The $\chi\psi$ Subunits of DNA Polymerase III Holoenzyme Bind to Single-stranded DNA-binding Protein (SSB) and Facilitate Replication of an SSB-coated Template*

(Received for publication, May 1, 1998)

Bradley P. Glover and Charles S. McHenry‡

From the Department of Biochemistry and Molecular Genetics, University of Colorado Health Sciences Center, Denver, Colorado 80262

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 273, No. 36, Issue of September 4, pp. 23476–23484, 1998
Printed in U.S.A.

A complex of the $\chi$ and $\psi$ proteins is required to confer resistance to high levels of glutamate on the DNA polymerase III holoenzyme-catalyzed reaction (Olson, M., Dallmann, H. G., and McHenry, C. (1995) J. Biol. Chem. 270, 29570–29577). We demonstrate that this salt resistance also requires templates to be coated with the Escherichia coli single-stranded DNA-binding protein (SSB). We show that this is the result of a direct $\chi\psi$–SSB interaction that is strengthened approximately 1000-fold when SSB is bound to DNA. On model oligonucleotide templates, DNA polymerase III core is inhibited by SSB. We show that the minimal polymerase assembly that will synthesize DNA on SSB-coated templates is polymerase III–$\tau$-$\psi\chi$ $\gamma$, the alternative product of the dnaX gene, will not replace $\tau$ in this reaction, indicating that $\tau$'s unique ability to bind to DNA polymerase III holding $\psi\chi$ in the same complex is essential. All of our findings are consistent with $\chi\psi$ strengthening DNA polymerase III holoenzyme interactions with the SSB-coated lagging strand at the replication fork, facilitating complex assembly and elongation.

The 10-subunit DNA polymerase III holoenzyme is the major replicative polymerase of Escherichia coli, responsible for synthesizing the entire bacterial chromosome. Like other replicases from eukaryotes and prokaryotes, the holoenzyme can be resolved into three primary functional units: a polymerase core ($\omega\varepsilon\theta$), a sliding clamp processivity factor ($\beta\kappa$), and a clamp assembly apparatus (DnaX complex, $\gamma_{2}\delta \delta' \chi_{1} \xi_{1}$). $\tau$ and $\gamma$ subunits are both products of the dnaX gene (1, 2); they comprise the ATPase that drives $\beta$ loading on a primed template and replication complex assembly (3–5). The $\gamma$ subunit is a truncated version of $\tau$ arising from a −1 ribosomal frameshift (6–9). The carboxyl-terminal extension of $\tau$, absent from $\gamma$, is responsible for dimerization of pol III (10–12) and binding to the DnaB helicase, effectively coupling all of the replicative activities of the fork into one complex (13, 14). The DnaX complex auxiliary subunits $\delta$ and $\delta'$ function in clamp assembly (11–17). The $\delta$-subunit directly contacts $\beta$ (16). $\chi$ and $\psi$ perform an ancillary, nonessential role in simple single-stranded assays (15–17). The presence of $\chi$ and $\psi$ makes the holoenzyme resistant to glutamate concentrations up to 800 mM and dramatically increases the affinity of DnaX for $\delta$ and $\delta'$, dropping the $K_D$ to a point where they saturate DnaX at physiological concentrations (17).

In addition to the holoenzyme, the polymerase can be resolved into three distinct subassemblies of decreasing complexity: pol III*, pol III′*, and pol III. The activity of pol III* core is limited in processivity and is inhibited by both SSB and physiological levels of spermidine (18). Association of $\tau$ forms the dimeric pol III* that is slightly more processive and resistant to spermidine inhibition but still sensitive to inhibition by SSB (18–21). Pol III* (pol III* + $\gamma_{2}\delta \delta' \chi_{1} \xi_{1}$) becomes resistant to SSB inhibition and exhibits an increased processivity.

DNA polymerases that are stimulated by their cognate SSBs also bind to them. T4 gp32 and T7 gene 2.5 protein SSBs stimulate and bind to T4 and T7 DNA polymerase, respectively (22–26). Human RP-A protein has been shown to interact directly with DNA polymerase a (27). In E. coli, SSB stimulates and binds to DNA polymerase II, but not DNA polymerase III (28). Since pol III* is stimulated by SSB and pol III* is inhibited, $\gamma$, $\delta$, $\delta'$, $\chi$, or $\psi$ would be expected to interact with SSB. Consistent with this hypothesis, Fradkin and Kornberg (29) have observed $\gamma$ complex binding to DNA that is SSB-dependent.

