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A Bipartite Polymerase–Processivity Factor Interaction: Only the Internal β Binding Site of the α Subunit is Required for Processive Replication by the DNA Polymerase III Holoenzyme

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Previously, we localized the β_2 interacting portion of the catalytic subunit (α) of DNA polymerase III to the C-terminal half, downstream of the polymerase active site. Since then, two different β_2 binding sites within this region have been proposed. An internal site includes amino acid residues 920–924 (QADMF) and an extreme C-terminal site includes amino acid residues 1154–1159 (QVELEF). To permit determination of their relative contributions, we made mutations in both sites and evaluated the biochemical, genetic, and protein binding properties of the mutant α subunits. All purified mutant α subunits retained near wild-type polymerase function, which was measured in non-processive gap-filling assays. Mutations in the internal site abolished the ability of mutant α subunits to participate in processive synthesis. Replacement of the five-residue internal sequence with AAAKK eliminated detectable binding to β_2. In addition, mutation of residues required for β_2 binding abolished the ability of the resulting polymerase to participate in chromosomal replication *in vivo*. In contrast, mutations in the C-terminal site exhibited near wild-type phenotypes. α Subunits with the C-terminal site completely removed could participate in processive DNA replication, could bind β_2, and, if induced to high level expression, could complement a temperature-sensitive conditional lethal *dnaE* mutation. C-terminal defects that only partially complemented correlated with a defect in binding to τ, not β_2. A C-terminal deletion only reduced β_2 binding fourfold; τ binding was decreased ca 400-fold. The context in which the β_2 binding site was presented made an enormous difference. Replacement of the internal site with a consensus β_2 binding sequence increased the affinity of the resulting α for β_2 over 100-fold, whereas the same modification at the C-terminal site did not significantly increase binding. The implications of multiple interactions between a replicase and its processivity factor, including applications to polymerase cycling and interchange with other polymerases and factors at the replication fork, are discussed.

Keywords: DNA polymerase III holoenzyme; beta binding site; replicase; processivity factor

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Abbreviations used: DnaX complex, a complex containing $DnaX_3\delta\delta'\chi\psi$ with DnaX being in the γ and/or τ form; τ complex, $\tau_3\delta\delta'\chi\psi$; Pol III holoenzyme, DNA polymerase III holoenzyme; IPTG, Isopropyl β-D-thiogalactoside; TCA, trichloroacetic acid; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; Ni^{2+}-NTA, nickel-nitrilotriacetic acid; BSA, bovine serum albumin; SSB, single-stranded binding protein; SPR, surface plasmon resonance; SA, streptavidin; RU, response unit; WT, wild-type.

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Introduction

The DNA polymerase III holoenzyme is a prototypical cellular replicase comprising a processivity factor (β_2), an AAA+ ATPase that binds the polymerase and assembles β_2 onto primed DNA (DnaX complex, $\tau_2\gamma\delta\delta'\chi\psi$) and a DNA polymerase (Pol III, $\alpha\varepsilon\theta$) that gains its specialized replicative properties, in part, from its ability to interact with other proteins at the replication fork.[1,2] The key role of β_2 as a processivity factor was first revealed by its ability to confer processivity upon DNA polymerase III if added in excess, permitting its assembly on DNA in the absence of the DnaX complex.[3] The mechanism used to bestow processivity was revealed by the X-ray crystal structure of β_2.[4] It is a ring-like molecule that encircles DNA and tethers the polymerase by protein–protein interactions.

dnaX, the structural gene for the ATPase subunit of the DnaX complex, encodes two proteins, γ and τ. γ Results from translational frameshifting, generating a shortened three domain protein that can function as a β_2 loader.[5–7] τ, The full-length translation product, contains the sequence of γ, a presumably unstructured tether and two additional domains that bind the DnaB helicase and the α subunit of Pol III. This C-terminal segment, C-τ, unique to τ was characterized by investigation of OmpT digestion of τ.[8,9] It has also been termed τ_C.[10]

The binding sites for τ and β_2 within the α subunit of Pol III were first defined by investigation of the binding properties of a series of tagged α subunits containing successively larger deletions from the N and C termini.[11,12] We found that deletion of 48 amino acid residues from the C terminus of α abolished τ binding, establishing the C terminus of α as part of an essential τ binding site. α Containing deletions of up to 542 amino acid residues from the N terminus retained nearly full τ binding. The same 48 residue C-terminal deletion bound β_2 only tenfold less tightly, suggesting that minor contributions to β_2 binding resided in this site. Deletion analysis suggested that the major β_2 binding site resided between residues 542 and 991.

Dixon and colleagues[13] identified a five amino acid sequence proposed to be the β_2 binding site of Pol III. Related sequences have been found in other β_2 binding proteins.[14–17] The sequence within Pol III was proposed to be residues 920–924 (QADMF). Peptide binding experiments indicated the importance of F924 and, to a lesser extent, Q920.[13,14] A survey of other β_2 binding proteins suggested a consensus sequence (QL(S/D)LF). Changing the A and M residues to the consensus L increased binding. The location of this proposed element was consistent with our localization of the principal β_2 binding site.

Subsequently, a related sequence was identified at the C terminus of α that also bound β_2.[10] The claim has been made that α binds β_2 mainly through the C-terminal 20 residues of α, and that these residues are required for function of α with β_2.[15] While the identification of a C-terminal β_2 binding element is consistent with our initial findings of a small diminution in β_2 binding upon deletion of the C terminus of α, the claim of its being the main binding site is inconsistent with our earlier results. Our studies indicated that the C terminus was responsible for only a small portion of the overall binding energy for β_2. We also found that α lacking its C terminus was still able to interact functionally with β_2, conferring processive synthesis.[12] An elegant model proposing competition of τ with β_2 for binding C-terminal sequences of α has been put forward as the basis of Pol III cycling upon completion of Okazaki fragment synthesis.[10,18] This hypothesis has firm experimental support and is likely to be a key feature enabling the Pol III holoenzyme to participate efficiently in discontinuous DNA replication. However, our earlier finding that the presence of τ decreased the binding of β_2 only twofold[12] would further suggest that the C-terminal element is not the strongest β_2 binding site.

Given these conflicting interpretations of experimental results and the resulting controversy, we chose to re-investigate our earlier observations. With the exception of our initial work, conclusions have been largely derived from studies of peptides representing the internal and C-terminal β_2 binding sites. We took advantage of these well-conducted and documented peptide studies and transferred the identified "mutations" into the context of the full-length α subunit. This permitted assessment of the relative contribution of the internal and C-terminal sites to the binding of β_2, to the reconstitution of Pol III holoenzyme reactions, and to the complementation of temperature-sensitive, conditional-lethal mutations in *dnaE*, the structural gene for the α subunit of Pol III.

Results

Rationale for construction of *dnaE* mutants

To explore the relative roles of two β_2 binding sites identified within the α subunit of Pol III, we built upon the work of the Dixon and O'Donnell laboratories, which have identified residues within each site that are important for the binding of synthetic peptides to β_2. Dixon and colleagues[13,14] demonstrated that changing the F residue of the internal β_2 binding site QADMF to either A or K abolished binding. Accordingly, we mutated F924 to K and A (mutants M1 and M5, respectively, in Figure 1) within the context of full-length α subunit. Mutation of the Q residue reduced binding of synthetic peptides, but it apparently was not absolutely required, since the trimeric peptide DLF could substitute for the pentameric QADMF sequence. Thus, we mutated the Q920 residue to A (M4). To completely abolish the internal binding site, we replaced the internal site with a sequence coding for AAAKK (M2). The second residue (A921) in the internal binding site appears

