DNA Polymerase α Subunit Residues and Interactions Required for Efficient Initiation Complex Formation Identified by a Genetic Screen*

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Running title: Genetic Screen for Initiation Complex Formation Defects

Keywords: Replicase, DNA polymerase III holoenzyme, initiation complex formation, genetic screen, protein-protein interaction, DNA polymerase, DNA replication, E. coli

Background: A genetic screen revealed interactions important for replication initiation.

Results: A PHP mutation ablated interaction with the ε subunit and distorted the active site. C-terminal mutations decreased binding by the clamp loader τ subunit.

Conclusion: The β binding domain functions in both β and τ binding.

Significance: This work advances our knowledge of replicase interactions critical for function.

ABSTRACT

Biophysical and structural studies have defined many of the interactions that occur between individual components or subassemblies of the bacterial replicase, DNA polymerase III holoenzyme (Pol III HE). Here, we extended our knowledge of residues and interactions that are important for the first step of the replicase reaction—the ATP-dependent formation of an initiation complex between the Pol III HE and primed DNA. We exploited a genetic selection using a dominant negative variant of the polymerase catalytic subunit that can effectively compete with wild-type Pol III α and form initiation complexes, but cannot elongate. Suppression of the dominant negative phenotype was achieved by secondary mutations that were ineffective in initiation complex formation. The corresponding proteins were purified and characterized. One class of mutant mapped to the PHP domain of Pol III α, ablating interaction with the ε proofreading subunit and distorting the polymerase active site in the adjacent polymerase domain. Another class of mutation, found near the C-terminus, interfered with τ binding. A third class mapped within the known β-binding domain, decreasing interaction with the β₂ processivity factor. Surprisingly, mutations within the β binding domain also ablated interaction with τ, suggesting a larger τ binding site than previously recognized.

The E. coli DNA polymerase III holoenzyme (Pol III HE)³ serves as a prototype for cellular replicases in all cellular systems (for a review, see (1)). It encompasses the standard tripartite composition found in all branches of life: (i) a DNA polymerase that is non-processive and exhibits no special properties by itself (2,3), (ii) a sliding clamp processivity factor, β₂, that encircles DNA and contacts the polymerase and ε proofreading subunit locking the enzyme into a processive complex (4-8) and (iii) a ‘clamp loader’ the DnaX complex (τγδδ'ψϕ) that exploits the energy of ATP hydrolysis to assemble β₂ onto a primer terminus and then chaperones the associated Pol III α subunit onto β₂ (9,10). Together, these three assemblies form a tight initiation complex on primed DNA in the absence of dNTPs (11). Upon addition of dNTPs, the Pol III HE advances rapidly and processively to synthesize at least 150 Kb of DNA without dissociating and, perhaps, the entire E. coli chromosome if a blocking lesion is not encountered (12,13).

Components of the DnaX complex are also involved in processive elongation. The presence of the τ subunit is required to protect the elongating complex from dissociation by removal of β₂ by exogenous DnaX complex
The presence of δ and δ', primarily characterized for their role in initiation complex formation are also required for processive elongation (15). The χ subunit contributes to processive elongation by interaction with SSB, stabilizing Pol III HE on the replication fork (16-20).

Significant information is available regarding subunit interactions within the Pol III HE derived from quantification of physical interactions with subassemblies in solution and by determination of the structure of subassemblies (7-9,21-33). More limited information is available regarding the dynamic interactions between Pol III HE subunits in the presence of all reaction components and the importance of these interactions at discrete reaction stages. To address this deficit in our understanding, we exploited a dominant negative Pol III α that contains a mutation in one of the critical acidic catalytic residues. Pol III α D403E can efficiently form initiation complexes but is unable to elongate (34). This results in sequestration of primer termini and blockage of competing wild-type Pol III HE (10).

This provided the basis for a genetic selection to isolate secondary mutations within dnaE D403E that caused loss of the dominant negative phenotype. We exploited this screen and identified several diverse mutations, spatially dispersed in three separate domains of Pol III α. Physical and enzymological characterization of these variant proteins provided additional insight regarding the positions of subunit interaction within Pol III α and the importance of these interactions in the initiation complex formation stage of the replicative reaction.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains**

Strains used in this study are listed in Table 1. BL21(DE3) was used for expression of T7 promoter-containing plasmids. Both BL21(DE3) and JCL60 were used for the selection of suppressor mutations. JCL60 and BL21 (DE3) contain the integrated λ(DE3) lysogen carrying the structural gene for T7 RNA polymerase under control of the lacUV5 promoter. JCL60 was prepared by integrating the λ(DE3) prophage into the E. coli TOP10 using a commercial λ(DE3) lysogenization kit.

**Plasmids**

The plasmids used in this study are listed in Table 1. pJCL5 contains dnaE D403E (plus N-terminal hexaHis and biotinylation tag) under control of the pBAD promoter (arabinose inducible). To prepare pJCL5, dnaE D403E was PCR amplified from pDFER.2 (34) using primers #JCL14 (5’ ATACCATGGCTGGTTGCCTGAAC) and #JCL17 (5’ ATAGAATTCTTATGCTAAAACCTCAG). The dnaE D403 fragment was digested with NcoI and EcoRI (3584 bp) and ligated into the NcoI and EcoRI sites of pBAD/HisA (3961 bp) creating pJCL3. A DNA fragment carrying the araC, pBAD promoter and dnaE D403E was removed from pJCL3 with EcoRI and SphI (4898 bp) and ligated into pET29c cut with EcoRI and SphI (4967bp, carrying kanR, lacI and both origins of replication) generating pJCL5.

pJCL5-lacZα-T7 is identical to pJCL5 except that it contains a T7 promoter upstream of the pBAD promoter and lacZα downstream of the dnaE D403 (Fig. 1). Overlap PCR (35) was used to place the lacZα downstream of the dnaE D403E. Primers S7269 (5’ ATACCATGGCTGGTTGCCTGAAC) and A9847 (5’ GTGTACACCTTTAGTCAAACTCCAGTTCCACC) were used to amplify a DNA fragment containing the 3’ end of dnaE D403E using pDFER.2 as a template (1645 bp). Primers S9833 (5’ TAAGGAGCTGACACATAGACCATGATTACCATTC) and A10047 (5’ GCGGCCGCTTATTAGCGCCATTCGCCATT CAGG) were used to amplify lacZα from E. coli MG1655 genomic DNA (210 bp). The overlap PCR fragment was generated using the outside primers S7269 and A10047 and the 1645 bp and 210 bp PCR fragments from above as templates. The resulting overlap PCR fragment (1842 bp fragment) was digested with BglII and NotI and ligated into the pJCL vector digested with BglII and NotI generating pJCL5-lacZα. The T7 promoter was placed upstream of the pBAD promoter in
pJCL5-\(\alpha\)-\(\beta\)-lacZ by overlap PCR. Primers S4843 (5’ GATCTCGACGCTCTCCCTTATGC) and AM2 (5’ TATAGTGAGTCGTATTACTTCTCTGAATGG CGGGAGT) were used to amplify the region upstream of pBAD promoter using pJCL5 as template (1110 bp). The primers SM2 (5’ ATACGACTCACTATAGGGAAACCAATTGT CCATATGGCA) and A6276 (5’ AGCCATGGTTAATTCCTCCTGTTAGCC) were used to amplify the region downstream of the pBAD promoter using pJCL5 as template (339 bp). The overlap PCR fragment was generated using the outside primers S4843 and AM2 using the 1110 bp and 339 bp PCR fragments from above as templates. The resulting overlap PCR fragment was digested with SphI and BamHI (1.25 kb) and ligated into pJCL5-\(\alpha\)-\(\beta\)-lacZ digested with SphI and BamHI (8.8 kb) to generate pJCL5-\(\alpha\)-\(\beta\)-T7.

