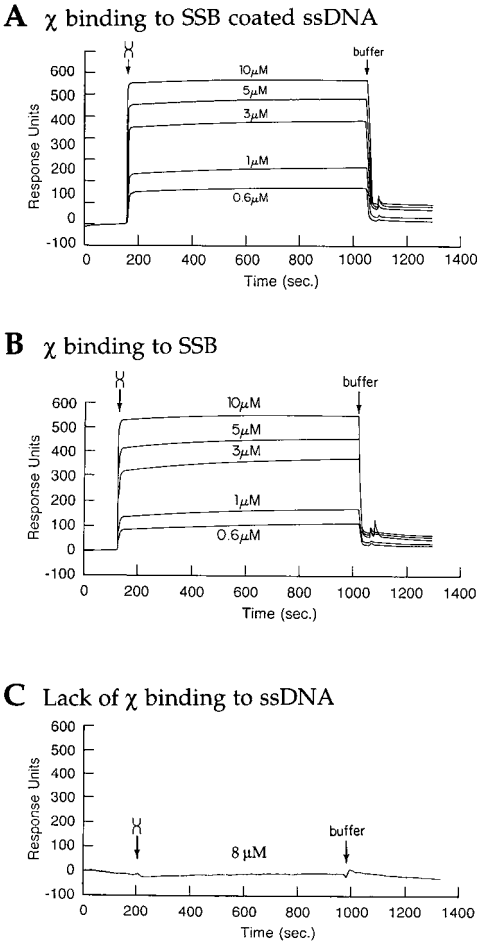


Fig. 8. χ binds SSB in the absence of ssDNA. Either SSB–oligo(dT_{65}), SSB or oligo(dT_{65}) were covalently attached to a sensor chip and solutions of χ were passed over the top. Arrows indicate the point at which χ is injected ('χ'), and the point at which injection of χ was terminated by the injection of buffer lacking χ ('buffer'). (**A**) Immobilized SSB–oligo(dT_{65}); (**B**) immobilized SSB; (**C**) immobilized oligo(dT_{65}).

Table I. The stoichiometry between χ and SSB–oligo(dT_{65})

χ concentration	χ/SSB_4 (plus DNA)	χ/SSB_4 (no DNA)
0.6 μM	1.04	0.44
1.0 μM	1.60	0.68
3.0 μM	2.88	1.48
5.0 μM	3.88	1.81
10 μM	4.04	2.18

The stoichiometry between χ and SSB–oligo(dT_{65}) was measured as follows. SSB was injected over a streptavidin sensor chip pre-coated with 319 RU of biotinylated oligo(dT_{65}), resulting in 754 RU of SSB bound to the ssDNA (12 fmol of SSB bound to 16.5 fmol of 65mer dT; assuming 120 fl volume in the flow cell and conversion factors of 100 RU equal to 1 mg/ml and 0.9 mg/ml for protein and DNA, respectively). The χ subunit was injected over the SSB–oligo(dT_{65}) complex in SPR buffer at concentrations of either 0.6, 1, 3, 5 or 10 μM. Sensor chips were regenerated after each injection as described in Materials and methods. The stoichiometry of χ to SSB was calculated from the plateau values at 15 min.

In Table I, the plateau values at the end of each 15 min injection were converted into fmol of χ bound to SSB. At the highest concentration of χ, the final RU indicates

~4 fmol of χ bound to one tetramer of SSB. This stoichiometry is consistent with the result of equilibrium gel filtration indicating approximately one molecule of χ to each SSB monomer.

In Figure 8B, SSB was immobilized directly to a sensor chip in the absence of DNA, then solutions of different concentrations of χ were passed over it. The results show that χ interacts with SSB in the absence of DNA. At the highest amount of χ used, the final increase in RU was 553, for a stoichiometry of 2.2 χ/SSB tetramer. This lower stoichiometry may indicate that χ interacts with SSB more weakly in the absence of ssDNA than in its presence. This would be consistent with the gel filtration experiments of Figure 7 in which a complex between χ and SSB was not observed unless ssDNA was present. However, in the SPR experiment of Figure 8B, the SSB is attached randomly to the sensor chip and thus some sites of interaction with χ may be occluded by its attachment to the chip.

In Figure 8C, χ was passed over a sensor chip to which the biotinylated oligo(dT_{65}) was immobilized to streptavidin. No signal was observed, indicating that χ does not interact strongly with ssDNA under these conditions.

SSB-113 does not support efficient initiation and elongation of replication at elevated ionic strength

Until now, the experiments of this report have shown that the χ subunit is the major contact between Pol III holoenzyme and SSB, and that holoenzyme lacking χ showed salt-dependent defects in elongation. This correlation suggests that χ functions to stabilize replication in high salt through an interaction with SSB. As these studies were in progress, we made an independent observation from another project which provided the necessary information to show that χ–SSB contact indeed underlies χ-mediated salt stability of holoenzyme action. This second project was a study of the SSB-113 mutant.

SSB-113 is a temperature-sensitive mutant for replication, and is also defective in recombination and repair at both permissive and non-permisive temperatures (reviewed in Chase, 1984; Meyer and Laine, 1990; Lohman and Ferrari, 1994). SSB-113 binds ssDNA as tightly as SSB, and only contains a single amino acid substitution (Ser for Pro) in the –1 position from the C-terminus (Chase *et al.*, 1984).

In Figure 9, SSB-113 was compared with SSB for its ability to support DNA synthesis by Pol III holoenzyme. Singly primed M13mp18 ssDNA was used as template and was coated with either SSB or SSB-113. The high processivity that is characteristic of this enzyme depends on coating of the ssDNA with SSB (Fay *et al.*, 1982). Hence, if SSB-113 is defective in action with Pol III holoenzyme, replication should be less efficient than a similar reaction using wild-type SSB. In Figure 9A, the initiation complex of the holoenzyme with DNA was formed in a pre-incubation at low ionic strength, then NaCl was added and replication was initiated upon adding the remaining two dNTPs. After 20 s, the reactions were quenched, and the extent of DNA synthesis was analyzed. The results show that in the presence of only 40 mM NaCl (the amount brought in by the protein preparations) replication proceeded similarly whether using SSB or

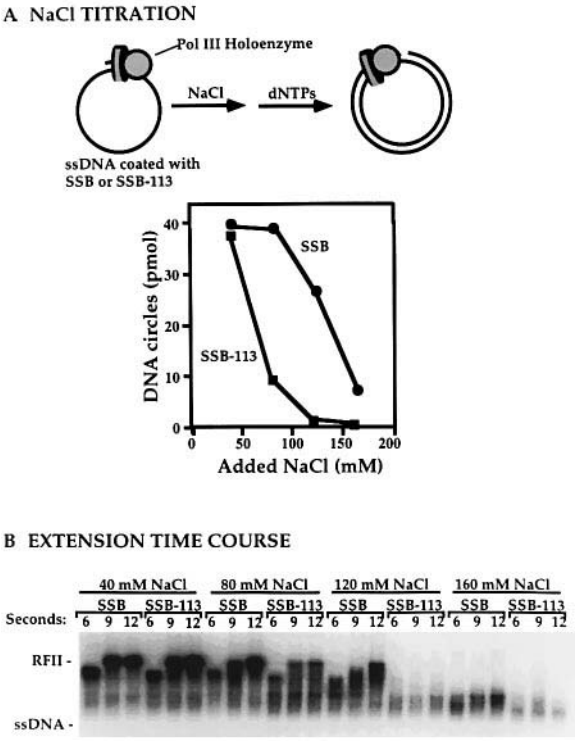


Fig. 9. SSB-113 does not confer resistance to NaCl on chain elongation by Pol III holoenzyme. Pol III holoenzyme was assembled onto primed M13mp18 ssDNA coated with either SSB or SSB-113. Following addition of NaCl, the dNTPs were added to initiate synthesis and timed aliquots were removed. (**A**) Quantitation of the 20 s time point. ●, results obtained using SSB; ■, results obtained using SSB-113. (**B**) Short time points were analyzed in a 0.8% native agarose gel followed by autoradiography. The positions of ssDNA and completed duplex circles (RFII) are indicated on the left. The amount of NaCl in the assay and use of SSB and SSB-113 are indicated at the top of the gel.