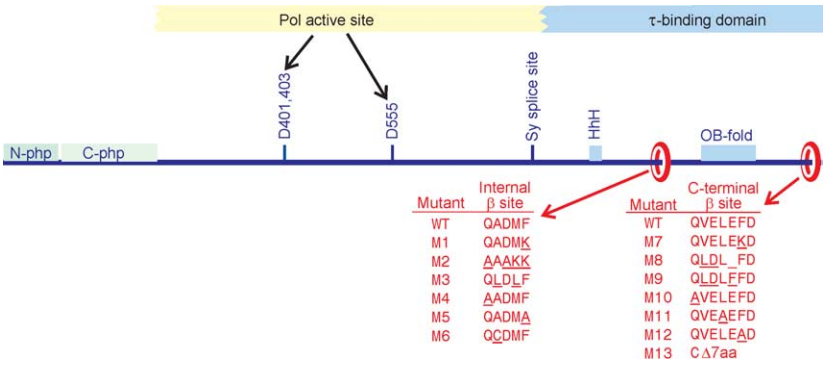

Figure 1. Modular organization of *dnaE*. Two candidate β_2 binding sites are indicated along with the mutations constructed to test their function. The C-terminal domain identified to bind τ[11,12] is indicated. This domain also contains two putative structural elements, the helix-hairpin-helix (HhH) (aa 835–850)[46] and the OB-fold[47] (aa 999–1073), both often involved in nucleic acid binding. Both β_2 binding sites reside within the τ-binding domain. Two putative phosphoesterase-like domains[48] are indicated at the N terminus. The polymerase domain is centrally located. It contains three essential catalytic acidic residues (D401, 403 and 555).[42] *DnaE* from *Synechocystis* is synthesized from *trans*-splicing of two proteins.[49] Assuming these fold into domains representing the mature protein, the splice site might define the interdomain boundary. It corresponds to residue 755 in the *E. coli* α sequence.

unimportant. We replaced it with a C to further test this hypothesis (M6). Dixon and colleagues, after surveying a number of β_2 binding proteins, derived the consensus sequence QL(D/S)LF and demonstrated that synthetic peptides containing this sequence in place of the internal β_2 binding site bound β_2 several-fold more strongly. Our mutant M3 represents this consensus sequence at the internal β_2 binding site of α.

O'Donnell and colleagues identified QVELEF, a hexameric variant of the Dixon consensus sequence with an E inserted between the C-terminal L and F residues. Mutation of the Q, L, or F to A abolished the ability to compete for interaction of full-length Pol III to β_2.[15] Accordingly, we constructed mutants M10, M11, and M12, respectively, to recreate these changes within α (Figure 1). We also mutated the F residue to K (mutant M7) to provide an analog to the F924K mutation in the internal site. To destroy the C-terminal sequence, we deleted the seven

Table 1. PCR primers used for construction of α mutants

Oligo	Purpose	Sequence (5′ to 3′)	Restriction site[a]
S6980	Flanking PCR primer M1-M6	ATTACCGATTTTGCGCCGCTTTACTGC	
A8356	Flanking PCR primer M1-M6	TACGCCTCCGACATAACGCTCAATCTCTTT	
SM1	M1 mutation	GCCGATATGAAAGGCGTGCTGGCCGAAGAGCC	
AM1	M1 mutation	CGCCTTTCATATCGGCCTGACCGATAGCTT	
SM2	M2 mutation	GCGGCCGCGAAAAAAGGCGTGCTGGCCGAAGAGCC	NotI
AM2	M2 mutation	CGCCTTTTTTCGCGGCCGCACCGATAGCTTCCGCTTTCG	NotI
SM3	M3 mutation	GCTGGATCTGTTCGGCGTGCTGGCCGAAGAGCC	PvuII
AM3	M3 mutation	CGCCGAACAGATCCAGCTGACCGATAGCTTCCGCT	PvuII
SM4	M4 mutation	GCGGCCGATATGTTCGGCGTGCTGGCCGAAGAGCC	EagI
AM4	M4 mutation	CGCCGAACATATCGGCCGCACCGATAGCTTCC	EagI
SM5	M5 mutation	CAGGCCGATATGGCCGGCGTGCTGGCCGAAGAGCC	NaeI
AM5	M5 mutation	CGCCGGCCATATCGGCCTGACCGATAGCTTCC	NaeI
SM6	M6 mutation	TGCGATATGTTCGGCGTGCTGGCCGAAGAGCC	
AM6	M6 mutation	CGCCGAACATATCGCACTGACCGATAGCTTCC	
S8035	Flanking PCR primer M7-M8	GTTGAACCGTCGCGTGCTGGAAAAACT	
ApDRK	Flanking PCR primer M7-M8	AACTAGTGGATCCGGTACCACCCAG	
SM7	M7 mutation	CTGGAGAAGGACTAATACAGGAATAC	
AM7	M7 mutation	GTATTAGTCCTTCTCCAGTTCCACCTGC	
SM8	M8 mutation	CTGGACCTGTTCGATTAATACAGGAATACTATGAGTCTG	
AM8	M8 mutation	TCGAACAGGTCCAGCTGCTCCGAACCAATGAGGCCACG	
S8501	Flanking PCR primer M9-M13	AACGTGAAACGTTAGGCCTG	
A9976	Flanking PCR primer M9-M13	AACTAGTGGATCCGGTACCA	
SM9	M9 mutation	CAGCTGGATCTCTTCTTTGACTAATACAGGA	PvuII
AM9	M9 mutation	GAAGAGATCCAGCTGCTCCGAACCAATGAGG	PvuII
SM10	M10 mutation	GAGGCGGTGGAACTCGAGTTTGACTAATACA	XhoI
AM10	M10 mutation	CTCGAGTTCCACCGCCTCCGAACCAATGAGG	XhoI
SM11	M11 mutation	GTGGAAGCGGAATTCGACTAATACAGGAATA	EcoRI
AM11	M11 mutation	GTCGAATTCCGCTTCCACCTGCTCCGAACCA	EcoRI
SM12	M12 mutation	GTGGAACTCGAGGCTGACTAATACAGGAATA	XhoI
AM12	M12 mutation	GTCAGCCTCGAGTTCCACCTGCTCCGAACCA	XhoI
SM13	M13 mutation	GGTTCGGAGTAATACAGGAATACTATG	
AM13	M13 mutation	CCTGTATTACTCCGAACCAATGAGGCC	

[a] In some cases, the oligonucleotide carrying the site-specific mutation contains a new restriction site that was used to facilitate the screening for mutant clones.

C-terminal amino acid residues of α that removed the entire proposed QVELEF binding sequence. We replaced the C-terminal site with the consensus sequence (M8), and to preserve the hexameric format of the C-terminal site, we also constructed the pseudo-consensus sequence QLDLFF (M9) (Table 1).

Expression, purification and characterization of mutant α subunits in simple gap-filling assays

To facilitate purification of multiple mutant proteins, we introduced the desired mutations (Figure 1; Table 1) into a *dnaE* construct fused to an N-terminal hexa-His tag and a site that is biotinylated *in vivo*. All were placed in an IPTG-inducible vector used in our previous studies to express full-length and mutant α subunits.[11,12] We found it necessary to express some mutants at 23 °C to avoid degradation and to maximize the amount of soluble protein. All mutants were purified by Ni^{2+}-NTA affinity chromatography. The resulting proteins were characterized in a simple assay that measures the ability of the polymerase to fill gaps in nuclease-activated DNA. This assay does not require replicase function or the presence of Pol III holoenzyme auxiliary subunits. All mutant α subunits exhibited specific activities within fivefold of wild-type α (Table 2). Part of the minor variation observed in specific activity was due to variations in the purity of different constructs, which ranged from 35% to 80% as judged by SDS-gels.

Only the internal β_2 binding site is essential for processive DNA replication

Function of Pol III on long single-stranded templates requires processive function and interaction with β_2.[3,19] Thus, these assays provide a means of determining the functional defect resulting from diminished interaction with β_2 in mutant α subunits. To correct for differences in activity that were attributable to differences in purity or to minor differences in intrinsic polymerase activity, we plotted the Pol III holoenzyme reconstitution activity against both β_2-independent gap-filling activity (Figure 2(a) and (c)) and absolute amount (Figure 2(b) and (d)) of the mutant α subunits. In either case, the conclusions remain the same.

Table 2. Gap-filling activities of purified mutant α fusion proteins

Mutant	Units/mg[a]	Fold reduction[b]
Wild-type	1.30×10^6	1.0
M1	7.14×10^5	1.8
M2	7.91×10^5	1.6
M3	1.20×10^6	1.1
M4	6.41×10^5	2.0
M5	9.31×10^5	1.4
M6	6.28×10^5	2.1
M7	9.28×10^5	1.4
M8	3.33×10^5	3.9
M9	2.83×10^5	4.6
M10	6.20×10^5	2.1
M11	5.40×10^5	2.4
M12	2.70×10^5	4.8
M13	4.50×10^5	2.9

[a] Specific activity of the indicated Ni^{2+}-NTA purified fractions of mutant polymerases using the gap-filling assay as described in Materials and Methods. The purity of mutant proteins varied from 35–80%. The specific activities above are not corrected for these differences in purity.
[b] Reduction in gap-filling activity of purified mutant α relative to wild-type.