Sde expression vectors (double mutant; dnaE D403E and sde8)

pJCL8 expresses dnaE D403E, A877E (sde8) from the T7 promoter. The sde8 mutation in pJCL8 was originally isolated from a genetic selection using the pJCL5 plasmid as the parental vector. The plasmid also contained an additional mutation (D792G) that was shown not involved in the suppression phenotype as separation of the D792G from the A877E did not alter the suppressor phenotype of A877E (data not shown). Consequently no further characterization of D792G was performed. To move the sde8 mutation into a clean T7 expression plasmid (away from D792G), overlap PCR was used to amplify dnaE D403E, A877E. Primers #JCL42 (5’ TTTCTCGAGCTGATCCGCACC) and #JCL44 (5’ ACGGTCAAACTCCCCGGACAT) plus #JCL43 (5’ ATGTCCGGGGAGTTTGACCGT) and #JCL45 (5’ ACGGTCAAACTCCCCGGACAT) were used to amplify two PCR fragments from the pDFER.2 plasmid bearing the dnaE D403E mutation. The overlap PCR fragment was generated by using the outside primers #JCL42 and #JCL45 and using the 2171 bp and 815 bp fragment from above as templates. The resulting overlap PCR fragment (2966 bp) was digested with EcoRI and HindIII and ligated into pDFER.2 (EcoRI and HindIII sites) generating pJCL8.

The plasmid pJCL21 plasmid expresses dnaE D403E, W1134C (sde50) from the T7 inducible promoter. The sde50 mutation in pJCL21 was originally isolated from a genetic selection using the pJCL5 plasmid as parental vector. To construct pJCL21, the pJCL5 plasmid harboring the sde50 mutation (dnaE D403E, W1134C) was digested with SphI/PstI plasmid to remove the pBAD promoter (1412 bp) and replaced with a DNA fragment bearing the T7 promoter from pET11.N0.1 (397 bp) generating pJCL21.

The plasmids pJCL24 and pJCL25 express dnaE D403E, L1157Q (sde387) and dnaE D403E, Δ881-927 (sde388) from the T7 inducible promoter, respectively. The sde387 and sde388 mutations in these plasmids were originally isolated from a genetic selection using pJCL5-\(\alpha\)-\(\beta\)-T7 plasmid as the parental vector. To construct pJCL24 and pJCL25, pJCL5-\(\alpha\)-\(\beta\)-T7 harboring either the sde387 or sde388 mutation was digested with SphI/PstI to remove the dual T7/pBAD promoter region (1432 bp), and replaced with a DNA fragment bearing the T7 promoter from pET11.N0.1 (397bp) generating pJCL24 and pJCL25.

Plasmid pJCL26 expresses the dnaE D403E, Q238K mutation (sde343) from the T7 promoter. The sde343 mutation in this plasmid was originally isolated from a genetic selection using the pJCL5-\(\alpha\)-\(\beta\)-T7 plasmid as the parental vector. In this case, sde343 mutation was moved into the pET11.N0.1 expression vector by overlap PCR. The 5’ region of dnaE bearing the Q238K mutation was amplified using oligos #JCL45 (5’ GGCCTGCAAGCGCGACCC) and #JCL46 (5’ ACGATATATTTCTGCAGCGC) using pDFER.2 as template. The region bearing the dnaE D403E mutation was amplified using oligos #JCL47 (5’ TCGCAGCCGCTGCTGTTG) and #JCL48 (5’ TACAGGCCCTAAGGTTCGCTCC). The overlap PCR fragment was generated using the outside primers #JCL45 and #JCL48 and using the 728 bp and 2176 bp PCR fragments from above as templates. The resulting overlap...
PCR fragment 2884 bp was digested with StuI and PstI (2873 bp) and ligated into the StuI and PstI sites of pDFER.2 (7187 bp) generating pJCL26.

Sde expression vectors (single sde mutation only)

The pET11.N0.1 plasmid fuses a hexaHis tag and a biotinylation sequence to the N-terminus of the Pol III α subunit under control of the T7 promoter (22,24). Site directed mutagenesis was performed by using the QuickChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent) using pET11.N0.1 as a template. For pET11.sde8, the WT sequence TCCGGGGCGTTTGACCGT was changed to TCCGGGGAGTTTGACCGT. For pET11.sde50, the WT sequence GCGACGTGGCGTGTC was changed to GCGACGTGCCGTGCT. For pET11.sde343, the WT sequence CCGCAGCAATATATGCGTAGC was changed to CCGCAGAAATATATGCGTAGC. For pET11.sde387, the WT sequence GTGGAACTGGAGTTT was changed to GTGGAACAGGAGTTT. For the pET11.sde388, the WT sequence GTGCTGGAA[AACTGATCATG ... TTCGGCCTGCTG]GCCGAAGAG was changed to GTGCTGGAA[Δ]GCCGAAGAG. All mutations were verified by sequencing.

Mutant Isolation and Characterization

The selections to isolate suppressors of dnaE D403E using the two plasmids pJCL5 and pJCL5-lacZα-T7 were identical except that pJCL5-lacZα-T7 contains a lacZα reporter (Fig. 1). The plasmids were transformed into BL21(DE3) or JCL60, respectively (Table 1: strains JCL52 and JCL68). The cells were grown to late-log phase in L-Broth + 25 μg/ml kanamycin and then plated onto LB + 25 μg/ml kanamycin + 0.2% arabinose plates to induce the pBAD promoter. Eight independent selections were performed with pJCL5-lacZα-T7 and 11 with pJCL5 to identify spontaneous suppressors. All resultant colonies were patched onto LB +25 μg/ml kanamycin + 0.2% arabinose ± 40 μg/ml X-gal (5-bromo-4-chloro-indolyl-β-D- galactopyranoside), to provide a visual indication of promoter integrity and polar effects caused by poor dnaE expression (Fig. 1). In suppressor selections harboring the pJCL5-lacZα-T7 plasmid: white colonies indicated mutated genes that were not expressed, whereas, blue colonies indicated an intact promoter and normal levels of dnaE expression. The plates were incubated at 37 °C for two days. Spontaneous suppressors arose at an average frequency of 1.6/10^5 for strain JCL52 and at an average frequency of 1/10^5 for strain JCL68.