SSB-113 (first data point). However, as the salt concentration was raised, DNA synthesis using SSB-113 diminished much more quickly than reactions using wild-type SSB to coat the template. These results are similar to those observed using Pol III* – χ in Figure 2.

In Figure 9B, the effect of SSB-113 on the speed and processivity of primer extension was examined at different salt concentrations by removing rapid time points and analyzing the products in a native agarose gel. The 6 s time point shows that the leading edge of the extension products at 40 and 80 mM NaCl using SSB-113 is in a similar position to those using SSB. This observation suggests that the speed of synthesis at 40 and 80 mM NaCl is not significantly altered on SSB-113-coated ssDNA. At 80 mM NaCl and above, templates coated with SSB-113 produce a smear, indicating that many of the polymerase molecules prematurely dissociate from the template. Hence, the inefficient elongation using SSB-113 at 80 mM NaCl would appear to lie in a decreased processivity rather than a decreased intrinsic speed of polymerization. At 120 and 180 mM NaCl, the extension products on both SSB-113- and wild-type SSB-coated DNA diminish significantly, although the amount of replication observed with SSB is greater than that observed using SSB-113 at both salt concentrations.

In Figure 10, primed M13mp18 ssDNA coated with SSB-113 was compared with the same template coated

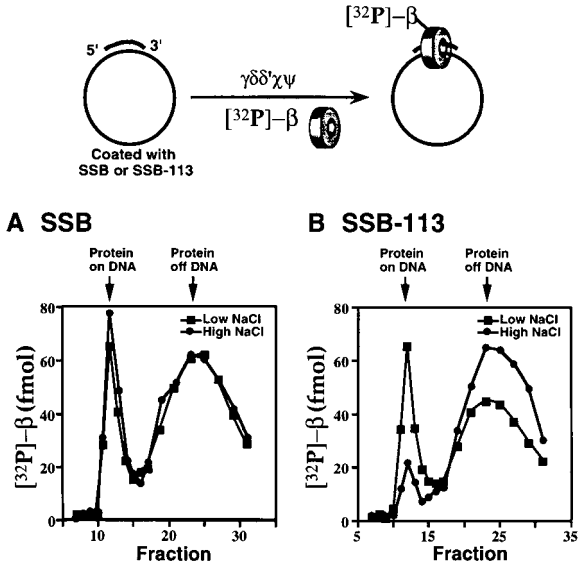


Fig. 10. SSB-113 does not confer resistance to NaCl on clamp loading by γ complex. [^{32}P]β subunit was assembled onto singly primed M13mp18 ssDNA coated with either SSB (**A**) or SSB-113 (**B**) in either 15 (■) or 135 mM (●) added NaCl. Reactions were gel filtered to resolve [^{32}P]β bound to DNA (fractions 10–15) from [^{32}P]β remaining free in solution (fractions 17–30).

with wild-type SSB when used as a substrate for [^{3}H]β assembly by γ complex. The results in Figure 10A show that similar amounts of [^{3}H]β clamps are assembled onto SSB-coated DNA at low and high salt. The results using SSB-113, in Figure 10B, show that at low salt a similar amount of [^{3}H]β is assembled onto SSB-113-coated DNA as on SSB-coated DNA. However, at high salt, the γ complex is much less efficient in assembling [^{3}H]β onto SSB-113-coated DNA compared with SSB-coated DNA.

The SSB-113 mutant cells are temperature sensitive for replication (Vales *et al.*, 1980), and thus these replication reactions would be expected to display temperature sensitivity as well. We have performed replication and clamp loading reactions at 30 and 42°C under several different NaCl concentrations, but the results are not temperature sensitive (data not shown). The temperature defect of SSB-113 in *in vitro* reactions using Pol III holoenzyme is explained further in the Discussion. Alternatively, the χ–SSB contact is involved in the repair- and recombination-defective phenotypes of SSB-113 that occur at all temperatures examined (Glassberg *et al.*, 1979; Vales *et al.*, 1980; Golub and Low, 1983; Lieberman and Witkin, 1983; Whittier and Chase, 1983; Laine and Meyer, 1992).

Table II shows another line of evidence that a specific interaction between the holoenzyme and SSB is functional in stabilizing holoenzyme action to added salt. The efficiency of DNA synthesis was compared at low and high NaCl using primed M13mp18 ssDNA coated with either *E.coli* SSB, *E.coli* SSB-113, the T4 SSB (gene 32 protein), the human SSB (RP-A) or no SSB. The results show that the *E.coli* SSB is the only one that stabilizes the holoenzyme activity in the presence of 120 mM NaCl. Use of *E.coli* SSB yielded 56% of the remaining activity compared with 17, 9 and 2.5% remaining activity for use of T4 gene 32 protein, human RP-A and *E.coli* SSB-113, respectively.

Table II. The effect of different SSBs on DNA replication by DNA polymerase III holoenzyme

SSB	DNA synthesis (pmol)	
	0 M NaCl	120 mM NaCl
None	6.5 ± 0.45	3.45 ± 0.85
E.coli SSB	55.3 ± 2.5	30.75 ± 4.85
E.coli SSB-113	63.65 ± 0.65	1.6 ± 0.2
T4 gene 32 protein	12.9 ± 0.7	2.15 ± 0.25
Human RP-A	14.0 ± 0.2	1.3 ± 0.2

Singly primed M13mp18 ssDNA was coated with the following SSBs: wild-type SSB from *E.coli*, SSB-113 from *E.coli*, gene 32 protein from phage T4 and three subunit RP-A from human. Replication was performed using Pol III* and β as described in Materials and methods. The results are an average of three experiments.

SSB-113 does not interact with χ

The deficiencies in replication using SSB-113 (Figures 9 and 10) are quite similar to those observed using Pol III* – χ (Figures 2–4). This similar behavior of both Pol III* – χ and SSB-113, combined with the knowledge that χ is the SSB interactive subunit of Pol III holoenzyme, suggested that the defect in SSB-113 may be based in lack of, or altered, interaction between SSB-113 and the χ subunit. In Figure 11, interaction between χ and either SSB or SSB-113 was examined by SPR.

In Figure 11A, biotin 5′ end-labeled oligo(dT_{100}) was immobilized to a sensor chip coated with streptavidin. Following this, the oligonucleotide was coated with either SSB or SSB-113. A solution of χ was injected over the immobilized SSB–ssDNA complex (first arrow), resulting in an increase in RU due to interaction of the χ with the SSB–ssDNA complex (solid line). Following this injection, the chip was washed with SPR buffer (second arrow) during which time the RU decreased due to dissociation of χ from the SSB–ssDNA complex. The amount of χ used was sufficient to saturate the SSB, and the observed stoichiometry of χ:SSB tetramer was 3.7:1 (similar to the stoichiometry of 4 χ per SSB tetramer in Figure 8) and 0.48 χ per SSB-113 tetramer. The K_d for this interaction was estimated from the SPR data to be 330 nM. This observed K_d value for interaction of χ with SSB is within 6-fold of the 53 nM K_d obtained by equilibrium gel filtration. The difference in K_d may be due to the higher concentration of NaCl and higher temperature used in the SPR analysis.

To determine whether χ interacts with SSB-113, SSB-113 was passed over the immobilized ssDNA (Figure 11A, dashed line), followed by injection of χ. Very little increase in RU was observed, indicating that the interaction between χ and SSB-113 is significantly reduced relative to wild-type SSB (dashed line; estimated $K_d \approx 16.8$ μM).