In this report, we demonstrate that $\chi\psi$ binds to SSB in the presence or absence of other subunits of the DnaX complex and that the interaction of SSB with $\chi\psi$ is responsible for the previously observed salt resistance conferred upon the holoenzyme by $\chi\psi$. We also demonstrate an additional function of $\chi\psi$ in forming a minimal polymerase (pol III* + $\chi\psi$) capable of replicating SSB-coated DNA.

EXPERIMENTAL PROCEDURES

Proteins—Pol III holoenzyme protein subunits were purified as follows: core (30), $\beta$ (31), $\tau$ and $\gamma$ (32), $\delta$ and $\delta'$ (by an unpublished procedure),$^2$ and $\chi\psi$ (17). The E. coli single-stranded DNA-binding protein was purified according to Griep and McHenry (20). Bovine serum albumin used in enzyme dilution buffer for replication assays was purchased from Intergen.

Nucleic Acids—Nonlabeled nucleotides and tritiated dTTP were purchased from Amersham Pharmacia Biotech, and the $^{32}$P-nucleotides were purchased from ICN. Oligonucleotides were synthesized by Synthetic Genetics Inc. The sequence of the 52-mer was 5'-TTGACGACATGCCGCAGCTGACACGCGCCGCTGAGGCTATCC-3'. The 102-mer's sequence was 5'-TTACGGTTATTTGGGGAATACCTTGATGGACTGCTGACTT-3'. The F52-mer was purchased from Intergen.

* This work was supported by National Institutes of Health Grant GM035869. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Genetics B-121, University of Colorado Health Sciences Center, 4200 E. Ninth Ave., Denver, CO 80262.

1 The abbreviations used are: holoenzyme, E. coli DNA polymerase III holoenzyme; core, E. coli DNA polymerase III core (A*E); DnaX complex, a complex containing either product of the dnaX gene ($\gamma$ or $\tau$) with associated $\delta$, $\delta'$, and $\psi$; $\gamma$-complex, a complex containing $\gamma$, $\delta$, $\delta'$, $\chi$, and $\psi$; SSB, E. coli single-stranded DNA binding protein; RU, response unit; MOPS, 4-morpholinepropanesulfonic acid.

2 M. Song, M. Olson, J. Carter, H. G. Dallmann, and C. S. McHenry, manuscript in preparation.
the same sequence as the 52-mer above but has a fluorescein attached to the 5’-OH. The 102-mer has a biotin molecule attached to the 5’-end.

3 The fluorescein has no functional purpose in this experiment; it was attached to support another investigation.

The 3’-ends of the F52-mer and 102-mer were modified to impart ε-exonuclease resistance to the oligonucleotides. In each, the third base from the 3’-end is a ribonucleotide connected to the penultimate base through a phosphorothioate linkage. These modifications increase the half-life of the oligonucleotide from 2 s to 18 min (33). M13Gori single-stranded DNA was prepared according to Johanson et al. (31).
Protein Determinations—Protein concentration was determined using the Pierce Coomassie Plus assay according to the manufacturer’s specifications. Bovine serum albumin (fat-free, Sigma) was used as an assay standard.

SDS-Polyacrylamide Electrophoresis—Superose 12 column fractions were loaded (20 μl) onto a 5–17.5% gradient SDS-polyacrylamide gel (0.75 × 18 × 16 cm) prepared according to Laemmli (34) and separated at 65 V overnight. Gels were stained with Coomassie Brilliant Blue G-250 in 20% methanol and 10% acetic acid with destaining being done in 10% methanol and 10% acetic acid.

BIAcore™ Binding Analysis—Molecular interactions were studied using an Amersham Pharmacia Biotech BIAcore™ instrument. CM5 research grade sensor chips (Amersham Pharmacia Biotech) were used in all experiments. Activation of the carboxymethyl dextran matrix was done using a 30-μl injection of a mixture of 0.2 mM 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide and 0.05 mM N-hydroxysuccinimide in water. Following the immobilization of the ligand, the matrix was inactivated by a 30-μl injection of 1 mM ethanolamine-HCl (pH 8.0). Streptavidin and bovine IgG were sequentially immobilized in 10 mM sodium acetate (pH 4.5) at 0.2 mg/ml and 0.1 mg/ml, respectively. SSB was immobilized in 10 mM sodium acetate (pH 4.0) at 0.05 mg/ml. An HBS buffer system (10 mM HEPES-KOH, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% P-20 detergent (Amersham Pharmacia Biotech)) was used in the immobilization of the biotinylated primer-template to the