To confirm the suppressor mutation was linked to the plasmid, the plasmid DNA was purified from each mutant using Qiagen mini prep kit, and the plasmid DNA backcrossed into the parent strain (JCL60) and tested for its ability to suppress the dominant negative phenotype of dnaE D403E and was expressed (blue when streaked on plates containing X-gal) (Fig. 1).

Immunoblots were performed on all plasmid-linked mutants to check for expression of full-length dnaE D403E, Sde protein (Fig. 1), 2 ml of cells were grown to late-log phase OD_600 ~0.8, induced with 1 mM IPTG in the presence of 20 μM D-biotin. 1 ml of cells was harvested by centrifugation, and lysed with 5 mg/ml lysozyme on ice for 20 min, 1 min at 37 °C. A total of 40 μl of the soluble protein, isolated after centrifugation, was separated on a 4-20% SDS-Page gel and run next to full-length purified Pol III α as a marker. The SDS-page gel was transferred to PVDF membrane, and incubated with a Streptavidin-Horse Radish Peroxidase conjugate (GE Life Sciences) at a 1:1500 dilution in Phosphate Buffered Saline (PBS) to present visualization of the biotin tag. The immunoblot was developed using enhanced chemiluminescence developer (GE Life sciences).

All full-length intragenic suppressors were sequenced on both strands to identify mutations using the primers #JCL37 (5' CGACATCTTTCGAAGC), #JCL22 (5' CTCAAAAGCGTATCAG), #JCL24 (5' CTGGAAAGCGTCTG), #JCL26 (5' GGCAAAACATCCGGTC), #JCL28 (5'
GGTTACGGATTTAAC), and #JC
L30 (5' CACCCTATCAACCCAG).

Purification of Sde Pol III α subunits

Purification of Sde mutant α proteins (lacking dnaE D403E).

BL21(DE3) strains containing overexpression pET11.sde plasmids were grown in 3.0 L of L-Broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl) + 100 μg/ml ampicillin at 23 °C at 220 rpm on a rotary shaker. It was necessary to express all mutants at 23 °C to avoid degradation and to maximize the amount of soluble protein. Cells were grown to an OD$_{600}$ = 0.5 to 0.6 and induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyronoside). At the time of induction, D-biotin and additional ampicillin were added to 10 μM and 100 μg/ml, respectively. Cells were harvested by centrifugation three hours after induction.

Cells were lysed as described (34) in the presence of 3 mg/ml lysozyme, 5 mM benzamidine, 1 mM PMSF, and 5 mM EDTA (34). The Pol III α-fusion protein was precipitated in 50% (w/v) ammonium sulfate, followed by Ni$^{2+}$-NTA column chromatography as described (24). After Ni$^{2+}$-NTA chromatography, peak fractions were monitored using the gap-filling assay and/or by 4-20% SDS-Page gels to detect full-length α protein. Pooled fractions were precipitated in 70% (w/v) ammonium sulfate and the pellet resuspended in 800 μl S-buffer (50 mM sodium phosphate (pH 7.8), 500 mM NaCl, 20% glycerol, 0.5 mM DTT). The sample was loaded onto an 80 ml (1 cm x 50 cm) Sephacryl S300 HR (GE Life sciences) gel filtration column pre-equilibrated with S-buffer. The column was run at a flow rate of 200 μl/min.

Purification of the Sde proteins (double mutant; dnaE D403E, sde).

Cells were grown, lysed, and prepared as described in the preceding paragraph. Protein pellets were resuspended in 10 ml Buffer S (50 mM sodium phosphate (pH 7.8), 500 mM NaCl, 20% glycerol) supplemented with 0.5 mM imidazole, 5 mM β-mercaptoethanol, and 1 mM PMSF. The protein was purified by a cobalt immobilized affinity chromatography using 1 ml bed volume of resin (TALON resin, Clontech). The column was washed with 20 column volumes of Buffer S + 0.5 mM imidazole followed by 10 column volumes of Buffer S + 1 mM imidazole at a flow rate of 0.2 ml/minute. The protein was eluted using a 10 column volume 1-50 mM imidazole gradient in Buffer S. Fractions were assayed for protein concentration, here and elsewhere, using the Coomassie Plus Bradford Assay Kit (Pierce). Purity of the protein, here and elsewhere, was assessed on 4-20% SDS-Page gels. Gap-filling assays for protein activity could not be used because the Pol III α D403E-sde proteins are catalytically inactive because of the dnaE D403E mutation. Pooled TALON column peak fractions (> 50% peak concentration) were diluted in Buffer I (50 mM imidazole (pH 6.5), 25 mM NaCl, 1 mM EDTA, 20% glycerol, 5 mM DTT) to a conductivity equivalent to Buffer I and loaded onto a BioRex 70 column, 1 ml bed volume, at a flow rate of 200 μl/min as previously described (36). The column was washed with four column volumes of Buffer I, and protein was eluted with a 10 column volume 25-300 mM NaCl gradient. Again purified α was analyzed as above.

Biochemical Assays

Pol III HE reconstitution assay

This assay measures the processive function of Pol III on long-single-stranded DNA templates. Assays were performed as described (34) with the following differences. Pol III HE replication assays (25 μl) were assembled on ice using the τ-complex (150 fmols; 6 nM τ$_{3δδ'3''}$ final), β$_2$ (150 fmols; 6 nM dimer final), DnaG primase (1 pmol; 40 nM final), DnaG primase (1 pmol; 40 nM final), SSB$_4$ (15 pmols; 600 nM final), mixed M13Gori DNA (58 fmol as ssDNA circle; 2.3 nM final), four rNTPs (0.2 mM each final), four dNTPs (48 μM each dATP, dCTP, dGTP final); 18 μM 3H dTTP; 100 cpm /pmol and 10 mM (final) magnesium acetate. The reaction was initiated by adding the WT or mutant Pol III α subunit as the last step and then immediately placing the reaction at 30 °C for 5 min.

Gap filling polymerase assay.
The gap-filling assay measures the ability of Pol I to fill in the gaps of 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 (34).

**Pol III HE competition assay**

This assay measures the ability of a mutant Pol III α to assemble at a primer terminus and thereby block catalysis by Pol III α. Pol III reaction mixtures were assembled on ice (34) using mutant Pol III α polymerase (25-150 fmol; 1-6 nM (final)). Reactions were initiated by adding the WT Pol III α (50 fmol; 2 nM (final)) then immediately placing the reaction at 30 °C for 5 min. Control experiments included heat denaturation of the competitors before addition to the assay. In these cases, no inhibition of the wild-type activity was observed.