Assay of the mutants predicted to have the most severe phenotype within the internal β_2 binding site inferred from published data from synthetic peptides (F924K (M1), F924A (M5) and complete internal β_2 binding site replacement (M2)) indicated complete destruction of Pol III holoenzyme activity (Figure 2(a) and (b)). Mutant M4 (Q920A), predicted on the basis of peptide data to have a partial phenotype, exhibits diminished functional affinity and requires greater quantities to achieve a level of activity significantly below WT (Figure 2(a) and (b)). Mutant M6 (A921C) as expected, exhibits wild-type (WT) α activity. Mutant M3, which contains a consensus β_2 binding site in place of the WT sequence, also functions as well as WT α (Figure 2(a) and (b)). The agreement of these results with the results predicted from synthetic peptides indicates that the internal β_2 binding site is essential for Pol III holoenzyme function.

Analysis of the mutants within the C-terminal β_2 binding site, predicted on the basis of synthetic peptide data to have a severe defect in β_2 interaction, yielded very different results. Mutants that led to loss of peptide function (F1159K (M7), F1159A (M12), Q1154A (M10), and L1157A(M11)) exhibited near wild-type levels of activity (Figure 2(c) and (d)). Replacement of the C-terminal β_2 binding site with the consensus motif (M8 and M9) yielded proteins with full functional activity (Figure 2(c) and (d)). Even the mutant with the C-terminal site completely removed (M13) exhibited slightly reduced, but near WT activity. At saturating levels, full activity was achieved, consistent with our initial results.[12] These results suggest a very limited, non-essential contribution of the C-terminal β_2 binding site to processive DNA replication.

More severe defects were observed in a previous study of α lacking seven C-terminal residues.[15] When surveying expression conditions, we found that several C-terminal mutants (F1159A (M12), CΔ7aa (M13)) when expressed at 30 °C were unstable and extra residues were removed from the C terminus proteolytically. Expression at 23 °C avoided this issue and resulted in a protein with higher endogenous gap-filling activity and nearly wild-type activity in reconstituted Pol III holoenzyme reactions. Thus, the apparent discrepancy with earlier work arises from the intactness of the proteins studied.

The internal binding site binds β_2 most tightly

We directly quantified the strength of the β_2–α

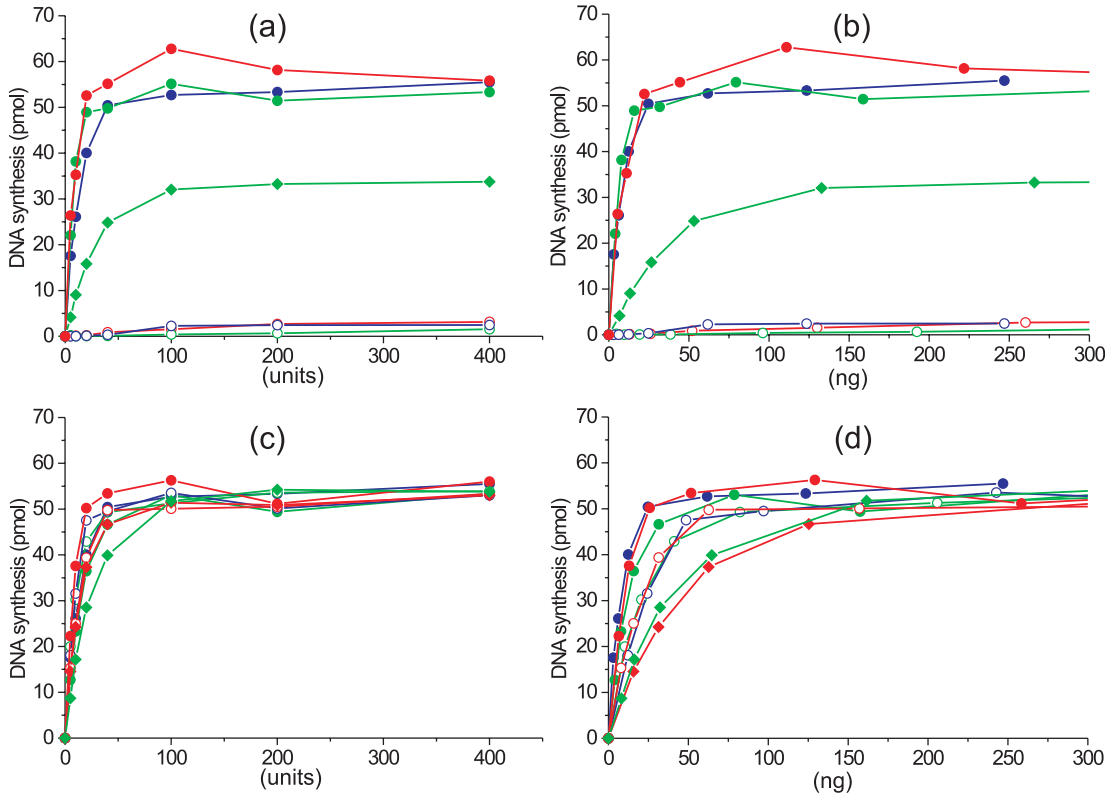

Figure 2. Activity of mutant α subunits in the Pol III holoenzyme reconstitution assay. Assays were performed as described in Materials and Methods. Data points presented are the average of two independent samples. (a) DNA synthesis catalyzed by internal site mutant α subunits plotted as a function of gap-filling activity. (b) Data from (a) plotted as a function of amount of protein added. In both (a) and (b): WT (blue ●); M1 (red ○); M2 (green ○); M3 (green ●); M4 (green ♦); M5 (blue ○); M6 (red ●). (c) DNA synthesis catalyzed by C-terminal site mutant α subunits plotted as a function of gap-filling activity. (d) Data from (c) plotted as a function of the amount of protein added. In both (c) and (d): WT (blue ●); M7 (green ●); M8 (green ○); M9 (blue ○); M10 (red ○); M11 (red ●); M12 (red ♦); M13 (green ♦).

interaction with our series of mutants using surface plasmon resonance (SPR). α Subunits were immobilized by interaction of their N-terminal biotin tag with streptavidin-coated BIAcore chips. β_2 Interacted with and dissociated from immobilized α too quickly to permit the more conventional kinetic method of analysis, so we used the equilibrium level of β_2 bound as a function of β_2 concentration to determine the K_D as we did previously for the χψ–SSB interaction.[20] By SPR, the K_D of wild-type α for β_2 was 0.8 μM, which is within the range previously estimated by a less precise gel filtration determination[12] (Figure 3(a) and (b); Table 3). Mutation of F924 to either A or K (M5 and M1, Figure 3(c) and (d); Table 3) resulted in ten and 16-fold reductions in binding affinity, a less severe change than observed in the holoenzyme reconstitution assays. The Q920A mutation resulted in a fivefold reduction in binding affinity. Changing all important residues within the internal β_2 binding site (QADMF→AAAKK) exhibited a synergistic effect of the combined mutations resulting in undetectable binding (Table 3). Substitution of the internal site with the consensus sequence QLDLF increased binding by a remarkable 120-fold. This is significantly more than expected by the 3.5-fold stronger binding observed with peptides. The stronger affinity is the result of a much slower dissociation rate, and a somewhat slower association rate, precluding the equilibrium analysis used for the other mutants. Thus, we determined the K_D by the standard kinetic method (Figure 3(e)).

Deletion of the entire C-terminal binding site resulted in only a 3.8-fold reduction in β_2 binding (Table 3), indicating a relatively minor contribution of the C-terminal element to β_2 binding, which is consistent with our earlier results.[12] Mutation of single residues shown to abolish peptide binding resulted in, at most, a twofold reduction in binding of the full-length protein. Insertion of the consensus β_2 binding sequence at the C terminus increased binding only 1.3-fold. These results indicate only a modest contribution of the C-terminal element to β_2 binding.