![Diagram](image-url)
streptavidin-derivatized chip. Protein-protein and protein-DNA interactions were then carried out in HKGM buffer (50 mM HEPES-KOH, pH 7.4, 100 mM potassium glutamate, 10 mM magnesium acetate, 0.005% P-20 detergent) at a flow rate of 5 ml/min at 20 °C. Ranges for apparent $K_D$ values were determined using BIAevaluation 2.1 software (Amersham Pharmacia Biotech). Injections of analyte over a streptavidin-derivatized flow cell were subtracted from the data to eliminate contributions due to minor refractive index changes. $R_{max}$ from each response curve in A was plotted against its corresponding $\chi\psi$ concentration. The $K_D$ was obtained from fitting the data to a rectangular hyperbola and determined to be 2.7 μM.

**Primer-Template Preparation**—5'-End-labeling of the 52-mer primer was done with [$\alpha$-32P]ATP using T4 polynucleotide kinase (Life Technologies, Inc.) in 250 mM imidazole (pH 6.4), 60 mM MgCl$_2$, 5 mM 2-mercaptoethanol, 0.35 mM ADP (5× concentrate) at 37 °C. All primer and templates were annealed in 50 mM HEPES, pH 7.4, 200 mM NaCl in a 1:1 ratio of primer to template. Annealing was done in an MJ Research Minicycler heating the mixture to 75 °C for 5 min and cooling to 25 °C at 0.5 °C/min.

**DNA Polymerization Assays**—Replication of the 52-mer/M13Gori primer-template was done under the following conditions. 25-μl assays contained 480 pmol of singly primed (with the 52-mer) M13 DNA (total

---

**Fig. 3. Determination of the $\chi\psi$-SSB $K_D$.** A, 7272 RU of SSB were immobilized on the sensor chip in 10 mM NaOAc (pH 4.0). Varying concentrations of $\chi\psi$ were passed over the immobilized SSB. Control injections of each $\chi\psi$ concentration over a sensor chip derivatized with 7086 RU of bovine serum albumin were subtracted from the data to eliminate contributions due to minor refractive index changes. B, The $R_{max}$ from each response curve in A was plotted against its corresponding $\chi\psi$ concentration. The $K_D$ was obtained from fitting the data to a rectangular hyperbola and determined to be 2.7 μM.
nucleotide); 500 fmol of $\alpha_2$; 600 fmol of $\delta$; 600 fmol of $\epsilon$; 500 fmol of $\psi$; 500 fmol of $\beta_2$; 900 nM SSB$_4$; 48 $\mu$M dATP, dCTP, and dGTP; 18 $\mu$M $[^3H]TTP$ (specific activity = 520 cpm/pmol TTP). Reactions were carried out in enzyme dilution buffer (50 mM HEPES-KOH, pH 7.5, 10% (v/v) glycerol, 10 mM magnesium acetate, 200 $\mu$g/ml bovine serum albumin, 0.02% (v/v) Tween 20) and incubated at 30 °C for 5 min. Assays were quenched by trichloroacetic acid precipitation.

52/102-mer primer-template assays contained 12 pmol of pol III core ($\alpha\epsilon\theta$), 3.2 pmol of $\tau$ or $\gamma$ complex, 16 pmol of $\beta$, and 8 pmol of SSB monomer. Typically, 1 pmol of primer-template was used in a 25-$\mu$l assay containing enzyme dilution buffer (50 mM HEPES-KOH, pH 7.5, 10% (v/v) glycerol, 0.1 M potassium glutamate, 10 mM dithiothreitol, 10 mM magnesium acetate, 200 $\mu$g/ml bovine serum albumin, 0.02% (v/v) Tween 20) and incubated at 30 °C for 5 min. Each assay involving elongation of $^{32}$P-labeled primer contained 18 $\mu$M TTP and 48 $\mu$M dATP, dCTP, and dGTP. $^{32}$P incorporation assays contained 18 $\mu$M $[^3H]TTP$ (specific activity = 800 cpm/pmol) and 48 $\mu$M dATP, dCTP, and dGTP. Reaction products were analyzed on 12% denaturing polyacrylamide gels and visualized on a Molecular Dynamics PhosphorImager. Quantitation of gel results were done using ImageQuant (Molecular Dynamics).