χ interacts with the C-terminus of SSB

The lack of interaction between χ and SSB-113 suggests that the C-terminus of SSB is necessary for this interaction. To determine whether the C-terminus is sufficient for interaction with χ, a biotinylated peptide corresponding to the C-terminal 15 amino acids of SSB was immobilized on a sensor chip coated with streptavidin. Upon flowing χ over the chip, a signal was observed indicating interaction with χ (Figure 11B). As expected, χ did not interact

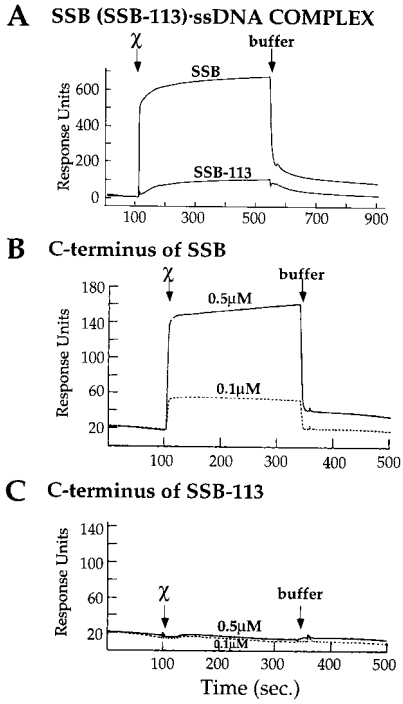


Fig. 11. SSB-113 does not interact with the χ subunit. The interaction of χ with either SSB or SSB-113 was studied using SRP analysis as described in Materials and methods. (**A**) A biotinylated DNA oligonucleotide was immobilized onto a streptavidin-coated sensor chip and then either SSB or SSB-113 was passed over the top. Then a solution of χ was passed over the SSB–ssDNA complex (first arrow) followed by SPR buffer (second arrow). Following each analysis, the sensor chip was regenerated as described in Materials and methods. (**B**) A biotinylated peptide derived from the last 15 amino acids of SSB was immobilized to a streptavidin-coated sensor chip followed by injections of χ at the indicated concentrations. (**C**) A biotinylated peptide derived from the last 15 amino acids of SSB-113 was immobilized to a streptavidin-coated sensor chip followed by injection of χ at the indicated concentrations. In (B) and (C), the first arrow marks the beginning of the χ injection and the second arrow indicates the start of the SPR buffer injection.

with a biotinylated 15 residue peptide corresponding to the C-terminus of SSB-113 (Figure 11C).

Discussion

Generality of interaction between the C-terminus of SSB and other proteins

This study, using Pol III holoenzyme subassemblies and pure subunits, shows that the χ subunit is the major contact between Pol III holoenzyme and SSB. In light of these results, one may explain the observation that elongation and proofreading by Pol III holoenzyme and Pol III* (they both contain γ complex) are stimulated by SSB whereas Pol III' (Pol III* that lacks γ complex) is either not affected or is inhibited (Fay *et al.*, 1981, 1982).

SSB plays an essential role in a variety of DNA metabolic processes including replication, recombination and repair (Chase, 1984; Meyer and Laine, 1990; Lohman and Ferrari, 1994). Accordingly, SSB appears to interact directly with other proteins. For example, using immobilized SSB as an affinity chromatography matrix, Perrino *et al.* demonstrated direct interactions between SSB and several other proteins ranging in size from 14 to 160 kDa (Perrino *et al.*, 1988). Further, there is preliminary evidence for direct interaction of SSB with Pol II (Molineux and Gefter, 1974), exonuclease I (Molineux and Gefter, 1975) and PriB (Low *et al.*, 1982). Therefore, it is not surprising that contact between SSB and Pol III holoenzyme can be added to this list. We have also detected a direct interaction between SSB and primase in the absence of DNA. In fact, the temperature-sensitive effect of SSB-113 is exhibited in reactions using Pol III holoenzyme on ssDNA that is primed by the action of primase. This reaction presently is under study and will be the subject of a future report.

Interaction between SSB and replication proteins extends beyond the *E.coli* system. A physical interaction of two other well-characterized SSBs (T4 gene 32 protein and T7 gene 2.5 protein) with their respective DNA polymerase subunits has been demonstrated (Cha and Alberts, 1988; Kim *et al.*, 1992). The T4 gene 32 protein has also been shown to interact with T4 primase (Cha and Alberts, 1988). The T7 gene 2.5 protein also interacts with the T7 gene 4 protein which contains both helicase and primase activity (Kim and Richardson, 1994). In the eukaryotic system, the human RP-A interacts with DNA polymerase α (Dornreiter *et al.*, 1992).

This study has localized a major site of interaction between χ and SSB to the C-terminus of SSB. Where is the C-terminus of SSB in the protein structure? The three-dimensional structure of the DNA-binding domain of *E.coli* SSB has been solved recently (Raghunathan *et al.*, 1997). This region, however, does not include the C-terminus of SSB. Perhaps a clue to the position of the C-terminus can be taken from the similarity of *E.coli* SSB to the T4 gene 32 protein. Phage T4 gene 32 protein is functionally similar to SSB; both bind to ssDNA and are essential for DNA replication. Furthermore, the C-terminus of gene 32 protein is essential for interaction with other proteins, including T4 DNA polymerase (Hurley *et al.*, 1993). Similarly to *E.coli* SSB, the C-terminus of gene 32 protein is not needed for DNA binding (Spicer *et al.*, 1979). The crystal structure shows that the C-terminus of gene 32 protein forms an α helix which is on the surface of the molecule and thus may be available for interaction with other proteins (Shamoo *et al.*, 1995).

Recently, the crystal structure of a 1:1 complex of the χ and ψ subunits has been solved (J.Gulbis, J.Finkelstein, M.O'Donnell and J.Kuriyan, unpublished). The structure of the χψ complex shows that the side of χ on the end of the rod-shaped χψ complex is highly basic. Perhaps this basic region of χ is the touchpoint to the acidic C-terminus of *E.coli* SSB. Further studies are planned to address this possibility.

An alignment of several SSBs is shown in Figure 12. It is interesting to note that the C-termini of these bacterial SSBs have acidic residues in common. Thus, it seems possible that the acidic C-terminus of these SSBs may be used to interact with other proteins. Support for this lies in observations that the C-terminus of both T4 gene 32 protein and T7 gene 2.5 protein are also highly acidic and are important for interaction with other proteins involved in DNA metabolism. The C-terminus of T4 gene 32 protein interacts with several proteins including the T4 primase and DNA polymerase (Burke *et al.*, 1980), T4 gene 59 protein (Morrical *et al.*, 1996) and the T4 UvsY member of the recombination machinery (Jiang *et al.*, 1993). It was also demonstrated that a deletion of the

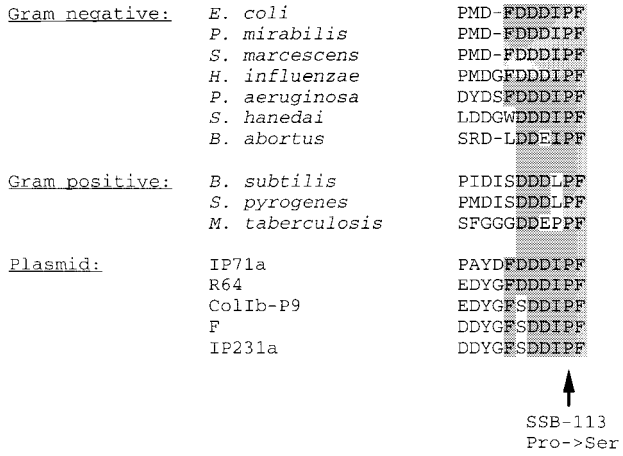


Fig. 12. Alignment of SSBs from diverse sources. The amino acid sequences of 15 SSB proteins from Gram-negative and Gram-positive bacteria and several plasmid-encoded SSBs are aligned. Amino acids which are conserved in 10 out of the 15 sequences are shaded.