The proposed C-terminal β_2 binding site plays a more important role in τ binding

To investigate whether the replication defects observed were, in part, a consequence of τ-binding defects, we measured τ binding to our series of α mutants. Examination of the change that gave the largest effect with the internal β_2 binding site (QADMF→AAAKK, M2) indicated that τ bound

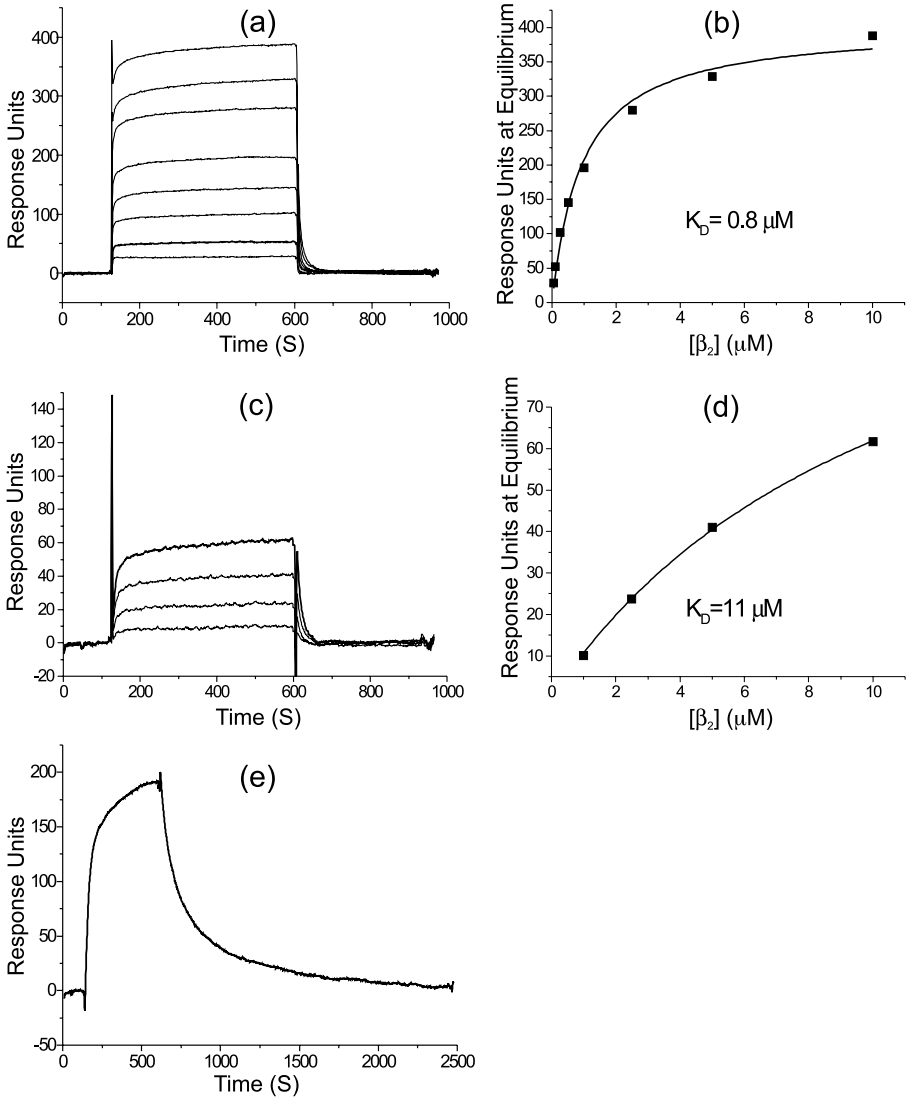

Figure 3. Binding of β_2 to immobilized α mutant subunits measured by SPR. Biotinylated α mutant proteins were captured on flow-cell surfaces through immobilized streptavidin as described in Materials and Methods. (a) Representative graph of overlayed sensorgrams of varying β_2 concentrations (50, 100, 250, 500, 1000, 2500, 5000, 10,000 nM) passed over immobilized WT α (~3500 RUs). The concentration of β_2 increases from lower to upper traces. (b) The R_{max} level of each sensorgram in (a) was plotted as a function of the β_2 concentration and fit to the 1:1 Langmuir model using BiaEvaluation 3.2 software. (c) Representative overlayed sensorgrams of varying concentrations of β_2 (1, 2.5, 5, 10 μM) passed over immobilized M1 mutant α protein (~3500 RUs). The concentration of β_2 increases from lower to upper traces. (d) The R_{max} from each sensorgram in (c) was plotted against the corresponding β_2 concentration. (e) A representative sensorgram of β_2 (10 nM) run over immobilized M3 mutant α (~3500 RUs).

just as tightly to M2 as to WT (Table 4). We interpret this to mean that τ does not interact directly with the internal β_2 binding site.

In contrast, complete deletion of the C-terminal sequence, which only diminishes the binding of β_2 about fourfold, results in a 440-fold reduction in τ binding (Table 4). A F1159K mutation (M7) only reduces τ binding fourfold, while a F1159A mutation (M12) reduces τ binding 740-fold. Interestingly, replacement of the C-terminal binding site with the consensus β_2 binding site QLDLF (M8) directly or with preservation of hexameric spacing (QLDLFF, M9) reduces τ binding 34-fold and 26-fold, respectively. A significant decrease in τ binding (36-fold) resulted from the L1157A (M11) mutation. Only a marginal reduction occurred as the consequence of a Q1154A mutation (M10, 1.4-fold) (Table 4). Thus, two of the three key residues reported to be important for β_2 binding to the C-terminal site[15] actually have a greater effect on τ binding in the context of the full-length protein. This is likely a consequence of the dominant role of the internal site in β_2 binding. The pleiotropic effect of the C-terminal mutations is consistent with the proposed competition of τ and β_2 for a common C-terminal binding site.[18]

Table 3. β_2 Binding affinity of mutant α subunits

Internal site mutant	aa 920–924	K_D (μM)	Affinity compared to WT
WT	QADMF	0.8±0.1	1
M1	QADMK	12.6±0.9	15.8-fold less
M2	AAAKK	≥100[a]	≧130-fold less
M3	QLDLF	0.0069±0.0028	120-fold greater
M4	AADMF	4.2 ± 0.4	5.3-fold less
M5	QADMA	8.0±1.6	Tenfold less
M6	QCDMF	1.6±0.1	Twofold less
C-terminal site mutant	aa 1154–1160		
WT	QVELEFD	0.8±0.1	1
M7	QVELEKD	1.4±0.1	1.8-fold less
M8	QLDL_FD	0.6±0.1	1.3-fold greater
M9	QLDLFFD	0.7±0.1	1.1-fold greater
M10	AVELEFD	1.1±0.1	1.4-fold less
M11	QVEAEFD	1.4±0.2	1.8-fold less
M12	QVELEAD	1.7±0.3	2.1-fold less
M13	CΔ7aa	3.0±0.2	3.8-fold less

The dissociation constant K_D was determined as described in Materials and Methods.
[a] The K_D of M2/β_2 binding could not be accurately determined due to its low affinity. Its K_D was set at the limit of detection.

Mutants in the internal, but not the C-terminal site, fail to complement *dnaE* temperature-sensitivity

To correlate our biochemical analysis with *in vivo* function, we measured the ability of our series of mutant α subunits to complement a temperature-sensitive *dnaE* mutation. We observed that both the F924A and F924K mutations and the mutation that removed the internal β_2 binding site (M2) failed to complement, even when induced to elevated levels by IPTG (Table 5). Consistent with the partial function of the Q920A mutation *in vitro*, mutant M4 rescued cells with a thermally inactivated chromosomally encoded α. The consensus replacement mutant (M3) also complemented.

Mutation of all single residues shown to be important for C-terminal peptide function yielded α subunits that could complement a *dnaE* temperature-sensitive mutation. The F1159K mutation (M7) complemented under all conditions, but the F1159A mutation (M12) only complemented partially, and only when induced with IPTG, yielding very small colonies after seven days growth (Table 5). The same phenotype was observed for the deletion mutation that removed the C-terminal β_2 binding site altogether (M13), indicating the importance of this element *in vivo*. Interestingly, the consensus replacement mutant that preserved the hexameric spacing of F1159 (M9) complemented under all conditions, but the consensus replacement mutant that switched to a pentameric motif (M8) failed to complement unless IPTG was added. However, IPTG complementation with mutant M8 could be distinguished from M12 and M13, since the former yielded normal-sized colonies within three days.