RESULTS

A Re-examination of the Salt Resistance Conferred to the $\tau$-Reconstituted DNA Polymerase III Holoenzyme by $\psi$—Previously, we demonstrated that $\psi$ confers salt resistance to a $\tau$-reconstituted DNA polymerase III holoenzyme but not a $\gamma$-reconstituted holoenzyme (17). The experiments in that report were all performed in the presence of SSB. We sought to ascertain whether SSB played a role in the observed salt effects. We bypassed the need for a DnaG primase and the accompanying SSB requirement by annealing a DNA 52-mer primer to an M13 template. Using this 52-mer/M13 primer-template, we reproduced the $\psi$-dependent salt resistance of a $\tau$-reconstituted holoenzyme. In the presence of $\psi$, the reconstituted holoenzyme retains >75% of its activity in the presence of 800 mM potassium glutamate. DNA replication of the reconstituted holoenzyme in the absence of $\psi$ is inhibited by high salt (Fig. 1A).

In order to determine whether the presence of SSB influenced salt resistance, we performed the same experiment in the absence of SSB (Fig. 1B). In the absence of SSB and added salt, the reconstituted holoenzyme was still able to replicate 41–43% of the template whether $\psi$ was present or not. As the salt concentration was increased, the activities of both the $\psi$-proficient and $\psi$-less $\tau$-reconstituted enzymes were inhibited equally (Fig. 1B). Thus, the salt resistance previously observed (17) required both $\psi$ and SSB. This result prompted us to examine whether SSB physically interacts with $\psi$.

**Gel Filtration Demonstrates That $\psi$ and SSB Interact Directly**—Incubation of equimolar amounts of $\psi$ and SSB$_4$ resulted in a complex of the components as revealed by Superose 12 gel filtration. The SSB-$\psi$ complex (Fig. 2C) elutes earlier than either of the individual proteins alone (Fig. 2, A and B), demonstrating that $\psi$ and SSB interact directly. The concentration of the $\psi$ and SSB$_4$ applied to the gel filtration column was 8.3 $\mu$M with the eluant being diluted 6-fold, suggesting that the $\psi$-SSB $K_D$ is probably in the micromolar range.

**SSB Binds to the $\gamma$-Complex via a $\psi$-SSB Interaction**—SSB interacts with the $\gamma$-complex only when $\psi$ is present. Complexes containing only $\gamma$, $\delta$, and $\delta'$ do not bind SSB (Fig. 2D). Minimally, $\gamma$ and $\psi$ are required for $\gamma$ to enter into a complex with SSB (Fig. 2E). The addition of $\delta$ and $\delta'$ to $\psi$ increases the affinity of $\gamma$ for $\psi$ (17) resulting in more $\psi$ in the high molecular weight complex and an accompanying larger amount of SSB (Fig. 2F). SSB also binds to the $\tau$-complex in a $\psi$-dependent manner (data not shown).

**Determination of the $\psi$-SSB $K_D$**—Having demonstrated that $\psi$ and SSB interact directly by gel filtration, we determined the $K_D$ for their interaction using the BIAcore™. Our initial investigation into the interaction between $\psi$ and SSB on the BIAcore found that the on and off rates for the interaction were too fast to be amenable to a kinetic analysis. Therefore, we utilized an equilibrium approach on the BIAcore to determine the dissociation constant. Various concentrations of $\psi$ were...
passed over SSB covalently attached to a CM5 sensor chip. Identical injections were made over a bovine serum albumin-derivatized chip, and the resulting sensograms were subtracted from the data as background. Fig. 3A shows an overlay plot of sensograms representing $\chi$ injections of varying concentrations. The $R_{\text{max}}$ from each sensogram was plotted as a function of the $\chi$ concentration (Fig. 3B). A hyperbolic relationship is observed, indicating that saturation was nearly achieved at concentrations equaling or exceeding 10 $\mu M$ $\chi$. The data were fit to a rectangular hyperbola ($RU_{\text{bound}} = [\chi][\text{RU}_{\text{max}}]/(\chi + K_D)$) generating a $K_D$ of 2.7 $\mu M$ for the interaction.

The $\chi$-SSB Interaction Increases the Affinity of the DnaX Complex for the DNA Primer-Template—Three oligonucleotides were synthesized for use in experiments in solution and on the BIAcore™. The F52-mer was annealed to the 5'-fluoresceinated end of the F52-mer being flush with the 3'-end of the 102-mer. This construct was immobilized on a streptavidin-derivatized BIAcore™ sensor chip for binding studies (Fig. 4A and B). The primer-template construct showed no dissociation or loss in RU over the time course of the experiments. SSB was then passed over the DNA, binding with a stoichiometry of 1:1.1 DNA to SSB (Fig. 4C). Under these ionic conditions, one SSB tetramer would be expected to bind to the exposed 50 nucleotides of single-stranded DNA (35).