C-terminus of T4 gene 32 protein has a severe effect on DNA replication *in vitro* and *in vivo* (Hurley *et al.*, 1993). Similarly, the C-terminus of T7 gene 2.5 protein is essential for physical and functional interaction with the T7 DNA polymerase (Kim and Richardson, 1994). Further, it was demonstrated that a mutant of *E.coli* SSB in which the last 10 amino acids were removed cannot substitute for the wild-type protein *in vivo* (Curth *et al.*, 1996).

Function of the χ–SSB contact in Pol III holoenzyme action

Pol III holoenzyme constituted in the absence of χ (Pol III – χ) appears less processive than the complete Pol III holoenzyme under conditions of elevated salt. Further, the β clamp loading activity of γ complex lacking χ is also less resistant to added NaCl than is the complete γ complex. These replication deficiencies of χ-less enzymes appear to be based on the loss of the χ–SSB contact. These studies formed the basis for examining the defect of SSB-113 at a molecular level. As summarized above, SSB-113 has a single amino acid replacement, and its interaction with χ is significantly reduced. Study of Pol III holoenzyme and γ complex on templates coated with SSB-113 showed the same low resistance to added NaCl as the enzymes lacking χ. This result is consistent with the χ–SSB contact having the dual function of aiding both chain elongation and clamp loading.

This dual role for χ suggests that it maintains contact with SSB during β assembly and during elongation. Previous studies have shown that the γ complex and core polymerase bind the same position on β and must switch their positions on the β ring for holoenzyme action (Naktinis *et al.*, 1996). First γ complex must bind β to assemble it onto DNA, and then core must interact with β for processivity in DNA synthesis (see Figure 13). There is only one χ subunit in γ complex, and only one γ complex in the holoenzyme (Onrust *et al.*, 1995b). Hence, if χ maintains contact with SSB during initiation and elongation, this single χ subunit must accommodate the switch between γ complex and core for β while maintaining contact with SSB. This point is illustrated in the scheme of Figure 13. In Figure 13A, the γ complex within the holoenzyme is shown interacting with SSB through the χ subunit of the χψ complex. The χψ complex is depicted as having a rod shape, as recently deduced from the crystal structure of the χψ complex (J.Gulbis, J.Finkelstein, M.O'Donnell and J.Kuriyan, unpublished). The γ complex is also shown opening β, and having contact with the primed template as suggested by the DNA-dependent ATPase activity of γδδ' and γ complex (Onrust *et al.*, 1991). At low ionic strength, the γ complex need not interact with SSB for efficient assembly of β onto DNA. However, at elevated ionic strength, the χ–SSB contact presumably enhances the β loading activity of γ complex by helping to keep γ complex localized to its site of action. In Figure 13B, the core polymerase (αεθ) has switched positions with the γ complex in order to interact with β for processive synthesis. In the shift from Figure 13A to B, the χψ rod, attached to γδδ', is depicted as rotating such that contact with SSB is maintained during the transition between initiation and elongation. Maintenance of this χ–SSB contact throughout the switch between γ complex and core polymerase is indicated by the fact that χ enhances the efficiency of chain elongation under conditions of elevated ionic strength.

Maintaining this contact between χ and SSB during elongation may help the processivity in elevated ionic strength by contributing to the forces that keep Pol III holoenzyme attached to the β clamp and primed template. However, the χ–SSB contact must be transient during elongation or else the holoenzyme could not move forward during primer extension. It appears from results in this study (e.g. Figure 7A and B) that the affinity of χ for SSB is enhanced by the presence of ssDNA. This difference in affinity for SSB on and off DNA may provide the basis for breaking and rejoining χ–SSB contact during holoenzyme movement. Hence, as the holoenzyme advances and the nearest SSB is displaced, χ will lose its affinity for the free SSB. This would allow Pol III holoenzyme to advance and χ could bind the next SSB on the ssDNA. Alternatively, χ itself may help destabilize the SSB–ssDNA complex, thereby acting as a 'molecular cowcatcher' for SSB displacement in advance of the holoenzyme. Further study is needed to determine whether either of these speculations are true or if some other mechanism is operative.

Use of χ at a replication fork

The two core polymerases within the holoenzyme coordinately synthesize the two strands of duplex DNA. The holoenzyme contains only one γ complex, and thus only one χ subunit. The question arises of on which strand the single χ subunit of Pol III holoenzyme functions. Within the replisome, the holoenzyme contacts the DnaB helicase, presumably leaving no room for SSB to bind before ssDNA is converted to duplex. In fact, leading strand synthesis has been shown to proceed as efficiently in the absence of SSB as in its presence (Mok and Marians, 1987). However, the lagging strand is synthesized discontinuously, leading to the generation of ssDNA and thus coating of the ssDNA by SSB. Further, primase acts upon this strand to form the RNA-primed sites upon which the γ complex assembles β clamps. These priming and clamp formation steps occur repeatedly once every 1–2 s. After this step, the Pol III holoenzyme must processively elong-

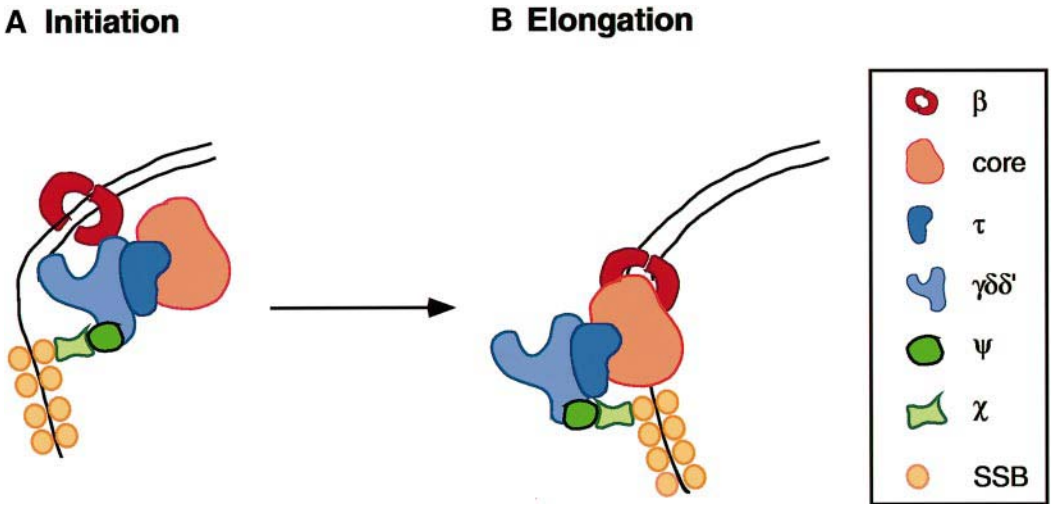


Fig. 13. Scheme for roles of χ in Pol III holoenzyme initiation and elongation. A single χ subunit is attached to the γδδ' subunits of γ complex through the ψ subunit. The τ dimer attaches one γ complex to two molecules of the core polymerase (for simplicity only one core and one τ subunit are shown in the diagram). The χ subunit, through its interaction with SSB, enhances γ complex action in loading β onto a primed site at physiological ionic strength. The scheme reflects this by showing contact between χ and SSB as β is being loaded onto primed DNA (**A**). The χ subunit also increases the processivity of Pol III holoenzyme during elongation at elevated ionic strength. The scheme reflects this by maintaining the χ–SSB contact as the core polymerase exchanges its position with γ complex for interaction with the β ring (**B**).

ate primers on SSB-coated lagging strand ssDNA (also aided by χ) to form full-length Okazaki fragments. Therefore, it seems likely that during replication fork propagation, the χ–SSB interaction is devoted mainly to action on the lagging strand. Action of a γ complex subunit on the lagging strand is in keeping with the numerous β clamps that must be assembled onto DNA during the discontinuous replication of the lagging strand.