Table 4. τ Binding affinity of mutant α subunits

Internal site mutant	aa 920–924	K_D (pM)	Affinity compared to WT
WT	QADMF	80	1
M2	AAAKK	84	1
C-terminal site mutant	aa 1154–1160		
WT	QVELEFD	80	1
M7	QVELEKD	330	Fourfold less
M8	QLDL_FD	2700	34-fold less
M9	QLDLFFD	2100	26-fold less
M10	AVELEFD	110	1.4-fold less
M11	QVEAEFD	2900	36-fold less
M12	QVELEAD	59,000	740-fold less
M13	CΔ7aa	35,000	440-fold less

The dissociation constant K_D was determined as described in Materials and Methods.

Table 5. Complementation of $dnaE^{ts}$ with *dnaE* β_2 binding site mutants

		Growth at 42 °C[a]		
IPTG concentration		0 mM	0.5 mM	1 mM
Internal site mutant	aa 920–924			
WT	QADMF	+	+	+
M1	QADMK	−	−	−
M2	AAAKK	−	−	−
M3	QLDLF	+	+	+
M4	AADMF	+	+	+
M5	QADMA	−	−	−
M6	QCDMF	+	+	+
C-terminal site mutant	aa 1154–1160			
WT	QVELEFD	+	+	+
M7	QVELEKD	+	+	+
M8	QLDL_FD	−	+	+
M9	QLDLFFD	+	+	+
M10	AVELEFD	+	+	+
M11	QVEAEFD	+	+	+
M12	QVELEAD	−	±	±
M13	CΔ7aa	−	±	±
Vector only				
pDRK-c		−	−	−

[a] A $dnaE^{ts}$ strain expressing mutant *dnaE*s were assayed for complementation as described in Materials and Methods. Growth at 42 °C after three days is designated +, whereas no growth after seven days is designated −. Because mutants M12 and M13 exhibited limited growth after seven days, exhibiting pinpoint colonies, they were designated −/+.

Discussion

Our initial work suggested that the C terminus of the α subunit of Pol III was required for τ binding but that a site more interior within the C-terminal half of α was primarily responsible for β_2 binding.[11,12] Deletion of the C-terminal 48 amino acid residues from α resulted in a relatively small diminution of β_2 binding that we initially misinterpreted, because of its small contribution, as being an ancillary portion of a common β_2 binding site. Since then, discrete pentameric and hexameric β_2 binding sequences located at residues 920–924 and 1154–1159 of the 1160 residue α subunit have been identified.[13–15] Synthetic peptides representing these sequences bind β_2 at a common site shared with δ, the primary β_2 interaction subunit of the DnaX complex "clamp loader".[21]

The relative importance of these two sequences has been a point of recent controversy.[15] Work using synthetic peptides led to the identification of residues that contributed to β_2 binding within each site.[13–15] We exploited this carefully documented work by placing each of the deleterious mutations identified in synthetic peptides into the context of full-length α with the expectation that, by comparison, we could establish the relative contributions of each of the sites.

This approach revealed an absolute requirement for the internal site (residues 920–924). Mutations within this region resulted in an α subunit that, although nearly fully active in non-processive gap filling activity, was completely inactive in Pol III holoenzyme function. These same mutations resulted in a reduced α–β_2 affinity. When multiple residues within the internal site were changed, β_2 binding was completely abolished, even though the C-terminal site remained intact. These results, as well as others presented here, demonstrate that the internal β_2 binding site of α drives β_2 binding and processive DNA replication, both in biochemical systems and *in vivo*. The C-terminal element plays, at most, a minor role in these processes, but could have important ancillary functions as discussed later.

Mutations in the C terminus led to only slight reduction in α function in Pol III holoenzyme activity and β_2 binding. Some changes that abolished the ability of synthetic peptides to bind β_2 resulted in an α subunit still able to function *in vivo* even if expressed at "leaky" levels from a highly repressed $pA1_{/04/03}$ promoter.[22] However, three mutations, deletion of the seven C-terminal residues, replacement of the C-terminal "hexameric" β_2 binding site with a "pentameric" consensus sequence, and mutation of F1159 to A, resulted in a gene that was only able to support chromosomal DNA replication upon transcriptional induction with IPTG. This indicates a functional contribution *in vivo* of the C-terminal element.

However, this result is complicated by a pleiotropic function of the C terminus. C-terminal α sequences bind both τ and β_2 in a competitive interaction.[18] Our results show that C-terminal α mutations that only partially complement *dnaE* temperature sensitivity (M12,13) exhibit the most severe defects in τ binding. Thus, the two C-terminal mutations that exhibit the most reduced biological function correlate with severe τ binding defects. Mutation of a residue that reduces β_2 binding in peptides (Q1154A, M10) but does not affect τ binding in the context of full-length α does not exhibit a defect in chromosomal replication.

An interesting observation made as part of our studies is the context sensitivity of sequence changes of the internal and C-terminal β_2 binding sites. Substitution of the internal site for the consensus β_2 binding site QLDLF resulted in a protein that bound β_2 greater than 100 times more strongly than the WT protein. Yet, placing this same sequence at the C terminus barely changed the affinity of α for β_2. This is likely due to the conformation in which the sequence is presented to β_2 in the two sites, but may also be caused by synergistic interaction with additional contacts outside the identified β_2 binding site.

The O'Donnell laboratory made the important observation that τ facilitates cycling of Pol III upon completion of an Okazaki fragment.[10,18] They put forward a model whereby τ and β_2 compete for binding to the C-terminal site and that upon encountering a nick, τ displaces β. However, we observed earlier that τ and β_2 can bind to α with little effect upon one another.[12] At most, the K_D values change twofold. This apparent discrepancy can be explained if the internal β_2 binding site provides most of the binding energy, but is not affected by τ binding. This would permit effective competition for the C-terminal sequences while maintaining a stable β_2–α interaction.

However, the internal site–β_2 interaction would also have to be destabilized to permit polymerase recycling. We previously observed that in a DNA-free state, the presence of the polymerase domain of α destabilized β_2–α interaction 20-fold.[12] If this negative effect was repressed when the polymerase was productively occupied on DNA and became operative upon encountering a nick, an alternative pathway would exist to enable polymerase recycling. Perhaps this mechanism explains why Pol III holoenzyme containing only the γ subunit, lacking τ, is capable of reduced but efficient Okazaki fragment synthesis during rolling circle replication.[23] The internal β_2 binding site is flanked by two potential DNA binding elements (Figure 1). These might serve as sensors, altering the conformation of the internal β_2 binding site depending upon the DNA binding state of the polymerase.

Cycling can also be triggered by synthesis of a new primer at the fork by DnaG primase, even before completion of ongoing Okazaki fragment synthesis.[24] Thus, full understanding of the modulation of polymerase–β_2 interactions during cycling will require the understanding of the integration of the requisite signaling for the DnaG-dependent pathway with those described[25] above.

We note parallels between our findings and possible mechanisms involved in eukaryotic DNA replication. Eukaryotic DNA polymerase δ holoenzyme has been shown to have up to three PCNA binding sites. A complex of only the large catalytic subunit and the second subunit (POL 33, CDC1 and p55 in *Saccharomyces cerevisiae, Schizosaccharomyces pombe* and humans, respectively) requires PCNA for processive DNA replication. Although some aspects remain controversial, binding of both subunits to PCNA has been reported. Binding of the catalytic subunit to PCNA occurs through its N terminus.[26] The second subunit has also been proposed to interact through its N terminus.[27] The third subunit (POL32 and CDC27 in *S. cerevisiae* and *S. pombe)* has been shown to interact with PCNA in solution by itself through a PCNA binding site found in the C-terminal domain.[28,29]

With the discovery of an important class of error-prone polymerases[15,17,30] and other proteins that interact with the β_2 protein,[15,16,31] initially identified as a cofactor for the DNA polymerase III holoenzyme,[32] considerable attention has been devoted to competition of these proteins for shared β_2 binding sites. The X-ray crystal structure of the C-terminal domain of DNA polymerase IV with β_2 shows that it is possible for both sites to be occupied simultaneously.[33] Even earlier, the notion of the multiple binding sites on β_2 and PCNA serving as a "tool belt" that might carry along factors with the replicase that might be needed for downstream transactions had been considered.[34] Thus, the demonstration of two binding sites within α for β_2 has significant importance, since, if both are indeed occupied simultaneously, interaction of other β_2 binding proteins would be precluded (Figure 4).