**Surface Plasmon Resonance**

A BIAcore 3000 instrument was used to quantify α-β2, α-ɛ, and α-τ interactions. Pol III α was immobilized on a Streptavidin chip by a biotin-streptavidin interaction (26,32,37). A flow rate of 5 µl/min in HKGM buffer (50 mM Hepes (pH 7.4), 100 mM potassium glutamate, 10 mM magnesium acetate, 0.005 % P-20 surfactant) at 25 °C was used for α-β2 and α-ɛ interaction. A flow rate of 25 µl/minute was used to measure α-τ interaction. All buffers were filtered and degassed before use. The Sensor Chip SA (BIAcore) was conditioned with three 1 min injections of 1 M NaCl, 50 mM NaOH prior to attachment of wild-type Pol III α or Sde subunits. For α-β2 and α-ɛ interactions, a total of 3500 Response units (RUs) of Pol III α was loaded per flowcell. For the α-τ interaction, a total of 500 RUs of α was loaded per flowcell. Flowcell 1 was not derivatized and was used as a control for background subtraction. Flowcells 2, 3, and 4, were used individually for different WT or mutant Pol III α subunits.

α-β2 binding: As observed previously (24), the on- and off-rates for β2 binding were too fast to measure. Thus, chip regeneration was not necessary. The dissociation constant K_D for α-β2 binding was determined by running a series of concentrations in quadruplicate of β2 (40 µl each of 50 nM, 100 nM, 250 nM, 500 nM, 1000 nM, 2500 nM, 5000 nM, and 10,000 nM) over the Sde mutant or WT α protein and determining the equilibrium level of β2 bound. The R_max level of each response curve was plotted as a function of the β2 concentration and fit to the 1:1 Langmuir model using BIAevaluation 4.1 software as described (24). The K_D values shown for the α-β2 interaction are derived from the average of the quadruplicate determinations.

α-ɛ binding: Binding conditions were similar to that in (37). Three different concentrations of ɛ subunit (40 µl of 500 nM, 1000 nM, and 1500 nM) were run over a Pol III α-derivatized flow cell. The dissociation constant K_D was calculated from measuring the ratio of the determined rate constants for k_off and k_on at each concentration injected using nonlinear regression analysis using SigmaPlot software. The K_D was derived by the equation K_D = k_off/k_on. ɛ was allowed to dissociate for 3 h sufficient for complete dissociation of α-ɛ complexes. Thus, regeneration of the chip surface was not required. K_D values shown were derived from the average of the K_D measured at each concentration for the individual α-ɛ interaction.

α-τ binding: as observed previously (24), τ binds to wild-type Pol III α with high affinity. We could not find conditions that would dissociate the τ from α without denaturing Pol III α. A total of 200 µl of τ (50 nM) was injected over the surface of the α-derivatized chip at a flow rate of 25 µl/minute (27) to prevent mass-transport effects seen in the response curves at lower flow rates 5 µl/min (data not shown). The concentration of τ was set at 50 nM so that a direct comparison of the dissociation constants could be made across the different WT- and mutant- α derivatized subunits (22,24). Dissociation was measured for 2000 seconds. Dissociation constants were determined by globally fitting the resulting sensorgrams to a 1:1 Langmuir model using BIAevaluation 4.1 software.
RESULTS

The availability of a dnaE D403E that encoded a protein that was inactive for elongation but competent for initiation complex formation provided a convenient genetic selection for secondary mutations that suppressed the dominant negative phenotype. These mutations would be expected to generate proteins that were defective in the initiation complex formation stage of the replicase reaction causing loss of their ability to compete with wild-type Pol III α.

We generated a plasmid that expressed dnaE D403E in trans under control of an arabinose-inducible promoter. Cells containing this plasmid were inviable in the presence of arabinose. Eleven independent selections were conducted to identify plasmid-borne spontaneous suppressor mutations. The most convenient selection protocol is diagramed in Fig. 1. Cells were grown in liquid culture in the absence of arabinose and then plated in the presence of arabinose to kill cells lacking suppressors of the dominant negative allele. Surviving cells were designated sde mutants (suppressor of dnaE). The presence of Xgal on the plates and the placement of the lacZα fragment downstream of dnaE D403E permitted elimination of those trivial mutations resulting from defects in the arabinose-inducible promoter, and polar mutations that caused decreased expression of dnaE. Blue colonies, indicating expression of lacZα, were grown, plasmid isolated, and backcrossed into JCL60 to confirm that the sde mutation was linked to the plasmid. Viable cells were grown, lysed, and protein extracts subjected to immunoblots to screen for those cells that expressed full length Pol III α, eliminating mutants that caused truncation. Plasmids expressing normal levels of full length Pol III α containing sde mutations were sequenced. Five containing diverse point mutations were subjected for further characterization. One deletion mutant that had lost the loop previously shown to be required for interaction of Pol III α with β₂ was also characterized. These are shown mapped onto the homologous residues within the crystal structure of Taq Pol III α (38) (Fig. 2).

To provide a biochemical check for the predicted phenotype, each of the selected sde mutants were expressed and the corresponding proteins purified. As expected, all were reduced in their ability to compete with wild-type Pol III α in Pol III HE reconstitution assays (Fig. 3).

Pol III α expression plasmids were constructed that contained sde mutations in the absence of the D403E mutation. This allowed potentially catalytically-active Sde proteins to be purified using polymerization assays and further biochemical and biophysical characterization. All were purified (Fig. 4).

Biochemical analysis indicated variable levels of reduction of overall Pol III HE activity in reconstituted Pol III HE assays (Table 2 and Fig. 5). Sde334 and Sde388 were both inactive. The remaining Sde proteins showed 3 to 7-fold reductions in the linear range of the assay (Table 2) and similar levels of overall activity at saturation (Fig. 5).

We determined the binding constants for these Sde proteins for interaction with the three subunits with which Pol III α interacts: β₂, ε and τ (Table 3 and Fig. 6). Only Sde proteins that contained mutations in the previously characterized β-binding domain exhibited significant (>2-fold) defects in interaction with β₂ free in solution. Sde388 which lacked the β binding loop did not interact with β₂, and a buried residue within the β-binding domain (Sde 8 (A877E) was reduced in binding affinity approximately three-fold (Table 3).

Sde8 (Q238K) is the only protein that showed a severe ε-binding defect. The Q238K mutation resides in a domain that has previously been characterized as the PHP domain of Pol III α that binds ε (37).

Sde50 and Sde387, both containing mutations (W1134C and L1157Q, respectively) within the C-terminus, containing sde mutations were sequenced. Five containing diverse point mutations were subjected for further characterization. One deletion mutant that had lost the loop previously shown to be required for interaction of Pol III α with β₂ was also characterized. These are shown mapped onto the
Sde388) also resulted in nearly complete ablation of \( \tau \) binding (Table 3).

**DISCUSSION**

Previous studies have characterized static interactions between Pol III HE subunits by biophysical measurements in solution and through structural studies. The goal of our study was to exploit a dominant negative variant of Pol III \( \alpha \) to identify suppressor mutations that made the resulting proteins less fit to compete with wild-type Pol III \( \alpha \) during the initiation complex formation stage of the replicase reaction. Several point mutations and a selected deletion of the \( \beta \) binding loop were selected for further study.