Materials and methods

Materials

Labeled deoxy- and ribonucleoside triphosphates and [^{3}H]$NaBH_4$ (50–75 Ci/mmol) were from Dupont-New England Nuclear; unlabeled deoxy- and ribonucleoside triphosphates were from Pharmacia-LKB; streptavidin was from Pierce; peptides were from Chiron Mimotopes U.S.; CM carboxymethyldextran matrix-coated sensor chips CM5 were from BiaCore Inc.; BioGel A15m was from Bio-Rad; proteins were purified as described: α, ε, γ and τ (Studwell and O'Donnell, 1990), β (Kong *et al*., 1992), δ and δ' (Dong *et al.*, 1993), χ and ψ (Xiao *et al.*, 1993a), θ (Studwell-Vaughan and O'Donnell, 1993), SSB (Weiner *et al.*, 1975), SSB-113 (purified from *E.coli* strain KLC792/pKAC21 kindly provided by T.Lohman) (Kelman, 1995) and M13 gpII (Meyer and Geider, 1979a). Phage T4 gene 32 protein was from Pharmacia-Upjohn. Buffer A is 20 mM Tris–HCl (pH 7.5), 0.5 mM EDTA, 2 mM dithiothreitol (DTT) and 20% glycerol. Buffer B consists of 25 mM Tris–HCl (pH 7.5), 40 mM NaCl, 10% glycerol. Buffer C is 20 mM Tris–HCl (pH 7.5), 8 mM $MgCl_2$, 100 mM NaCl, 5% glycerol, 2 mM DTT and 50 μg/ml bovine serum albumin (BSA). Buffer D is 20 mM Tris–HCl (pH 7.5) and 5% glycerol. β clamping buffer was 20 mM Tris–HCl (pH 7.5), 8 mM $MgCl_2$, 0.5 mM ATP, 60 μM dCTP, 60 μM dGTP, 5 mM DTT, 0.1 mM EDTA and 4% glycerol. Replication buffer was β clamping buffer plus 40 μg/ml BSA. SPR buffer is 10 mM HEPES–HCl (pH 7.4), 150 mM NaCl and 0.005% Tween-20.

DNAs

M13mp18 was phenol extracted from phage and purified by two successive bandings (one downward and one upward) in cesium chloride gradients as described (Turner and O'Donnell, 1994). M13mp18 ssDNA was primed with a DNA 30mer (map position 6817–6846) as described (Studwell and O'Donnell, 1990). Synthetic DNA oligomers were synthesized by the Oligos Etc. company. These oligonucleotides were: biotinylated oligo(dT_{65}) and oligo(dT_{100}), oligo(dT_{65}), oligo(dT_{35}) and the 87mer (5′-CGCAATGCAGCACATCCCCCTTTCGCCAGCTGGCGTCGTACGCCGGGCAATATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGA).

Reconstituted protein complexes

γδδ'χψ, γδδ'ψ and γδδ' were reconstituted by incubating 1.7 mg of γ, 1.25 mg of δ, 1.0 mg of δ', ± 0.420 mg of ψ, ±0.52 mg of χ for 1 h at 15°C in 2 ml of buffer A. The complexes were then purified using a 1 ml mono-Q column as previously described (Onrust *et al.*, 1995a). Pol III* and Pol III' were reconstituted and purified as previously described (Studwell-Vaughan and O'Donnell, 1991; Onrust *et al.*, 1995b). Pol III* – χ was reconstituted as described for Pol III* except that the χ subunit was omitted from the reaction. Protein concentrations were determined from their extinction coefficient at 280 nm calculated from their amino acid composition, except for γ complex and Pol III* which were quantitated by the Protein Assay (Bio-Rad) method using BSA as a standard.

Radioactive proteins and complexes

Subunits. Proteins were labeled by reductive methylation as described (Kelman *et al.*, 1995) to specific activities of: χ, 70 c.p.m./fmol; θ, 31 c.p.m./fmol; δ', 22 c.p.m./fmol; and β, 117 c.p.m./fmol (as dimer). A derivative of β, β^{PK}, containing a seven residue recognition sequence for the catalytic subunit of cAMP-dependent protein kinase, was radiolabeled using [α-^{32}P]ATP and used in this study; it retains full activity in clamp loading and replication (Stukenberg *et al.*, 1994). The activities of [^{3}H]θ and [^{3}H]δ' were within 90% of that of the unlabeled subunit, as determined by constituting them into the nine subunit Pol III* followed by assaying replication activity with β on SSB-coated singly primed M13mp18 ssDNA (Stukenberg *et al.*, 1994). The [^{3}H]β retained 100% activity with Pol III* as judged by assaying replication activity with Pol III* on SSB-coated singly primed M13mp18 ssDNA (Stukenberg *et al.*, 1994).

Complexes. [^{3}H]Pol III' was constituted using [^{3}H]θ and purified from free subunits as described (Onrust *et al.*, 1995b). [^{3}H]γδδ'ψχ (γ complex), [^{3}H]γδδ'ψ and [^{3}H]γδδ' were constituted and purified from free subunits as described above under 'Reconstituted protein complexes', with the substitution of [^{3}H]δ' for the unlabeled δ'.

Gel filtration

Gel filtration analysis of protein–M13mp18 ssDNA complexes was performed at 4°C. Reactions were incubated for 5 min in 100 μl of buffer A at 37°C, then were loaded onto 5 ml BioGel A15m (Bio-Rad) columns equilibrated in buffer A containing 50 μg/ml BSA. The amount of Pol III* and its subassemblies, as well as the amount of ssDNA and SSB used in each analysis is described in the legends to Figures 5 and 6. Fractions of 200 μl were collected, and the amount of [^{3}H]protein in

each fraction was quantitated by scintillation counting of 150 µl aliquots of each fraction. Typical recovery of total radioactivity was 70–90%.

Gel filtration analyses of χ and SSB in the presence or absence of 87mer DNA oligonucleotide were performed at 5°C using an FPLC HR 10/30 Superose-12 column (Pharmacia-LKB) equilibrated in buffer B. χ (1.4 nmol as monomer) was incubated with SSB (0.7 nmol as tetramer) with or without 6 nmol of 87mer oligonucleotide in a total volume of 100 µl for 5 min at 37°C, then loaded onto the column. After the first 6 ml, fractions of 170 µl were collected and analyzed by 15% SDS–polyacrylamide gels (100 µl per lane) stained with Coomassie Brilliant Blue R-250.

Equilibrium gel filtration

The equilibrium gel filtration technique (Hummel and Dreyer, 1962) was used to determine the dissociation constant between χ and SSB, in the presence of oligo(dT_{65}). An FPLC HR 10/30 Superose-12 gel filtration column (Pharmacia-LKB) was equilibrated in buffer D containing 1 µM χ (0.1% of which was [^{3}H]χ) at 4°C. Then 2.5 nmol of oligo(dT_{65}) (without biotin) and 0.85 nmol of SSB (as monomer) were incubated in the same buffer (also containing 1 µM [^{3}H]χ) in a final volume of 100 µl for 5 min at 37°C. Following the incubation, the sample was loaded onto the column. After the first 5.2 ml, fractions of 190 µl were collected and the amount of [^{3}H]protein in the fractions was quantitated by scintillation counting of 150 µl aliquots. The K_d value was calculated as follows: the pmol of χ that eluted in the peak with the SSB–DNA complex (i.e. χ–SSB–DNA) was calculated from the specific activity of χ to be 807 pmol. The amount of SSB–DNA free is the total SSB–DNA added (850 pmol) minus the amount of χ bound to SSB–DNA (850 pmol – 807 pmol = 43 pmol). The K_d value was calculated as follows: K_d = ([χ][SSB–DNA free])/([χ–SSB–DNA]) = 1 µM (43/807) = 53 nM.