One possible untested consequence of the C-terminal τ/β_2 competition model is that, if τ binds the C terminus of α upon encountering blocking damage, the C-terminal β_2 binding site is released. This might permit association of alternative polymerases, like DNA polymerases IV or V, with β_2 at the revealed site. Determination of which polymerase associates might be determined by additional contacts with proteins associated with the damaged fork or by the relative levels of these proteins present in the cell because of the status of SOS induction.[35–38] Polymerase switching could occur without complete release of Pol III, which could remain associated with β_2 through association *via* its internal site. Indeed, the Benkovic laboratory has elegantly demonstrated that T4 DNA polymerase can actively associate with the trimeric T4 sliding clamp and displace the initial polymerase during ongoing replication.[25] Such a mechanism would permit facile replacement by Pol III at the primer terminus once the damaged site is bypassed. These intriguing possibilities will require experimental testing in future work, including a determination of whether Pol III containing a C-terminal binding defect permits easier access of other polymerases to the fork.

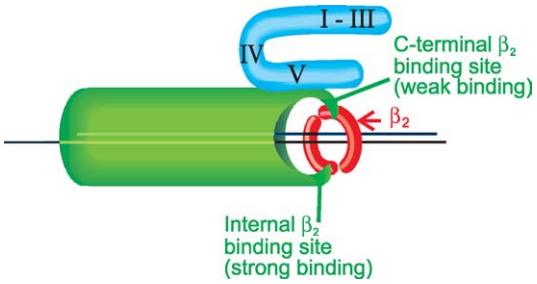

Figure 4. A bipartite association of the α subunit of Pol III and β_2. Pol III is associated with β_2 through both internal and C-terminal binding sites. Contact between two β binding sites within α and the two α binding sites within dimeric β would "saturate" all β_2 binding sites, precluding association with other polymerases. Even though the affinity of the C-terminal element of α for β is not strong enough to generate an interaction under physiological protein concentrations, the local concentration of the C-terminal element would be enormously enhanced once an interaction of the internal α element is established. As discussed in the text, a competition of the C terminus of τ, most likely domain V, for the β_2 binding site or an internal communication between the polymerase active site and the internal β_2 binding site might reveal one or both sites on β_2, permitting intrusion by other DNA polymerases. Polymerase cycling, upon completion of an Okazaki fragment, might be enabled by one or both of these mechanisms.

Materials and Methods

Lysozyme was purchased from Worthington Biochemical. Superflow Ni^{2+}-NTA resin was purchased from Qiagen. P-20 surfactant and streptavidin (SA) chips were purchased from Biacore.

Buffer N is 50 mM sodium phosphate (pH 7.8), 500 mM NaCl, 10% (v/v) glycerol, 0.5 mM DTT, 1 mM imidazole. Buffer S is 50 mM sodium phosphate (pH 7.8), 500 mM NaCl, 20% glycerol, 0.5 mM DTT. HKGM buffer is 50 mM Hepes (pH 7.4), 100 mM potassium glutamate, 10 mM MgOAc, 0.005% P-20 surfactant.

Strains

Escherichia coli strain DH5α (F^- ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *hsd*R17 (r_k^-, m_k^+) *pho*A *sup*E44 λ^- *thi*1 *gyr*A96 *rel*A1) was used for cloning procedures and for plasmid propagation. *E. coli* strain BL21(DE3) (F^- *omp*T *dcm hsd*S(r_B^-, m_B^-) *gal* λ(DE3)) was used for protein expression. *E. coli* strain UTH2 (F^- *thr leu* B_1^- *thy lac str*R *met ton*A *su*II$^+$ *srl*$^-$ 1300::Tn*10 rec*A56 *dnaE*486) was used for complementation analysis.[39]

Plasmid construction and site-specific mutagenesis

Two plasmids were used for expression of the various *dnaE* mutations: pET11-N and pDRKc.[11] The pET11-N plasmid fuses a hexaHis tag and a biotinylation sequence to the N terminus of the protein of interest. The pDRKc vector contains a pA1 promoter (IPTG inducible) upstream of the gene of interest.

pPRD3, a vector that expresses the full-length native *dnaE* gene from the pA1 promoter, was constructed by recombinant methods. A nearly full-length PstI to BamHI

dnaE fragment (lacking several codons from the N terminus) from pET11-N(O)[11] was cloned into the PstI to BamHI sites of pBluescript KS+ (Stratagene), generating pPRD1. To create pPRD2, the PstI to ApaLI region of pPRD1 was replaced with an oligonucleotide encoding an optimized consensus ribosome binding site and the first ten codons of *dnaE*, which were changed to the most prevalent isoaccepting tRNAs. The sequence reads (PstI site, ribosome binding site, first ten amino acid residues, ApaLI):CTGCAGAAGGAGGTTATATATAATGAGCGAG CCGCGCTTCGTTCACCTGCGCGTGCA. The full-length *dnaE* EcoRI to XbaI fragment from pPRD2 was cloned into the EcoRI to XbaI sites of pDRKc,[11] generating pPRD3.

The pET11-N(O) series of mutants M1 through M13 were constructed by an overlap extension polymerase chain reaction (PCR) procedure.[40] Oligonucleotide pairs were complementary either to sequences flanking the region of the desired mutation or to the sequence we desired to mutate and encoded the mutation (see Table 1). Two rounds of PCR were performed. In the first round, a reaction was performed with a single flanking primer and a mutant primer. Either pET11-N(O) or pPRD3 was used as the DNA template. The second round was run using the two overlapping primary mutant PCR fragments and the outside primers only. Restriction digests were performed on PCR-amplified fragments from the second round and ligated into the expression vector pET11-N(O).

The pPRD3 (pA1 promoter driven *dnaE* expression) series of mutants M1 through M13 was constructed by moving restriction fragments bearing the original mutation from pET11-N(O) series into pPRD3. All mutations were verified by sequencing and/or diagnostic restriction digests. Many of the mutations were marked with restriction sites to facilitate screening (Table 1).

Complementation analysis

Strain UTH2 (*dnaE486*-ts) was transformed with the pPRD3 series of mutant plasmids. Transformants were selected on L-plates containing ampicillin at 100 μg/ml at 30 °C. Individual colonies were assayed for complementation by streaking them out in quadruplicate onto selective plates containing 0 mM, 0.5 mM, or 1 mM IPTG at 42 °C. The growth of individual colonies after three days indicated that the mutant plasmid could complement the temperature-sensitive defect.

Expression and purification of mutant α subunits

Strains containing overexpression plasmids were grown as described[11] with the following exceptions. Strains were grown in 1.5 liters of L-Broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 0.005% (w/v) thymine (pH 7.0)+100 μg/ml ampicillin) at 23 °C at 220 rpm on a rotary shaker. At the time of induction, *d*-biotin and additional ampicillin were added to 10 μM and 100 μg/ml, respectively. Cells were harvested three hours after induction.

Cells were lysed as described[41,42] in the presence of 3 mg/ml lysozyme, 5 mM benzamidine, 1 mM PMSF, and 5 mM EDTA. The α fusion protein was precipitated with ammonium sulfate, followed by a Ni^{2+}-NTA column as described[11] with the following exceptions. The column was washed with 20 volumes of buffer S+1 mM imidazole (pH 7.8). Proteins were eluted with 12 column volumes of a 1 mM–100 mM imidazole (pH 7.8) gradient in buffer S. After chromatography, activity of the α subunit was monitored using the gap-filling assay. Activity eluted approximately halfway through the gradient.