Sde343, containing a mutation in the PHP domain (Q238K) that binds \( \varepsilon \), was inert for \( \varepsilon \) binding and was catalytically inactive. This mutation occurs adjacent to residue 237 of the Pol III \( \alpha \) PHP domain which has been shown in crosslinking studies to interact with the flexible C-terminal sequence of \( \varepsilon \) (39). Previously, we observed defects in polymerase activity created by point mutations (34) and deletions (22) within the PHP binding domain. An \( \varepsilon \)-\( \beta_2 \) interaction makes significant contributions to the stability of initiation complexes (7,8) and may contribute to the observed phenotype. An alternative explanation could be that the sde phenotype is caused by a deformation of the active site of the enzyme, diminishing its ability to interact with the primer terminus and form initiation complexes.

A loop presented by the \( \beta \)-binding domain of Pol III \( \alpha \) has been shown to be an important site of physical and functional interaction of the polymerase with \( \beta_2 \) to form a processive replication complex (6,22-24,32). Sde388, containing a deletion of this loop is unable to bind \( \beta_2 \) in solution and presumably is unable to form a stable initiation complex, consistent with the loss of the dominant negative phenotype of the Sde388 derivative of dnaE D403E. A mutation in a buried internal residue (Sde8; A877E) within the \( \beta \) binding domain leads to a significant, but lesser decrease in static \( \beta_2 \) binding in solution. Structural studies have demonstrated a significant conformational change in Pol III \( \alpha \) upon initiation complex formation where the \( \beta_2 \) binding domain rotates by ca. 20°, bringing it in alignment with the path of exiting duplex DNA from the polymerase (6).

We have demonstrated that in the absence of DNA that the N-terminal domains containing the PHP domain and the palm and thumb domains that form the polymerase active site hold the \( \beta \)-binding domain in a low affinity state (32). It is possible that the A877E mutation puts the \( \beta \) binding domain in a low affinity state or hampers the conformational change in the presence of primed DNA during the Pol III HE initiation complex formation reaction. However, this notion is speculative and will require further experimentation to test this hypothesis.

We have demonstrated that the C-terminus of Pol III \( \alpha \) is involved in binding of the \( \tau \) subunit of the clamp loader (22,24). C-terminal mutations L1157A and F1159A reduce the affinity of Pol III \( \alpha \) for \( \tau \) 36- and 740-fold, respectively (24). Structures of Taq Pol III \( \alpha \), which has a different fold and is thought to be non-homologous to E. coli Pol III \( \alpha \) in this region, also binds Taq \( \tau \) through its C-terminus (40). Thus, our finding sde mutations (L1157Q and W1134C) in the C-terminus is consistent with a \( \tau \) binding defect and extends our knowledge of the \( \tau \) binding site on Pol III \( \alpha \). We note that the residues mutated in sde50 and 387 define the edges of a ridge that contain exposed hydrophobic residues that might also be involved in \( \tau \) interaction. Interaction of Pol III \( \alpha \) with \( \tau \) is required for chaperoning Pol III \( \alpha \) onto nascently loaded \( \beta_2 \) and a physiologically relevant rate of initiation complex formation (10,41), consistent with the observed phenotype.

Information is also available regarding the \( \tau \) partner in the binding interaction. We had demonstrated that a deletion of the C-terminal domain V of \( \tau \) ablated binding to \( \alpha_\varepsilon \), identifying it as the sole \( \alpha \) binding domain (26). A structure is available for the C-terminal domain of \( \tau \) (42). The Pol III \( \alpha \) binding sequences of \( \tau \) have been localized to the extreme C-terminus of \( \tau \) in a region that is unstructured in solution in the absence of binding partners (42).

More surprising was the observation of a \( \tau \) binding defect in the two sde mutants (\( \Delta \)881-
927 and A877E) that are located in the β2 binding domain. The β binding domain and the C-terminal domain that binds τ are independently folding domains, so we do not expect this result to be due to a folding defect. Deletion of the C-terminus has a minimal effect on β2 binding and other mutations within the β binding domain do not have a deleterious effect on τ binding (24). In deletion studies, we have observed that deletion of N-terminal sequences that include the polymerase portion of Pol III α (palm, thumb and partial fingers deletion) interferes with τ binding (22). It is possible that additional τ interactions occur beyond the C-terminal domain. The observed decrease in τ binding is even more severe than that of β2 in the sde8 (A877E) mutation. We note that in the structure of Taq Pol III α bound to the Taq τ C-terminal domain that a contact was observed with the Taq Pol III α β2 binding domain (40). The authors of this study were uncertain if the interactions observed were due to crystal packing forces or a functional contact. Our findings suggest that the β binding domain is important for physical interaction of τ and of functional importance in the initiation complex formation. Together, these mutants increase our knowledge of the complex protein interactions within the DNA replicase required for replication initiation.
REFERENCES

1. McHenry, C. S. (2011) DNA replicases from a bacterial perspective. *Annu Rev Biochem* **80**, 403-436.

2. McHenry, C. S. and Crow, W. (1979) DNA Polymerase III of *Escherichia coli*: Purification and Identification of Subunits. *J. Biol. Chem.* **254**, 1748-1753.

3. Fay, P. J., Johanson, K. O., McHenry, C. S., and Bambara, R. A. (1981) Size classes of products synthesized processively by DNA polymerase III and DNA polymerase III holoenzyme of *Escherichia coli*. *J. Biol. Chem.* **256**, 976-983.

4. Crute, J. J., LaDuca, R. J., Johanson, K. O., McHenry, C. S., and Bambara, R. A. (1983) Excess β subunit can bypass the ATP requirement for highly processive synthesis by the *Escherichia coli* DNA polymerase III holoenzyme. *J. Biol. Chem.* **258**, 11344-11349.

5. Kong, X. P., Onrust, R., O'Donnell, M. E., and 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.

6. Wing, R. A., Bailey, S., and Steitz, T. A. (2008) Insights into the replisome from the structure of a ternary complex of the DNA polymerase III α-subunit. *J. Mol. Biol.* **382**, 859-869.

7. Jergic, S., Horan, N. P., Elshenawy, M. M., Mason, C. E., Urathamakul, T., Ozawa, K., Robinson, A., Goudsmits, J. M., Wang, Y., Pan, X., Beck, J. L., van Oijen, A. M., Huber, T., Hamdan, S. M., and Dixon, N. E. (2013) A direct proofreader-clamp interaction stabilizes the Pol III replicase in the polymerization mode. *EMBO J* **32**, 1322-1333.

8. Toste-Rego, A., Holding, A. N., Kent, H., and Lamers, M. H. (2013) Architecture of the Pol III-clamp-exonuclease complex reveals key roles of the exonuclease subunit in processive DNA synthesis and repair. *EMBO J* **32**, 1334-1343.

9. Jeruzalmi, D., O'Donnell, M. E., and Kuriyan, J. (2001) Crystal structure of the processivity clamp loader gamma complex of *E. coli* DNA polymerase III. *Cell* **106**, 429-441.