We next wanted to ascertain the role of the $\chi$-SSB interaction during the association of the DnaX complex with SSB-coated primer-template. Either 400 nM $\tau$-complex ($\tau\delta\delta'\chi\psi$ or $\tau\delta\delta'$) complex was passed over primer-template in the presence or absence of SSB prebound to the DNA (Fig. 5, A–D). The $\tau$-complex does not significantly interact with the primer-template in the absence of SSB (stoichiometry < 1:0.04) (Fig. 4A). In the presence of SSB and absence of $\chi\psi$, the clamp loader complex does not interact stably with primer-template (stoichiometry < 1:0.04) (Fig. 5D). It is only when both SSB and $\chi\psi$ are present that the $\tau$-complex interacts with the F52/102-mer (stoichiometry 1:1 DNA to $\tau$-complex) (Fig. 5B). In a parallel experiment using a range of protein concentrations, we determined the apparent $K_D$ of the $\tau$- and $\gamma$-complex for SSB-coated DNA to be 3 and 9 nM, respectively. This range is approximately 1000-fold lower than the value determined for $\chi$ and SSB, suggesting that the presence of the DNA and auxiliary subunits significantly strengthens the interaction. The binding and subsequent stable complex formed when the $\tau$-complex interacts with the SSB-coated primer-template prompted us to test whether the $\gamma$-complex functions in a similar manner. Four experiments performed using the $\gamma$-complex (Fig. 6) in place of the $\tau$-complex demonstrated that significant binding only occurred in the presence of both $\chi$ and SSB alone, suggesting that the presence of the DNA and auxiliary subunits significantly strengthens the interaction. The binding and subsequent stable complex formed when the $\gamma$-complex interacts with the SSB-coated primer-template.

The $\chi$-SSB Interaction Facilitates Primer Elongation by Pol III—In order to assign a functional significance to the $\chi$-SSB interaction, we investigated its role in DNA replication on short defined primer-templates. Fig. 7 demonstrates that various holoenzyme subassemblies can elongate the F52/102-mer primer-template. Bands represent incorporation of $[\alpha^{32}\text{P}]\text{dTTP}$ arising from DNA synthesis. In the absence of auxiliary subunits, pol III can elongate a small percentage of the primer to full length (Fig. 7, lane 1). This elongation is prevented when SSB coats the primer-template (lane 2). The SSB-coated F52/102-

---

**Fig. 5.** $\chi\psi$ mediates a strong interaction of the $\tau$-complex with an SSB-coated primer-template. 400 nM $\tau\delta\delta'\chi\psi$ or $\tau\delta\delta'$ was injected over the SSB-coated or uncoated F52/102-mer™ immobilized on the sensor chip via a biotin-streptavidin linkage (Fig. 3). All proteins were diluted in HKGM buffer plus 100 $\mu M$ ATP. In order to dissociate all bound protein on the F52/102-mer, a 35-$\mu l$ pulse of 1.5 M guanidine, 50 mM HEPES, 0.005% P20 detergent (Amersham Pharmacia Biotech) guanidine regeneration was done between each analysis on the primer-template. Regeneration and removal of all bound protein was effective, since the increased experimental signal was returned to the initial RU value for the DNA primer-template. A, 400 nM $\tau\delta\delta'\chi\psi$ was injected over primer-template. B, the same conditions as A except that the primer-template was coated (1:1 stoichiometry SSB to F52/102-mer) with SSB. C, 400 nM $\tau\delta\delta'$ over the primer-template. D, the same conditions as C except that the primer-template was coated with SSB.
mer can be elongated significantly by \( \tau \) and pol III only when \( \chi \psi \) is present (compare lanes 3 and 4). The addition of \( \beta \) in the presence of the \( \tau \)-clamp assembly complex facilitates elongation in the presence and absence (compare lanes 5 and 6) of SSB on the DNA. In the presence of \( \beta \) and absence of the \( \tau \)-complex, no elongation is observed in the presence of SSB (lane 7).