Biochemical assays

The gap-filling assay measures the ability of a polymerase to fill in the gaps in a nuclease-activated calf thymus DNA as a template. It does not require interaction with the other Pol III holoenzyme subunits. Gap-filling assays were performed as described.[42,43]

The Pol III holoenzyme reconstitution assay measures the processive function of Pol III on long single-stranded DNA templates. Assays were performed as described[42,43] with the following differences. Holoenzyme replication assays were assembled on ice using the τ-complex (100 fmol as $\tau_3\delta\delta'\chi\psi$), β_2 (150 fmol as dimer), mixed with M13G*ori* DNA (58 fmol as circle), *DnaG* primase (1.5 pmol), SSB (60 pmol, monomer), four rNTPs (0.2 mM each), four dNTPs (48 μM each dATP, dCTP, dGTP; 18 μM dTTP;100 cpm ^{3}H/pmol dNTPs) and 10 mM magnesium acetate. The reaction was initiated by adding the wild-type or mutant α subunit as the last step and then immediately placing the reaction at 30 °C for five minutes. To assure reproducibility from assay to assay, a mixture of all the components (except α) was prepared and quickly frozen in liquid nitrogen for short-term storage. The assays were performed by thawing the mixture and then adding the α subunit. Control experiments demonstrated that the freezing of the mixture had no effect on the activity of the assay. To directly compare different mutant α subunits, we titrated the subunits based on their gap-filling activities.

Protein concentrations were determined by the method of Bradford[44] using the Coomassie Plus Bradford Assay Reagent (Pierce) according to the manufacturer's instructions. Bovine serum albumin (BSA) was used as the protein standard. Protein concentrations were measured in the linear region of the protein standard curve.

Surface plasmon resonance

A Biacore 3000 instrument was used to quantify α–β_2 and α–τ interactions. α (WT or mutant) was immobilized on a SA chip by a biotin–streptavidin interaction. Typically, 3000–3500 response units of α were loaded per flow cell. A flow rate of 5 μl/minute in HKGM buffer at 20 °C was used as previously determined for the α–β_2 interaction.[12] All buffers were filtered and degassed before use. The SA chip was pre-conditioned with three one-minute injections of 5 μl of 1 M NaCl/50 mM NaOH per the manufacturer's instructions. Flow cell 1 was not derivatized and was used as a control surface for background subtraction. After completion of a β_2 injection, the response quickly returned to baseline; thus, regeneration buffers were not required. For the α–β_2 interaction, the kinetic analysis of the k_{off} and the k_{on} rates could not be accurately determined for most of the mutants because they were too fast (beyond the limitations of the instrument). Thus, the dissociation constant K_D for α–β_2 binding was determined by running a series of concentrations in quadruplicate of β_2 (40 μl from 50 nM to 10,000 nM) over the indicated mutant α protein at a flow rate of 5 μl/minute. The R_{max} level of each response curve was plotted as a function of the β_2 concentration and fit to the 1:1 Langmuir model using BiaEvaluation 3.2 software as described.[45]

The binding affinity of M3α for β_2 was significantly greater than that of the other α subunits studied. In this

case, β_2 did not rapidly establish equilibrium, but associated more slowly than other α subunits and dissociated much more slowly. Thus, the K_D was calculated from the ratio of the determined rate constants for k_{off} and k_{on}. Several different concentrations of β_2 were run over the SA chip at different response units of α loaded onto the chips: 500 RUs, 1000 RUs, and 3500 RUs. Accurate off-rates were determined in quadruplicate for β_2 at concentrations of 10 nM, 50 nM, and 1000 nM. They did not significantly differ (6.1×10^{-3} ($\pm1.0\times10^{-3}$) s^{-1}). Accurate on-rates in quadruplicate were only determined for lowest 10 nM β_2 concentration at α response units of 500, 1000, and 3500. They did not significantly differ (8.8×10^{5} ($\pm3.7\times10^{5}$) 1 M^{-1} s^{-1}). The K_D was derived by the equation $K_D = k_{off}/k_{on}$.

In determinations of τ binding to α, we could not find regeneration conditions that would dissociate the bound τ from α completely without denaturing α. Thus, an individual SA chip was used for each binding experiment. Flow cell 1 was not derivatized and was used as a control surface for background subtraction. For individual flow cells 2, 3, and 4, 400–500 response units of the mutant α subunit were immobilized onto each. Three different concentrations of the τ subunit were injected (40 μl) over each separate flow cell at a flow rate of 5 μl/minute in buffer HKGM. τ Binding was tested at concentrations of 5 nM, 10 nM, and 20 nM for α mutants bearing the internal β_2 binding site mutations and at concentrations of 50 nM, 100 nM, and 200 nM for α mutants bearing the C-terminal β_2 binding site mutations. Dissociation was allowed to take place for 30 minutes. The dissociation constants were determined by fitting the resulting sensorgrams to a 1:1 Langmuir model using BiaEvaluation 3.2 software.

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References

1. McHenry, C. S. (2003). Chromosomal replicases as asymmetric dimers: studies of subunit arrangement and functional consequences. *Mol. Microbiol.* **49**, 1157–1165.
2. Kelman, Z. & O'Donnell, M. E. (1995). DNA polymerase III holoenzyme: structure and function of a chromosomal replicating machine. *Annu. Rev. Biochem.* **64**, 171–200.
3. LaDuca, R. J., Crute, J. J., McHenry, C. S. & Bambara, R. A. (1986). The β subunit of the *Escherichia coli* DNA polymerase III holoenzyme interacts functionally with the catalytic core in the absence of other subunits. *J. Biol. Chem.* **261**, 7550–7557.
4. Kong, X. P., Onrust, R., O'Donnell, M. E. & Kuriyan, J. (1992). Three-dimensional structure of the β subunit of *E. coli* DNA polymerase III holoenzyme: a sliding DNA clamp. *Cell*, **69**, 425–437.
5. Blinkowa, A. L. & Walker, J. R. (1990). Programmed ribosomal frameshifting generates the *Escherichia coli* DNA polymerase III γ subunit from within the τ subunit reading frame. *Nucl. Acids Res.* **18**, 1725–1729.
6. Tsuchihashi, Z. & Kornberg, A. (1990). Translational frameshifting generates the γ subunit of DNA polymerase III holoenzyme. *Proc. Natl Acad. Sci. USA*, **87**, 2516–2520.
7. Flower, A. M. & McHenry, C. S. (1990). The γ subunit of DNA polymerase III holoenzyme of *Escherichia coli* is produced by ribosomal frameshifting. *Proc. Natl Acad. Sci. USA*, **87**, 3713–3717.
8. Dallmann, H. G., Kim, S., Pritchard, A. E., Marians, K. J. & McHenry, C. S. (2000). Characterization of the unique C-terminus of the *Escherichia coli* τ DnaX protein: monomeric C-τ binds α and DnaB and can partially replace τ in reconstituted replication forks. *J. Biol. Chem.* **275**, 15512–15519.
9. Gao, D. & McHenry, C. S. (2001). τ Binds and organizes *Escherichia coli* replication proteins through distinct domains: partial proteolysis of terminally tagged τ to determine candidate domains and to assign domain v as the α binding domain. *J. Biol. Chem.* **276**, 4433–4440.
10. Leu, F. P., Georgescu, R. & O'Donnell, M. E. (2003). Mechanism of the *E. coli* τ processivity switch during lagging-strand synthesis. *Mol. Cell*, **11**, 315–327.
11. Kim, D. R. & McHenry, C. S. (1996). Biotin tagging deletion analysis of domain limits involved in protein–macromolecular interactions: mapping the τ binding domain of the dna polymerase III α subunit. *J. Biol. Chem.* **271**, 20690–20698.
12. Kim, D. R. & McHenry, C. S. (1996). Identification of the β-binding domain of the α subunit of *Escherichia coli* polymerase III holoenzyme. *J. Biol. Chem.* **271**, 20699–20704.
13. Dalrymple, B. P., Kongsuwan, K., Wijffels, G., Dixon, N. E. & Jennings, P. A. (2001). A universal protein–protein interaction motif in the eubacterial DNA replication and repair systems. *Proc. Natl Acad. Sci. USA*, **98**, 11627–11632.
14. Wijffels, G., Dalrymple, B. P., Prosselkov, P., Kongsuwan, K., Epa, V. C., Lilley, P. E. *et al.* (2004). Inhibition of protein interactions with the β2 sliding clamp of *Escherichia coli* DNA polymerase III by peptides from β2-binding proteins. *Biochemistry*, **43**, 5661–5671.
15. Lopez de Saro, F. J., Georgescu, R. E., Goodman, M. F. & O'Donnell, M. E. (2003). Competitive processivity-clamp usage by DNA polymerases during DNA replication and repair. *EMBO J.* **22**, 6408–6418.
16. Kurz, M., Dalrymple, B., Wijffels, G. & Kongsuwan, K. (2004). Interaction of the sliding clamp β-subunit and Hda, a DnaA-related protein. *J. Bacteriol.* **186**, 3508–3515.
17. Lenne-Samuel, N., Wagner, J., Etienne, H. & Fuchs, R. P. P. (2002). The processivity factor β controls DNA polymerase IV traffic during spontaneous mutagenesis and translesion synthesis *in vivo*. *EMBO Rep.* **3**, 45–49.
18. Lopez de Saro, F. J., Georgescu, R. E. & O'Donnell, M. E. (2003). A peptide switch regulates DNA polymerase processivity. *Proc. Natl Acad. Sci. USA*, **100**, 14689–14694.
19. McHenry, C. S. & Johanson, K. O. (1981). Purification of the beta subunit of the DNA polymerase III holo enzyme of *Escherichia coli* and analysis of its function in the initiation of DNA elongation. Meeting on