10. Downey, C. D. and McHenry, C. S. (2010) Chaperoning of a replicative polymerase onto a newly-assembled DNA-bound sliding clamp by the clamp loader. *Mol. Cell* **37**, 481-491.
11. Johanson, K. O. and McHenry, C. S. (1982) The β subunit of the DNA polymerase III holoenzyme becomes inaccessible to antibody after formation of an initiation complex with primed DNA. *J. Biol. Chem.* **257**, 12310-12315.

12. Mok, M. and Marians, K. J. (1987) Formation of rolling-circle molecules during φX174 complementary strand DNA replication. *J. Biol. Chem.* **262**, 2304-2309.

13. Mok, M. and Marians, K. J. (1987) The *Escherichia coli* preprimosome and DNA B helicase can form replication forks that move at the same rate. *J. Biol. Chem.* **262**, 16644-16654.

14. Kim, S., Dallmann, H. G., McHenry, C. S., and Marians, K. J. (1996) τ protects β in the leading-strand polymerase complex at the replication fork. *J. Biol. Chem.* **271**, 4315-4318.

15. Song, M. S., Pham, P. T., Olson, M., Carter, J. R., Franden, M. A., Schaaper, R. M., and McHenry, C. S. (2001) The δ and δ' subunits of the DNA polymerase III holoenzyme are essential for initiation complex formation and processive elongation. *J. Biol. Chem.* **276**, 35165-35175.

16. Glover, B. P. and McHenry, C. S. (1998) The χψ subunits of DNA polymerase III holoenzyme bind to single-stranded DNA-binding protein (SSB) and facilitate replication of a SSB-coated template. *J. Biol. Chem.* **273**, 23476-23484.

17. Kelman, Z., Yuzhakov, A., Andjelkovic, J., and O'Donnell, M. E. (1998) Devoted to the lagging strand - the χ subunit of DNA polymerase III holoenzyme contacts SSB to promote processive elongation and sliding clamp assembly. *EMBO J* **17**, 2436-2449.

18. Yuan, Q. and McHenry, C. S. (2009) Strand displacement by DNA polymerase III occurs through a τ-ψ-χ link to SSB coating the lagging strand template. *J. Biol. Chem.* **284**, 31672-31679.

19. Marceau, A. H., Bahng, S., Massoni, S. C., George, N. P., Sandler, S. J., Marians, K. J., and Keck, J. L. (2011) Structure of the SSB-DNA polymerase III interface and its role in DNA replication. *EMBO J* **30**, 4236-4247.

20. Witte, G., Urbanke, C., and Curth, U. (2003) DNA polymerase III chi subunit ties single-stranded DNA binding protein to the bacterial replication machinery. *Nucleic Acids Res* **31**, 4434-4440.

21. Jeruzalmi, D., Yurieva, O., Zhao, Y., Young, M., Stewart, J., Hingorani, M., O'Donnell, M. E., and Kuriyan, J. (2001) Mechanism of processivity clamp opening by the δ subunit wrench of the clamp loader complex of *E. coli* DNA polymerase III. *Cell* **106**, 417-428.
22. Kim, D. R. and McHenry, C. S. (1996) Biotin tagging deletion analysis of domain limits involved in protein-macromolecular interactions: Mapping the $\tau$ binding domain of the DNA polymerase III $\alpha$ subunit. *J. Biol. Chem.* **271**, 20690-20698.

23. Dalrymple, B. P., Kongsuwan, K., Wijffels, G., Dixon, N. E., and Jennings, P. A. (2001) A universal protein-protein interaction motif in the eubacterial DNA replication and repair systems. *Proc. Natl. Acad. Sci. U. S. A* **98**, 11627-11632.

24. Dohrmann, P. R. and McHenry, C. S. (2005) A bipartite polymerase-processivity factor interaction: Only the internal $\beta$ binding site of the $\alpha$ subunit is required for processive replication by the DNA polymerase III holoenzyme. *J. Mol. Biol.* **350**, 228-239.

25. Glover, B. P. and McHenry, C. S. (2000) The DnaX-binding subunits $\delta'$ and $\psi$ are bound to $\gamma$ and not $\tau$ in the DNA polymerase III holoenzyme. *J. Biol. Chem.* **275**, 3017-3020.

26. Gao, D. and McHenry, C. S. (2001) $\tau$ binds and organizes *Escherichia coli* replication proteins through distinct domains: partial proteolysis of terminally tagged $\tau$ to determine candidate domains and to assign domain V as the $\alpha$ binding domain. *J. Biol. Chem.* **276**, 4433-4440.

27. Gao, D. and McHenry, C. S. (2001) $\tau$ binds and organizes *Escherichia coli* replication proteins through distinct domains. Domain IV, located within the unique C terminus of $\tau$, binds the replication fork helicase, DnaB. *J. Biol. Chem.* **276**, 4441-4446.

28. Gao, D. and McHenry, C. S. (2001) $\tau$ binds and organizes *Escherichia coli* replication proteins through distinct domains. Domain III, shared by $\gamma$ and $\tau$, binds $\delta\delta'$ and $\chi\psi$. *J. Biol. Chem.* **276**, 4447-4453.

29. Glover, B. P., Pritchard, A. E., and McHenry, C. S. (2001) $\tau$ binds and organizes *Escherichia coli* replication proteins through distinct domains. Domain III, shared by $\gamma$ and $\tau$, oligomerizes DnaX. *J. Biol. Chem.* **276**, 35842-35846.

30. Song, M. S., Dallmann, H. G., and McHenry, C. S. (2001) Carboxyl-terminal domain III of the $\delta'$ subunit of the DNA polymerase III holoenzyme binds $\delta$. *J. Biol. Chem.* **276**, 40668-40679.

31. Song, M. S. and McHenry, C. S. (2001) Carboxyl-terminal domain III of the $\delta'$ subunit of DNA polymerase III holoenzyme binds DnaX and supports cooperative DnaX-complex assembly. *J. Biol. Chem.* **276**, 48709-48715.

32. Kim, D. R. and McHenry, C. S. (1996) Identification of the $\beta$-binding domain of the $\alpha$ subunit of *Escherichia coli* polymerase III holoenzyme. *J. Biol. Chem.* **271**, 20699-20704.
33. Keniry, M. A., Park, A. Y., Owen, E. A., Hamdan, S. M., Pintacuda, G., Otting, G., and Dixon, N. E. (2006) Structure of the θ subunit of *Escherichia coli* DNA polymerase III in complex with the ε subunit. *J Bacteriol* **188**, 4464-4473.

34. Pritchard, A. E. and McHenry, C. S. (1999) Identification of the acidic residues in the active site of DNA polymerase III. *J. Mol. Biol.* **285**, 1067-1080.

35. Higuchi, R., Krummel, B., and Saiki, R. K. (1988) A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucleic Acids Res* **16**, 7351-7367.

36. Kim, D. R. and McHenry, C. S. (1996) *In vivo* assembly of overproduced DNA polymerase III: Overproduction, purification, and characterization of the α, α-ε, and α-ε-θ subunits. *J. Biol. Chem.* **271**, 20681-20689.