The experiments presented in Fig. 7 prompted us to determine the minimum requirements necessary in order to overcome the SSB inhibition of pol III. We end-labeled the 52-mer with \( ^{32} \)P and incorporated unlabeled dNTPs with various combinations of holoenzyme subunits (Fig. 8). The trend seen in the experiment reported in Fig. 7 was also observed here; pol III is inhibited by SSB on the primer-template (Fig. 8, compare lanes 1 and 2). \( \tau \) and \( \chi \psi \) by themselves (lanes 3 and 4) produce replication products identical to those generated by the inhibited pol III core and do not support full-length product formation. The addition of \( \delta^\prime \) to \( \tau \) produces replication products identical to \( \tau \) alone (lane 6). The concerted action of both \( \tau \) and \( \chi \psi \) allows full-length primer extension (lane 5). Additionally, we sought to determine if \( \tau \)'s ability to interact with the core polymerase played a role in the elongation process. In comparing the ability of the \( \tau \)- and \( \g \)-complex to facilitate the primer elongation we found that the \( \g \)-subunit is unable to facilitate primer extension in the presence of \( \chi \psi \) (compare lanes 7 and 8). The presence of \( \tau \) rescues \( \g \) in the presence of \( \chi \psi \) and permits pol III to replicate through SSB to full-length product (lane 9). Quantitation of the full-length product arising from each sub-assembly reaction was done by integrating each lane and examining the resultant percentages (Table I). In the presence of pol III and SSB, 100% full-length product formation occurs when, minimally, both \( \tau \) and \( \chi \psi \) are present.

**DISCUSSION**

The interaction of a single-stranded DNA-binding protein with its cognate polymerase has been well documented (22, 24, 27, 28, 36, 37). The benefits arising from this interaction are
alone or in a protein complex. Thus, xcin enzyme initiation complex assembly and elongation.

Amounts of subunits used in the various experiments were 3.2 pmol of xct, xct, xct, and xct, 12 pmol of core, and 2 pmol of SSB xct. One pmol of the xct-labeled xct2/102-mer was elongated in an assay containing xct xct, xct, xct, xct, xct, xct, and xct in enzyme dilution buffer (xct xct, xct, 10% xct/glycerol, 0.1 M potassium glutamate, 10 mM dithiothreitol, 10 mM magnesium acetate, 200 xct/ml bovine serum albumin, 0.02% xct/ Tween 20) and incubated at xct °C for 5 min. Here, xct refers to xct-labeled xct-mer, while xct refers to molecular weight standards. Replication products were separated on a 12% denaturing polyacrylamide gel and visualized on a Molecular Dynamics PhosphorImager.

TABLE I

The xct xct interaction relieves SSB inhibition of xct

| Subassembly | Percentage of full-length product |
|-------------|----------------------------------|
| xct         | %                                |
| Pol III     | 100                              |
| Pol III SSB | 10                               |
| Pol III SSB xct | 9.3                              |
| Pol III SSB xct | 5.3                              |
| Pol III SSB xct, xct | 100                              |
| Pol III SSB xct, xct | 21                               |
| Pol III SSB xct, xct, xct | 110                              |
| Pol III SSB xct, xct, xct, xct | 29                               |
| Pol III SSB xct, xct, xct, xct, xct | 116                              |

*a From the gel in Fig. 8.

*b Percentage of elongation was determined by dividing the percentage of fully elongated product from each subassembly by the percentage of fully elongated product from Pol III alone.

obvious, since any interaction promoting the co-localization of an enzyme and its substrate is probably a favorable one. The observations that xct and not xct is stimulated by SSB (18) and that xct complex binds to single-stranded DNA coated with SSB, but not to uncoated single-stranded DNA (29), suggest that a component of the xct complex xct, xct, xct, xct binds to xct.

We directly demonstrate, by gel filtration and surface plasmon resonance, that xct and no other holoenzyme component forms a tight complex with SSB. Thus, the SSB stimulation of higher forms of DNA polymerase III probably arises from this interaction. The DnaX gene products form a complex with xct (17, 38). SSB binds to and gel filters with xct whether it exists alone or in a protein complex. Thus, xct can bind to SSB in the presence of its partners in the DnaX complex and holoenzyme. This shows that binding of SSB and DnaX are not mutually exclusive, consistent with an xct-xct interaction during holoenzyme initiation complex assembly and elongation.

The interaction between the DnaX complex subunits xct and SSB prompted us to investigate whether this interaction could strengthen the interaction between the complex and the DNA. The xct-SSB interaction brings together entities that must interact for both clamp loading and replication to occur. Considering the relatively high equilibrium constant for the proteins in the absence of DNA, xct and SSB probably do not interact significantly free in the cell. It is only when SSB is bound to the DNA primer-template and the clamp loader associates with the primer terminus that the xct-SSB interaction occurs. The apparent Kx for the xct-SSB interaction is significantly (approximately 1000-fold) lower under conditions where a DNA template and auxiliary subunits are present. The relatively low affinity of free SSB for xct probably prevents SSB from acting as a competitive inhibitor of xct-SSB-DNA binding, enabling holoenzyme to be targeted to the replication fork.