Surface plasmon resonance (SPR)

In the study of χ binding SSB–ssDNA complex (Figure 8A), streptavidin [0.1 mg/ml in 10 mM sodium acetate (pH 4.5)] was immobilized onto the CM carboxymethyldextran matrix-coated sensor chip CM5 by carbodiimide covalent linkage following the manufacturer's instructions (BiaCore Inc.) in a 30 µl injection at 5 µl/min. Resulting RU values were in the range of 1600–2000 RU. Then, 30 µl of biotinylated oligo(dT_{65}) (1 µM) was immobilized upon a 6 min injection over the streptavidin-coated chip, resulting in an additional 319 RU. Following this, 30 µl of 150 nM SSB (as tetramer) was passed over the immobilized DNA, resulting in a further increase of 754 RU (Figure 8C was performed as above but SSB was not included in the experiment). To immobilize SSB in the absence of ssDNA (Figure 8B), SSB was coupled to the CM chip using carbodiimide coupling in a 30 µl injection containing 150 nM SSB (as tetramer) in 10 mM sodium acetate (pH 4.5). The final RU of immobilized SSB was 1148 RU. Before the χ subunit was passed over the immobilized SSB–oligo(dT_{65}), SSB and oligo(dT_{65}), χ was first dialyzed against SPR buffer at 1 mg/ml and then diluted to the indicated concentrations (0.6, 1, 3, 5 or 10 µM) with SPR buffer. The solutions of χ were injected at a flow rate of 2 µl/min for 15 min, after which equilibrium was nearly achieved. After completing each analysis of χ over immobilized SSB, the surface of the chip was regenerated by injecting 10 µl of 0.1 M glycine (pH 9.5) to disrupt protein–protein complexes (5 µl/min). To disrupt χ–SSB–oligo(dT_{65}) complexes, 0.05% SDS in SPR buffer was used followed by injection of SSB and then the next solution of χ. The SDS treatment did not disrupt biotinylated oligonucleotide bound to immobilized streptavidin (i.e. at least 98% of the capacity of the immobilized biotinylated DNA to bind SSB was preserved).

In experiments comparing the ability of χ to bind SSB and SSB-113 (Figure 11A), biotinylated oligo(dT_{100}) was bound to streptavidin immobilized on the sensor chip as described above. Before injecting SSB and SSB-113 over the immobilized DNA, the proteins were pre-dialyzed at 1 mg/ml against SPR buffer. Then, either SSB (8 nM as tetramer) or SSB-113 (25 nM as tetramer) was injected for 2 min at a flow rate of 5 µl/min over the immobilized DNA. The interaction between the ssDNA and SSB resulted in 890 and 1040 RU for SSB and SSB-113, respectively. This was followed by a 7.5 min injection of χ (3 µM pre-dialyzed in SPR buffer) at 5 µl/min. After completing each analysis, the chip was regenerated with SDS treatment as described above.

To examine interaction of χ with the C-terminus of SSB and SSB-113 (Figure 11B and C), biotinylated peptides derived from the last 15 amino acids of wild-type SSB (biotin-P-S-N-E-P-P-M-D-F-D-D-D-I-P-F-COOH) or SSB-113 (biotin-P-S-N-E-P-P-M-D-F-D-D-D-I-S-F-COOH) were injected over a sensor chip pre-coated with streptavidin. The interactions between the biotinylated peptides and streptavidin resulted in 67 and 99 RU for SSB and SSB-113, respectively. The χ subunit, pre-dialyzed against SPR buffer, was diluted to concentrations of 0.1 and 0.5 µM and then injected over immobilized SSB at a flow rate of 5 µl/min for 4 min. After completing each analysis, the surface of the chip was regenerated by injecting 10 µl of 0.1 M glycine (pH 9.5) to disrupt protein–peptide complexes (5 µl/min). The regeneration released non-covalently bound protein, and preserved at least 95% of the capacity of the immobilized peptide to bind solution phase analyte.

β clamping reactions using γ complex and γδδ'ψ

β clamping reactions of Figure 4 contained 300 fmol of circular DNA [either singly primed M13mp18 ssDNA or singly nicked circular duplex, (as described below)], 250 fmol of γ complex (50 ng) or γδδ'ψ (42 ng), 500 fmol of [^{3}H]β (40 ng) and 13 µg of SSB in 100 µl of β clamping buffer. Reactions were incubated for 5 min at 37°C in the presence or absence of 160 mM potassium glutamate, then applied to 5 ml BioGel A15m columns (Bio-Rad) pre-equilibrated with buffer C containing 50 µg/ml BSA. Fractions of 200 µl were collected, and [^{3}H]β was quantitated by analyzing 150 µl of the indicated fraction by liquid scintillation counting.

Assays were performed using either singly primed M13pm18 ssDNA or nicked plasmid DNA. Singly primed M13mp18 ssDNA was prepared as described (Studwell and O'Donnell, 1990). Singly nicked circular duplex DNA (RFII) was prepared from pBluescript (SK+) form I DNA as follows. pBluescript plasmid was purified by banding twice in cesium chloride equilibrium density gradients. The pBluescript plasmid was singly nicked with M13 gpII protein for 20 min at 37°C as described (Meyer and Geider, 1979b). The gpII nicks once in the phage origin in 50–60% of the plasmid molecules without remaining covalently attached to the DNA (Meyer and Geider, 1979b).

The β clamping reactions of Figure 10 were performed as described above with the following exceptions: reactions (50 µl) contained 200 fmol of singly primed M13mp18 ssDNA coated with 5.8 µg of either SSB or SSB-113, 1 pmol of [^{32}P]β, and 40 ng of γ complex. Reactions were performed in the presence of either 15 or 135 mM added NaCl.

Replication assays

The replication assays of Figures 2 and 3 contained 63 ng (32 fmol) of singly primed M13mp18 ssDNA, 0.8 µg of SSB, 27 ng (332 fmol as dimer) of β and 88 fmol of Pol III*, or Pol III* lacking χ, in a final volume of 25 µl of replication buffer. Reactions were first pre-incubated for 5 min at 37°C in the absence of added NaCl to form the initiation complex of Pol III holoenzyme on the primed DNA. Following the pre-incubation, NaCl was added (using a 2 M stock) to concentrations of either 0, 40, 80, 120, 160 or 200 mM, and 60 µM dATP and 20 µM [α-^{32}P]dTTP (3000 c.p.m./pmol) were added to initiate DNA synthesis. In the experiment of Figure 2, reactions were quenched after 20 s at 37°C and DNA synthesis was quantitated using DE81 paper as described (Rowen and Kornberg, 1979). The time points in Figure 3 were each performed as individual reactions as described above, except that they were quenched at the times indicated in the figure. Products were analyzed in a 0.7% native agarose gel followed by autoradiography. Reactions of Figure 9A were performed as described above except the ssDNA was coated with 0.8 µg of either SSB or SSB-113, and 75 fmol of Pol III* was used. Due to the NaCl in the SSB-113 preparation, the initial NaCl concentration was 40 mM (and an equal amount of NaCl was added to the wild-type SSB in experiments comparing SSB-113 with SSB). Following the pre-incubation, NaCl was added (either no addition, or addition of 40, 80 or 120 mM NaCl) to give final concentrations of 40, 80, 120 and 160 mM NaCl. Replication was initiated upon addition of 60 µM dATP and 20 µM [α-^{32}P]dTTP (3000 c.p.m./pmol), and reactions were quenched with an equal volume of 1% SDS and 40 mM EDTA at 6, 9, 12 and 20 s. The 20 s time points are presented in the analysis of Figure 9A, and the 6, 9 and 12 s reactions were analyzed in a 0.7% native agarose gel followed by autoradiography in Figure 9B.

Acknowledgements

We are grateful to Dr Tim Lohman for the SSB-113 overexpression plasmid and for a sample of F-SSB encoded by F′. This work was supported by a grant from the National Institutes of Health (GM38839).