37. Wieczorek, A. and McHenry, C. S. (2006) The NH(2)-terminal php domain of the α subunit of the *E. coli* replicase binds the ε proofreading subunit. *J. Biol. Chem.* **281**, 12561-12567.

38. Bailey, S., Wing, R. A., and Steitz, T. A. (2006) The structure of *T. aquaticus* DNA polymerase III is distinct from eukaryotic replicative DNA polymerases. *Cell* **126**, 893-904.

39. Ozawa, K., Horan, N. P., Robinson, A., Yagi, H., Hill, F. R., Jergic, S., Xu, Z. Q., Loscha, K. V., Li, N., Tehei, M., Oakley, A. J., Otting, G., Huber, T., and Dixon, N. E. (2013) Proofreading exonuclease on a tether: the complex between the *E. coli* DNA polymerase III subunits alpha, epsilon, theta and beta reveals a highly flexible arrangement of the proofreading domain. *Nucleic Acids Res* **41**, 5354-5367.

40. Liu, B., Lin, J., and Steitz, T. A. (2013) Structure of the Pol III α-τ(c)-DNA complex suggests an atomic model of the replisome. *Structure* **21**, 658-664.

41. Downey, C. D., Crooke, E., and McHenry, C. S. (2011) Polymerase Chaperoning and Multiple ATPase Sites Enable the *E. coli* DNA Polymerase III Holoenzyme to Rapidly Form Initiation Complexes. *J. Mol. Biol.* **412**, 340-353.

42. Su, X. C., Jergic, S., Keniry, M. A., Dixon, N. E., and Otting, G. (2007) Solution structure of domains IVa and V of the τ subunit of *Escherichia coli* DNA polymerase III and interaction with the α subunit. *Nucleic Acids Res.* **35**, 2825-2832.
Footnotes:

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3 The Abbreviations used are Pol, polymerase; HE, holoenzyme; SSB, single-stranded DNA binding protein; Xgal, 5-bromo-4-chloro-indolyl-β-D-galactopyranoside.
Figure legends

Figure 1. Diagramatic representation of the genetic selection used to isolate intragenic suppressors of dnaE D403E (sde mutations). Procedures are described under Materials and Methods. The 24 of 29 mutants not selected for further study were either revertants, duplicates of mutants already obtained or deletions, insertions or frameshifts in the C-terminus that we already knew interfered with $\tau$ binding (24).

Figure 2. Positions of sde mutations on Taq $\alpha$ structure (38). Mutations are mapped to the corresponding position of Taq DnaE $\alpha$. The PHP domain is shown in yellow and contains sde343 (Q238K) mutation. The palm domain is shown in purple. The thumb domain is shown in green. The fingers domain is shown in blue and contains both the sde8 (A877E) and sde388 (A881-927) mutations (loop shown for $\alpha$-helix in cartoon format). The C-terminal domains comprised of an OB-fold domain (bright red) and an extreme C-terminal domain (dark red). The extreme C-terminal domain contains both sde387 (L1157Q) and sde50 (W1134C) mutations.

Figure 3. Suppression of the dnaE D403E dominant negative phenotype measured by competition holoenzyme reconstitution assays in vitro. The concentration of WT Pol III $\alpha$ was 2 nM. DnaE D403E (black ●); Sde8 (D403E, A877E, red ▲); Sde50 (D403E, W1134C, green ▼); Sde343 (D403E, Q238K, gray ●); Sde387 (D403E, L1157Q, orange ▲); Sde388 (D403E, A881-927, blue *). The experiment shown was conducted obtaining single data points, but is qualitatively similar to other experiments conducted at different Pol III levels.

Figure 4. Analysis of purified WT and Sde Pol III $\alpha$ subunits by 4-20% SDS-PAGE. 2 $\mu$g of Pol III $\alpha$ was loaded onto a 4-20% SDS gel as indicated. Proteins were visualized by coomassie blue staining. The specific activity (units/mg using the gap filling assay that measures intrinsic polymerase activity only) for the purified proteins were $5 \times 10^6$ (WT), $1.7 \times 10^6$ (Sde8), $2.6 \times 10^5$ (Sde50), 0 (not detectable) (Sde343), $1.7 \times 10^5$ (Sde387), and $1.7 \times 10^3$ (Sde388). All mutants, including Sde388 (A881-927) were selected from the screen developed to support this work.

Figure 5. Activity of Sde Pol III $\alpha$ subunits in the Pol III HE reconstitution assay. Data points presented are the average of two independent samples. DNA synthesis by Sde Pol III $\alpha$ is plotted as a function of concentration of the Sde Pol III $\alpha$ added. DnaE WT (black ■); Sde8 (A877E, red ▲); Sde50 (W1134C, green ▼); Sde343 (Q238K, gray ●); Sde387 (L1157Q, orange ▲); Sde388 (A881-927, blue *). Note: the plots for both Sde343 (Q238K, gray ●), and Sde388 (A881-927, blue *) are coincident and overlap.

Figure 6. Binding of $\beta_2$, $\varepsilon$, and $\tau$ to immobilized Sde Pol III $\alpha$ subunits measured by Surface Plasmon Resonance (SPR). Biotinylated Sde Pol III $\alpha$ subunits were captured on flow-cell surfaces through immobilized streptavidin. The dissociation constants $K_D$'s for $\alpha$-$\beta_2$, $\alpha$-$\varepsilon$, and $\alpha$-$\tau$ interactions were determined as described under Materials and Methods. (a) Representative overlayed sensograms of $\beta_2$ at one concentration (500 nM) passed over the indicated Sde mutant $\alpha$ to demonstrate relative affinities of the mutant subunits. (b) Representative overlayed sensograms of $\varepsilon$ at one concentration (1000 nM) passed over the indicated attached Sde subunits. (c) Overlayed sensograms of $\tau$ (50 nM) passed over the indicated attached Sde mutant $\alpha$ subunits. In (a), (b), and (c), DnaE WT (black); Sde8 (A877E, red); Sde50 (W1134C, green); Sde343 (Q238K, gray); Sde387 (L1157Q, orange); Sde388 (A881-927, blue).
## TABLE 1.
### Strain and plasmids used in this work