We have demonstrated that the DnaX complex binds to SSB via xct on an SSB-coated primer-template. The xct complex binds to the SSB/DNA with nearly twice the stoichiometry of the xct complex, suggesting perhaps that the C-terminal portion of xct provides some added stability over xct, enabling interactions away from the primer terminus. Further experiments will be necessary in order to resolve the reason for the higher stoichiometry.

The ability of the xct-SSB interaction to link a polymerase subassembly and a DNA template led us to investigate its functional role in replication. The xct-SSB interaction enables pol III xct and not pol III or pol III to replicate through an SSB-coated DNA template. The minimal assembly that can replicate on an SSB template is pol III-xct-xct, which can interact with xct, but not pol III, is unable to replace xct in the assembly, consistent with xct needing to be held in the proximity of the polymerase to stimulate it during the replicative reaction. This provides further evidence for our observations that the DnaX complex does not cycle during replication and is a required component for elongation.

In a previous study, we found that xct relieved the salt inhibition of a xct-reconstituted holoenzyme and not a xct-reconstituted one that lacked xct (17). Here, we reproduced this effect and added the finding that salt resistance occurs only when xct and SSB are both present. The most direct explanation that is consistent with all of our observations is that xct interacts with SSB during replication, stabilizing the complex, and that at elevated salt, when electrostatic interactions are diminished, this interaction becomes dominant in stabilizing polymerase-template-primer interactions. It would follow that a significant portion of the xct-SSB binding energy derives from hydrophobic interactions. In a review article, O'Donnell and colleagues (38) refer to an unpublished observation that xct and SSB interact. This finding would extend our own to identifying the xct component of the xct pair as the interacting subunit.

Our results show that xct is important for stabilizing and interaction with a primer-template in the presence of SSB, especially at elevated salt, but show that the interaction is not required for efficient replication, at least on single-stranded templates. If xct is loaded by a DnaX complex lacking xct, efficient elongation can still be achieved on an SSB-coated template. This would suggest that a signal that triggers SSB to release during replication resides in a component other than xct, perhaps the polymerase itself.

Since there is only one copy of xct dimeric DNA polymerase III holoenzyme, xct probably functions in the lagging strand half, stabilizing interactions with the SSB-coated template during elongation and in establishing new initiation complexes on nascent primers.

Note Added in Proof—After this manuscript was prepared for publication, a report appeared (Kelman, Z., Yuzhakov, A., Andjelkovic, J., and O'Donnell, M. (1998) EMBO J. 17, 2436–2449) that overlapped with a subset of our findings. Based on the behavior of reactions reconstituted with a mutant SSB, it was inferred that an SSB-xct interaction was responsible for the observed salt resistance of elongation com-

FIG. 8. Minimal holoenzyme subunits required for overcoming SSB inhibition. Amounts of subunits used in the various experiments were 3.2 pmol of xct, xct, xct, and xct, 12 pmol of core, and 2 pmol of SSB xct. One pmol of the xct-labeled xct2/102-mer was elongated in an assay containing xct xct, xct, xct, xct, xct, xct, and xct in enzyme dilution buffer (xct xct, xct, 10% xct/glycerol, 0.1 M potassium glutamate, 10 mM dithiothreitol, 10 mM magnesium acetate, 200 xct/ml bovine serum albumin, 0.02% xct/ Tween 20) and incubated at xct °C for 5 min. Here, xct refers to xct-labeled xct-mer, while xct refers to molecular weight standards. Replication products were separated on a 12% denaturing polyacrylamide gel and visualized on a Molecular Dynamics PhosphorImager.

TABLE I

The xct xct interaction relieves SSB inhibition of xct

| Subassembly | Percentage of full-length product |
|-------------|----------------------------------|
| xct         | %                                |
| Pol III     | 100                              |
| Pol III SSB | 10                               |
| Pol III SSB xct | 9.3                              |
| Pol III SSB xct | 5.3                              |
| Pol III SSB xct, xct | 100                              |
| Pol III SSB xct, xct | 21                               |
| Pol III SSB xct, xct, xct | 110                              |
| Pol III SSB xct, xct, xct, xct | 29                               |
| Pol III SSB xct, xct, xct, xct, xct | 116                              |

*a From the gel in Fig. 8.

*b Percentage of elongation was determined by dividing the percentage of fully elongated product from each subassembly by the percentage of fully elongated product from Pol III alone.
plexes. This report also localized the site of interaction to the carboxyl terminus of SSB.