structure and DNA–protein interactions of replication origins presented at the Icn-University of California at Los Angeles. *Struct. Cell Biochem.* (Suppl. 5), 343.
20. Glover, B. P. & McHenry, C. S. (1998). The χψ subunits of DNA polymerase III holoenzyme bind to single-stranded DNA-binding protein (SSB) and facilitate replication of an SSB-coated template. *J. Biol. Chem.* **273**, 23476–23484.
21. Jeruzalmi, D., Yurieva, O., Zhao, Y., Young, M., Stewart, J., Hingorani, M. *et al*. (2001). Mechanism of processivity clamp opening by the delta subunit wrench of the clamp loader complex of *E. coli* DNA polymerase III. *Cell*, **106**, 417–428.
22. Lanzer, M. L. & Bujard, H. (1988). Promoters largely determine the efficiency of repressor action. *Proc. Natl Acad. Sci. USA*, **85**, 8973–8977.
23. Kim, S., Dallmann, H. G., McHenry, C. S. & Marians, K. J. (1996). τ Protects β in the leading-strand polymerase complex at the replication fork. *J. Biol. Chem.* **271**, 4315–4318.
24. Li, X. & Marians, K. J. (2000). Two distinct triggers for cycling of the lagging strand polymerase at the replication fork. *J. Biol. Chem.* **275**, 34757–34765.
25. Yang, J., Zhuang, Z., Roccasecca, R. M., Trakselis, M. A. & Benkovic, S. J. (2004). The dynamic processivity of the T4 DNA polymerase during replication. *Proc. Natl Acad. Sci. USA*, **101**, 8289–8294.
26. Zhang, P., Mo, J. Y., Perez, A., Leon, A., Liu, L., Mazloum, N. *et al*. (1999). Direct interaction of proliferating cell nuclear antigen with the p125 catalytic subunit of mammalian DNA polymerase δ. *J. Biol. Chem.* **274**, 26647–26653.
27. Lu, X., Tan, C. K., Zhou, J. Q., You, M., Carastro, L. M., Downey, K. M. & So, A. G. (2002). Direct interaction of proliferating cell nuclear antigen with the small subunit of DNA polymerase delta. *J. Biol. Chem.* **277**, 24340–24345.
28. Johansson, E., Garg, P. & Burgers, P. M. (2004). The Pol32 subunit of DNA polymerase δ contains separable domains for processive replication and proliferating cell nuclear antigen (PCNA) binding. *J. Biol. Chem.* **279**, 1907–1915.
29. Bermudez, V. P., MacNeill, S. A., Tappin, I. & Hurwitz, J. (2002). The influence of the Cdc27 subunit on the properties of the *Schizosaccharomyces pombe* DNA polymerase delta. *J. Biol. Chem.* **277**, 36853–36862.
30. Sutton, M. D., Kim, M. & Walker, G. C. (2001). Genetic and biochemical characterization of a novel umuD mutation: insights into a mechanism for UmuD self-cleavage. *J. Bacteriol.* **183**, 347–357.
31. Hughes, A. J., Jr, Bryan, S. K., Chen, H., Moses, R. E. & McHenry, C. S. (1991). *Escherichia coli* DNA polymerase II is stimulated by DNA polymerase III holoenzyme auxiliary subunits. *J. Biol. Chem.* **266**, 4568–4573.
32. McHenry, C. S. & Kornberg, A. (1977). DNA polymerase III holoenzyme of *Escherichia coli* purfication and resolution into subunits. *J. Biol. Chem.* **252**, 6478–6484.
33. Bunting, K. A., Roe, S. M. & Pearl, L. H. (2003). Structural basis for recruitment of translesion DNA polymerase Pol IV/DinB to the β-clamp. *EMBO J.* **22**, 5883–5892.
34. Pages, V. & Fuchs, R. P. P. (2002). How DNA lesions are turned into mutations within cells? *Oncogene*, **21**, 8957–8966.
35. Burnouf, D. Y., Olieric, V., Wagner, J., Fujii, S., Reinbolt, J., Fuchs, R. P. & Dumas, P. (2004). Structural and biochemical analysis of sliding clamp/ligand interactions suggest a competition between replicative and translesion DNA polymerases. *J. Mol. Biol.* **335**, 1187–1197.
36. Pham, P., Rangarajan, S., Woodgate, R. & Goodman, M. F. (2001). Roles of DNA polymerases V and II in SOS-induced error-prone and error-free repair in *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **98**, 8350–8354.
37. Sutton, M. D., Narumi, I. & Walker, G. C. (2002). Posttranslational modification of the umuD-encoded subunit of *Escherichia coli* DNA polymerase V regulates its interactions with the β processivity clamp. *Proc. Natl Acad. Sci. USA*, **99**, 5307–5312.
38. Walker, G. C. (1998). Skiing the black diamond slope: progress on the biochemistry of translesion DNA synthesis. *Proc. Natl Acad. Sci. USA*, **95**, 10348–10350.
39. Welch, M. M. & McHenry, C. S. (1982). Cloning and identification of the product of the dnaE gene of *Escherichia coli*. *J. Bacteriol.* **152**, 351–356.
40. Horton, R. M., Ho, S. N., Pullen, J. K., Hunt, H. D., Cai, Z. & Pease, L. R. (1993). Gene splicing by overlap extension. *Methods Enzymol.* **217**, 270–279.
41. Cull, M. G. & McHenry, C. S. (1995). Purification of *Escherichia coli* DNA polymerase III holoenzyme. *Methods Enzymol.* **262**, 22–35.
42. Pritchard, A. E. & McHenry, C. S. (1999). Identification of the acidic residues in the active site of DNA polymerase III. *J. Mol. Biol.* **285**, 1067–1080.
43. McHenry, C. S. & Crow, W. (1979). DNA polymerase III of *Escherichia coli*: purification and identification of subunits. *J. Biol. Chem.* **254**, 1748–1753.
44. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.
45. Glover, B. P. & McHenry, C. S. (1998). The χψ subunits of DNA polymerase III holoenzyme bind to single-stranded DNA-binding protein (SSB) and facilitate replication of an SSB-coated template. *J. Biol. Chem.* **273**, 23476–23484.
46. Doherty, A. J., Serpell, L. C. & Ponting, C. P. (1996). The helix-hairpin-helix DNA-binding motif: a structural basis for non-sequence-specific recognition of DNA. *Nucl. Acids Res.* **24**, 2488–2497.
47. Theobald, D. L., Mitton-Fry, R. M. & Wuttke, D. S. (2003). Nucleic acid recognition by OB-fold proteins. *Annu. Rev. Biophys. Biomol. Struct.* **32**, 115–133.
48. Aravind, L. & Koonin, E. V. (1998). Phosphoesterase domains associated with DNA polymerases of diverse origins. *Nucl. Acids Res.* **26**, 3746–3752.
49. Evans, T. C., Jr, Martin, D., Kolly, R., Panne, D., Sun, L., Ghosh, I. *et al*. (2000). Protein trans-splicing and cyclization by a naturally split intein from the dnaE Gene of *Synechocystis* species PCC6803. *J. Biol. Chem.* **275**, 9091–9094.

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