| Strain/Plasmid | Relevant genotype | Reference or Source | DMSO # |
|---------------|-------------------|---------------------|---------|
| TOP10         | F⁻ mcrA, Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(araA-leu)7697 galU galK rpsL endA1 nupG recA1 φ80lacZΔM15 araD139Δ(araA-leu)7697 | Invitrogen.com | 2090 |
| JCL60         | TOP10 ΔDE3        | This Work           | 1697   |
| BL21(DE3)     | F⁻ dcm ompT hsdS gal λ(DE3) | EMDMillipore.com | 2089   |
| MG1655        | F⁻ λ ilvG rfb50 rph1 | ATCC.org | 2083   |
| JCL52         | BL21(DE3)/pJCL5   | This Work           | 2084   |
| JCL68         | JCL60/pJCL5-lacZα-T7 | This Work           | 2085   |
| JCL59         | BL21(DE3)/pJCL8   | This Work           | 2085   |
| JCL95         | BL21(DE3)/pJCL21  | This Work           | 2079   |
| JCL98         | BL21(DE3)/pJCL24  | This Work           |       |
| JCL99         | BL21(DE3)/pJCL25  | This Work           |       |
| JCL120        | BL21(DE3)/pJCL26  | This Work           |       |
| PDEC118       | BL21(DE3)/pET11.N0.1 | (22)               | 1464   |
| PDEC119       | BL21(DE3)/pET11.sde343 | This Work           | 2272   |
| PDEC120       | BL21(DE3)/pET11.sde8 | This Work           | 2273   |
| PDEC121       | BL21(DE3)/pET11.sde50 | This Work           | 2274   |
| PDEC122       | BL21(DE3)/pET11.sde387 | This Work           | 2275   |
| PDEC123       | BL21(DE3)/pET11.sde388 | This Work           | 2276   |
| pBAD/hasa     | pBAD              | Invitrogen.com      |       |
| pDFER.2       | pET11.N0.dnaE D403E | (22)               | 1432   |
| pET29c        | T7 expression vector | EMDMillipore.com |       |
| pJCL3         | pBAD-dnaE D403E (sub-clone) | This Work           | 1686   |
| pJCL5         | pBAD-dnaE D403E    | This Work           | 1696   |
| pJCL5-lacZα-T7| pBAD/pT7-dnaE D403E-lacZα | This Work           | 2080   |
| pJCL8         | T7-(dnaE D403E, A887E) (sde8) | This Work           | 2089   |
| pJCL21        | T7-(dnaE D403E, W1134C) (sde50) | This Work           | 2119   |
| pJCL24        | T7-(dnaE D403E, L1157Q) (sde387) | This Work           | 2122   |
| pJCL25        | T7-(dnaE D403E, Δ881-927) (sde388) | This Work           | 2123   |
| pJCL26        | T7-(dnaE D403E, Q238K) (sde343) | This Work           | 2125   |
| pET11.N0.1    | T7-dnaE (N-term 6-His, biotin) | (22)               | 2252   |
| pET11.sde8    | T7-dnaE A877E      | This Work           | 2254   |
| pET11.sde50   | T7-dnaE W1134C    | This Work           | 2255   |
| pET11.sde343  | T7-dnaE Q238K    | This Work           | 2253   |
| pET11.sde387  | T7-dnaE L1157Q    | This Work           | 2256   |
| pET11.sde388  | T7-dnaE Δ881-927  | This Work           | 2257   |
TABLE 2.
Activity of Sde mutant α subunits in the Pol III holoenzyme reconstitution assay.

| Mutant Protein | Units/mg^a | Fold Reduction^b |
|----------------|------------|------------------|
| DnaE WT        | 7.12 x 10^6 | 0                |
| Sde8 A877E     | 1.08 x 10^6 | 7                |
| Sde50 W1134C   | 2.78 x 10^6 | 3                |
| Sde343 Q238K   | N/D        | >700             |
| Sde387 L1157Q  | 1.98 x 10^6 | 4                |
| Sde388 Δ881-927| N/D        | >100             |

^a Specific activity of the indicated mutant polymerases using the Pol III holoenzyme reconstitution assay as described under Materials and Methods. The units/mg was determined in the linear region of the curve.

^b Reduction in Pol III HE reconstitution activity of purified mutant α relative to wild-type. For Sde343, the reduction was set at the limit of detection.
TABLE 3.
Sde Pol III α affinity for β2, ε and τ

| Mutant protein       | k\text{on} (M\text{−}1 s\text{−}1) | k\text{off} (s\text{−}1) | K_D (µM) | Affinity compared to WT (%) | Maximum stoichiometry achieved^b |
|----------------------|----------------------------------|--------------------------|----------|-----------------------------|---------------------------------|
| **β2 binding**       |                                  |                          |          |                             |                                 |
| DnaE WT              |                                  |                          |          |                             |                                 |
| Sde8 A877E           | 7.4 x 10^4 (± 1.1)               | 5.3 x 10^4 (± 0.8)       | 7.2 (± 0.4) nM | 100                          | 1.00                            |
| Sde8 A877E           | 7.3 x 10^4 (± 1.4)               | 8.5 x 10^4 (± 1.4)       | 11.7 (± 3.9) nM | 62                           | 0.60                            |
| Sde50 W1134C         | 6.6 x 10^4 (± 0.4)               | 6.6 x 10^4 (± 0.8)       | 10.0 (± 0.7) nM | 72                           | 0.63                            |
| Sde343 Q238K         | N/D                              |                          |          |                             |                                 |
| Sde387 L1157Q        | 7.2 x 10^4 (± 0.3)               | 5.3 x 10^4 (± 0.7)       | 7.3 (± 0.8) nM | 99                           | 0.62                            |
| Sde388 Δ881-927      | 5.6 x 10^4 (± 0.5)               | 8.9 x 10^4 (± 0.2)       | 15.8 (± 1.7) nM | 46                           | 0.57                            |
| **ε binding**        |                                  |                          |          |                             |                                 |
| DnaE WT              |                                  |                          |          |                             |                                 |
| Sde8 A877E           |                                  |                          |          |                             |                                 |
| Sde50 W1134C         |                                  |                          |          |                             |                                 |
| Sde343 Q238K         |                                  |                          |          |                             |                                 |
| Sde388 Δ881-927      |                                  |                          |          |                             |                                 |
| **τ-binding**        |                                  |                          |          |                             |                                 |
| DnaE WT              |                                  |                          |          |                             |                                 |
| Sde8 A877E^b         |                                  |                          |          |                             |                                 |
| Sde50 W1134C         |                                  |                          |          |                             |                                 |
| Sde343 Q238K         |                                  |                          |          |                             |                                 |
| Sde387 L1157Q        |                                  |                          |          |                             |                                 |
| Sde388 Δ881-927      |                                  |                          |          |                             |                                 |

^a N/D = not detectable.

^b The values in this column represent the maximal stoichiometry achieved between β2, ε or τ relative to Pol III α bound to the chip. Note that for β2 and some of the other proteins with weak binding Sde variants that the level of analyte was not saturating.
Figure 1

Transfer to growth media (0.2% arabinose, 25 μg/ml kanamycin)
Express DnaE-D403E from pBAD promoter

Dead dominant-negative dnaE-D403E
Alive suppressor of dnaE-D403E = sde (1093 total)

Colony-purify mutants and plate on 0.2% arabinose + 40 μg/ml Xgal
White Decreased expression
Blue (540 total)

Isolate plasmid, transform back into E. coli JCL60
to test for plasmid linkage

Dead not plasmid linked, extragenic suppressor
Alive Plasmid linked (513 total)

Immunoblot
Screen for expression of full-length Pol III \( \alpha \) (29 total)

Sequence plasmid DNA

Reversion of dnaE403E
5 intragenic suppressors selected for further study
sde8; sde50; sde387; sde343; sde388
Figure 2

Figure 3