REFERENCES

1. Kodaira, M., Biswas, S. B., and Kornberg, A. (1983) Mol. Gen. Genet. 192, 80–86
2. Mullin, D. A., Woldringh, C. L., Henson, J. M., and Walker, J. R. (1983) Mol. Gen. Genet. 192, 73–79
3. Maki, S., and Kornberg, A. (1988) J. Biol. Chem. 263, 6555–6560
4. Wickner, S. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 3511–3515
5. Lee, S.-H., and Walker, J. R. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2713–2717
6. McHenry, C. S., Grieb, M. A., Tomasiewicz, H., and Bradley, M. (1990) Molecular Mechanism in DNA Replication and Recombination (Richardson, C. C., and Lehman, I. R., eds) pp. 115–126, Alan Liss, Inc., New York
7. Tsuchihashi, Z., and Kornberg, A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2516–2520
8. Blum, D. A., and Walker, J. R. (1990) Nucleic Acids Res. 18, 1725–1729
9. Flower, A. M., and McHenry, C. S. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3713–3717
10. Onrust, R., Finkelstein, J., Turner, J., Naktinis, V., and O’Donnell, M. (1995) J. Biol. Chem. 270, 13366–13377
11. Dallmann, H. G., and McHenry, C. S. (1995) J. Biol. Chem. 270, 29563–29569
12. McHenry, C. S. (1982) J. Biol. Chem. 257, 16644–16654
13. Kim, S., Dallmann, H. G., McHenry, C. S., and Marians, K. J. (1996) J. Biol. Chem., 271, 643–650
14. Onrust, R., Finkelstein, J., Naktinis, V., Turner, J., Fang, L., and O’Donnell, M. (1995) J. Biol. Chem. 270, 13358–13365
15. Olsen, M. W., Dallmann, H. G., and McHenry, C. S. (1995) J. Biol. Chem. 270, 29570–29577
16. Fay, P. J., Johanson, K. O., McHenry, C. S., and Bambara, R. A. (1981) J. Biol. Chem. 256, 976–983
17. Fay, P. J., Johanson, K. O., McHenry, C. S., and Bambara, R. A. (1982) J. Biol. Chem. 257, 5692–5699
18. Grieb, M. A., and McHenry, C. S. (1989) J. Biol. Chem. 264, 11294–11301
19. Reems, J. A., and McHenry, C. S. (1994) J. Biol. Chem. 269, 33091–33096
20. Hurley, J. M., Chervitz, S. A., Jarvis, T. C., Singer, B. S., and Gold, L. (1993) J. Mol. Biol. 229, 398–418
21. Cha, T. A., and Alberts, B. M. (1988) Cancer Cells 6, 1–10
22. Huberman, J. A., Kornberg, A., and Alberts, B. M. (1971) J. Mol. Biol. 62, 39–52
23. Kim, Y. T., Tabor, S., Churchich, J. E., Richardson, C. C. (1992) J. Biol. Chem. 267, 15032–15040
24. Kim, Y. T., and Richardson, C. C. (1994) J. Biol. Chem. 269, 5270–5278
25. Dornreiter, I., Erdile, L. F., Gilbert, I. U., von Winkler, D., Kelly, T. J., and Fanning, E. (1992) EMBO J. 11, 769–776
26. Molinexx, I. J., and Gefter, M. L. (1974) Proc. Natl Acad. Sci. U. S. A. 71, 3858–3862
27. Fradkin, L. G., and Kornberg, A. (1992) J. Biol. Chem. 267, 10318–10322
28. McHenry, C. S., and Crow, W. (1979) J. Biol. Chem. 254, 1748–1753
29. Johanson, K. O., Haynes, T. E., and McHenry, C. S. (1986) J. Biol. Chem. 261, 11460–11465
30. Dallmann, H. G., Thimmig, R. L., and McHenry, C. S. (1995) J. Biol. Chem. 270, 29555–29562
31. Grieb, M. A., Reems, J., Franden, M. A., and McHenry, C. S. (1990) Biochemistry 29, 9006–9014
32. Laemmli, U. K. (1970) Nature 227, 680–685
33. Lohman, T. M., and Ferrari, M. E. (1994) Annu. Rev. Biochem. 63, 527–570
34. Kim, Y. T., Tabor, S., Churchich, J. E. and Richardson, C. C. (1992) J. Biol. Chem. 267, 15032–15040
35. Nakai, H., and Richardson, C. C. (1988) J. Biol. Chem. 263, 9831–9839
36. Xiao, H., Dong, Z., and O’Donnell, M. (1993) J. Biol. Chem. 268, 11779–11784
37. Kelman, Z., and O’Donnell, M. (1995) Annu. Rev. Biochem. 64, 171–200