References

Burke,R.L., Alberts,B.M. and Hosoda,J. (1980) Proteolytic removal of the COOH terminus of the T4 gene 32 helix-destabilizing protein

alters the T4 *in vitro* replication complex. *J. Biol. Chem.*, **255**, 11484–11493.
Cha,T.A. and Alberts,B.M. (1988) *In vitro* studies of the T4 bacteriophage DNA replication system. *Cancer Cells*, **6**, 1–10.
Chase,J.W. (1984) The role of *E.coli* single-stranded DNA binding protein in DNA metabolism. *BioEssays*, **1**, 218–222.
Chase,J.W. and Williams,K.R. (1986) F sex factor encodes a single-stranded DNA binding protein (SSB) with extensive sequence homology to *Escherichia coli* SSB. *Proc. Natl Acad. Sci. USA*, **80**, 5480–5484.
Chase,J.W., Merrill,B.M. and Williams,K.R. (1983) Single-stranded DNA binding proteins required for DNA replication. *Annu. Rev. Biochem.*, **55**, 103–136.
Chase,J.W., L'Italien,J.J., Murphy,J.B., Spicer,E.K. and Williams,K.R. (1984) Characterization of the *Escherichia coli* SSB-113 mutant single-stranded DNA-binding protein. *J. Biol. Chem.*, **259**, 805–814.
Curth,U., Genschel,J, Urbanke,C. and Greipel,J. (1996) *In vitro* and *in vivo* function of the C-terminus of *Escherichia coli* single-stranded DNA binding protein. *Nucleic Acids Res.*, **24**, 2706–2711.
Dallmann,H.G. and McHenry,C.S. (1995) DnaX complex of *Escherichia coli* DNA polymerase III holoenzyme. Physical characterization of the DnaX subunits and complexes. *J. Biol. Chem.*, **270**, 29563–29569.
Dong,Z., Onrust,R., Skangalis,M. and O'Donnell,M. (1993) DNA polymerase III accessory proteins. I. *holA* and *holB* encoding δ and δ'. *J. Biol. Chem.*, **268**, 11758–11765.
Dornreiter,I., Erdile,L.F., Gilbert,I.U., von Winkler,D., Kelly,T.J. and Fanning,E. (1992) Interaction of DNA polymerase α-primase with cellular replication protein A and SV40 T antigen. *EMBO J.*, **11**, 769–776.
Fay,P.J., Johanson,K.O., McHenry,C.S. and Bambara,R.A. (1981) Size classes of products synthesized processively by DNA polymerase III and DNA polymerase III holoenzyme of *Escherichia coli*. *J. Biol. Chem.*, **256**, 976–983.
Fay,P.J., Johanson,K.O., McHenry,C.S. and Bambara,R.A. (1982) Size classes of products synthesized processively by two subassemblies of *Escherichia coli* DNA polymerase III holoenzyme. *J. Biol. Chem.*, **257**, 5692–5699.
Fradkin,L.G. and Kornberg,A. (1992) Prereplicative complexes of components of DNA polymerase III holoenzyme of *Escherichia coli*. *J. Biol. Chem.*, **267**, 10318–10322.
Glassberg,J., Meyer,R.R. and Kornberg,A. (1979) Mutant single-strand binding protein of *Escherichia coli*: genetic and physiological characterization. *J. Bacteriol.*, **140**, 14–19.
Golub,E.I. and Low,K.B. (1983) Indirect stimulation of genetic recombination. *Proc. Natl Acad. Sci. USA*, **80**, 1401–1405.
Granzow,R. and Reed,R. (1992) Interactions in the fourth dimension. *Biotechnology*, **10**, 390–393.
Hummel,J.P. and Dreyer,W.J. (1962) Measurement of protein-binding phenomena by gel filtration. *Biochim. Biophys. Acta*, **63**, 530–532.
Hurley,J.M., Chervitz,S.A., Jarvia,T.C., Singer,B.S. and Gold,L. (1993) Assembly of the bacteriophage T4 replication machine requires the acidic carboxy terminus of gene 32 protein. *J. Mol. Biol.*, **229**, 398–418.
Jiang,H., Giedroc,D. and Kodadek,T. (1993) The role of protein–protein interactions in the assembly of the presynaptic filament for T4 homologous recombination. *J. Biol. Chem.*, **268**, 7904–7911.
Kelman,Z. (1995) Interactions between DNA polymerases and other cellular proteins. Ph.D. Thesis, Cornell University.
Kelman,Z. and O'Donnell,M. (1995) DNA polymerase III holoenzyme: structure and function of a chromosomal replicating machine. *Annu. Rev. Biochem.*, **64**, 171–200.
Kelman,Z., Naktinis,V. and O'Donnell,M. (1995) Radiolabeling of proteins for biochemical studies. *Methods Enzymol.*, **262**, 430–442.
Kim,Y.T. and Richardson,C.C. (1994) Acidic carboxyl-terminal domain of gene 2.5 protein of bacteriophage T7 is essential for protein–protein interactions. *J. Biol. Chem.*, **269**, 5270–5278.
Kim,Y.T., Tabor,S., Churchich,J.E. and Richardson,C.C. (1992) Interaction of gene 2.5 protein and DNA polymerase of bacteriophage T7. *J. Biol. Chem.*, **267**, 15032–15040.
Kong,X.-P., Onrust,R., O'Donnell,M. and Kuriyan,J. (1992) Three dimensional structure of the β subunit of *Escherichia coli* DNA polymerase III holoenzyme: a sliding DNA clamp. *Cell*, **69**, 425–437.
Kornberg,A. and Baker,T. (1992) *DNA Replication*. 2nd edn. W.H.Freeman and Company, New York.
LaDuca,R.J., Fay,P.J., Chuang,C., McHenry,C.S. and Bambara,R.A. (1983) Site-specific pausing of deoxyribonucleic acid synthesis catalyzed by four forms of *Escherichia coli* DNA polymerase III. *Biochemistry*, **22**, 5177–5188.
Laine,P.S. and Meyer,R.R. (1992) Interaction of the heat shock protein GroEL of *Escherichia coli* with single-stranded DNA-binding protein: suppression of *SSB-113* by *groEL46*. *J. Bacteriol.*, **174**, 3204–3211.
Lieberman,H.B. and Witkin,E.M. (1983) DNA degradation, UV sensitivity and SOS-mediated mutagenesis in strains of *Escherichia coli* deficient in single-strand DNA binding protein: effects of mutations and treatments that alter levels of exonuclease V or RecA protein. *Mol. Gen. Genet.,* **190**, 92–100.
Lohman,T.M. and Ferrari,M.E. (1994) *Escherichia coli* single-stranded DNA-binding protein: multiple DNA-binding modes and cooperativities. *Annu. Rev. Biochem.*, **63**, 527–570.
Low,R.L., Shlomai,J. and Kornberg,A. (1982) Protein n, a primosomal DNA replication protein of *Escherichia coli*. Purification and characterization. *J. Biol. Chem.*, **257**, 6242–6250.
Maki,S. and Kornberg,A. (1988) DNA polymerase III holoenzyme of *Escherichia coli*. II. A novel complex including the γ subunit essential for processive synthesis. *J. Biol. Chem*., **263**, 6555–6560.
Maki,H., Maki,S. and Kornberg,A. (1988) DNA polymerase III holoenzyme of *Escherichia coli*. IV. The holoenzyme is an asymmetric dimer with twin active sites. *J. Biol. Chem.*, **263**, 6570–6578.
McHenry,C.S. (1982) Purification and characterization of DNA polymerase III'. Identification of τ as a subunit of the DNA polymerase III holoenzyme. *J. Biol. Chem*., **257**, 2657–2663.
McHenry,C.S. and Crow,W. (1979) DNA polymerase III of *Escherichia coli*. Purification and identification of subunits. *J. Biol. Chem.*, **254**, 1748–1753.
Meyer,T.F. and Geider,K. (1979a) Bacteriophage fd gene II-protein. I. Purification, involvement in RF replication, and the expression of gene II. *J. Biol. Chem.*, **254**, 12636–12641.
Meyer,T.F. and Geider,K. (1979b) Bacteriophage fd gene II-protein. II. Specific cleavage and relaxation of supercoiled RF from filamentous phages. *J. Biol. Chem.*, **254**, 12642–12646.
Meyer,R.R. and Laine,P.W. (1990) The single-stranded DNA-binding protein of *Escherichia coli*. *Microbiol. Rev.*, **54**, 342–380.
Mok,M. and Marians,K.J. (1987) The *Escherichia coli* preprimosome and DnaB helicase can form replication forks that move at the same rate. *J. Biol. Chem.*, **262**, 16644–16654.
Molineux,I.J. and Gefter,M.L. (1974) Properties of the *Escherichia coli* DNA binding (unwinding) protein: interaction with DNA polymerase and DNA. *Proc. Natl Acad. Sci. USA*, **71**, 3858–3862.
Molineux,I.J. and Gefter,M.L. (1975) Properties of the *Escherichia coli* DNA-binding (unwinding) protein interaction with nucleolytic enzymes and DNA. *J. Mol. Biol.*, **98**, 811–825.
Morrical,S.W., Beernink,H.T.H., Dash,A. and Hempstead,K. (1996) The gene 59 protein of bacteriophage T4. *J. Biol. Chem.*, **271**, 20198–20207.
Naktinis,V., Onrust,R. Fang,L. and O'Donnell,M. (1995) Assembly of a chromosomal replication machine: two DNA polymerases, a clamp loader and sliding clamps in one holoenzyme particle. II. Intermediate complex between the clamp loader and its clamp. *J. Biol. Chem.*, **270**, 13358–13365.
Naktinis,V., Turner,J. and O'Donnell,M. (1996) A molecular switch in a replication machine defined by an internal competition for protein rings. *Cell*, **84**, 137–145.
O'Donnell, M. (1987). Accessory proteins bind a primed template and mediate rapid cycling of DNA polymerase III holoenzyme from *Escherichia coli*. *J. Biol. Chem*., **262**, 16558–16565.
O'Donnell,M. and Studwell,P.S. (1990) Total reconstitution of DNA polymerase III holoenzyme reveals dual accessory protein clamps. *J. Biol. Chem.*, **265**, 1179–1187.
Onrust,R., Stukenberg,P.T. and O'Donnell, M. (1991) Analysis of the ATPase subassembly which initiates processive DNA synthesis by DNA polymerase III holoenzyme. *J. Biol. Chem.*, **266**, 21681–21686.
Onrust,R., Finkelstein,J., Naktinis,V., Turner,J., Fang,L. and O'Donnell,M. (1995a) Assembly of a chromosomal replication machine: two DNA polymerases, a clamp loader and sliding clamps in one holoenzyme particle. I. Organization of the clamp loader. *J. Biol. Chem.*, **270**, 13348–13357.
Onrust,R., Finkelstein,J., Turner,J., Naktinis,V. and O'Donnell,M. (1995b) Assembly of a chromosomal replication machine: two DNA polymerases, a clamp loader and sliding clamps in one holoenzyme particle. III. Interface between two polymerases and the clamp loader. *J. Biol. Chem.*, **270**, 13366–13377.
Perrino,F.W., Meyer,R.R., Bobst,A.M. and Rein,D.C. (1988) Interaction of a folded chromosome-associated protein with single-stranded DNA-binding protein of *Escherichia coli*, identified by affinity chromatography. *J. Biol. Chem.*, **263**, 11833–11839.

Raghunathan,S., Ricard,C.S., Lohman,T.M. and Waksman,G. (1997) Crystal structure of the homo-tetrameric DNA binding domain of single-stranded DNA-binding protein determined by multiwavelength X-ray diffraction on the selenomethionyl protein at 2.9-Å resolution. *Proc. Natl Acad. Sci. USA*, **94**, 6652–6657.

Scott,J.F., Eisenberg,S., Bertsch,L.L. and Kornberg,A. (1977) A mechanism of duplex DNA replication revealed by enzymatic studies of phage ϕX174: catalytic strand separation in advance of replication. *Proc. Natl Acad. Sci. USA*, **74**, 193–197.

Shamoo,Y., Friedman,A.M., Parsons,M.R., Konigsberg,W.H. and Steitz,T.A. (1995) Crystal structure of a replication fork single-stranded DNA binding protein (T4 gp32) complexed to DNA. *Nature*, **376**, 362–366.

Spicer,E.K., Williams,K.R. and Konigsberg,W.H. (1979) T4 gene 32 protein trypsin-generated fragments. *J. Biol. Chem.*, **254**, 6433–6436.

Studwell,P.S. and O'Donnell,M. (1990) Processive replication is contingent on the exonuclease subunit of DNA polymerase III holoenzyme. *J. Biol. Chem.*, **265**, 1171–1178.

Studwell-Vaughan,P.S. and O'Donnell,M. (1991) Constitution of the twin polymerase of DNA polymerase III holoenzyme. *J. Biol. Chem*., **266**, 19833–19841.

Studwell-Vaughan,P.S. and O'Donnell,M. (1993) DNA polymerase III accessory proteins. V. θ encoded by *holE*. *J. Biol. Chem.*, **268**, 11785–11791.

Stukenberg,P.T. and O'Donnell,M. (1995) Assembly of a chromosomal replication machine: two DNA polymerases, a clamp loader and sliding clamps in one holoenzyme particle. V. Four different polymerase–clamp complexes on DNA. *J. Biol. Chem.*, **270**, 13384–13391.

Stukenberg,P.T., Studwell-Vaughan,P.S. and O'Donnell,M. (1991) Mechanism of the sliding β-clamp of DNA polymerase III holoenzyme. *J. Biol. Chem.*, **266**, 11328–11334.

Stukenberg,P.T., Turner,J. and O'Donnell,M. (1994) An explanation for lagging strand replication: polymerase hopping among DNA sliding clamps. *Cell*, **78**, 877–887.

Tsuchihashi,Z. and Kornberg,A. (1989) ATP interactions of the τ and γ subunits of DNA polymerase III holoenzyme of *Escherichia coli*. *J. Biol. Chem.*, **264**, 17790–17795.

Turner,J. and O'Donnell,M. (1994) Cycling of *E.coli* DNA polymerase III from one sliding clamp to another—a model for the lagging strand. *Methods Enzymol.*, **262**, 442–449.

Vales,L.D., Chase,J.W. and Murphy,J.B. (1980) Effect of *ssbA1* and *lexC113* mutations on lambda prophage induction, bacteriophage growth, and cell survival. *J. Bacteriol.*, **143**, 887–896.

Weiner,J.H., Bersch,L.L. and Kornberg,A. (1975) The deoxyribonucleic acid unwinding protein of *Escherichia coli*. Properties and functions in replication. *J. Biol. Chem.*, **259**, 1972–1980.

Whittier,R.F. and Chase,J.W. (1983) DNA repair properties of *Escherichia coli tif-1*, *recAo281* and *lexA1* strains deficient in single-strand DNA binding protein. *Mol. Gen. Genet.*, **190**, 101–111.

Xiao,H., Crombie,R., Dong,Z., Onrust,R. and O'Donnell,M. (1993a) DNA polymerase III accessory proteins. III. *holC* and *holD* encoding χ and ψ. *J. Biol. Chem.*, **268**, 11773–11778.

Xiao,H., Dong,Z. and O'Donnell,M. (1993b) DNA polymerase III accessory proteins. IV. Characterization of χ and ψ. *J. Biol. Chem.*, **268**, 11779–11784.

Received January 2, 1998; revised and accepted February 16, 1998