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Inhibition of Protein Interactions with the β_2 Sliding Clamp of *Escherichia coli* DNA Polymerase III by Peptides from β_2 Binding Proteins

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OVEREXPRESSION OF THE α SUBUNIT OF DNA POLYMERASE III

Plasmid pRW203 is a derivative of vector pNS2 (*1*) containing a 6595-bp *Bam*HI fragment from the *E. coli* chromosome bearing the *rnhB*, *dnaE* and *accA* genes. This plasmid and the *tac* promoter vector ptac-85 (*2*) were generous gifts from Hatch Echols and Philip Marsh, respectively. The bacteriophage λ promoter vector pCE30 was as described (*3*). The *E. coli recA* strain AN1459 (*4*) was used routinely as host for plasmids. Strains carrying pCE30 derivatives were grown at 30 ˚C in Luria-Bertani (LB) media containing 50 mg/L ampicillin. Other strains were grown at 37 ˚C, unless specified. Overproduction of the a subunit was assessed by SDS-PAGE (7%) analysis of lysed whole cells that had been grown to A_{595} = 0.5–0.6 at 30 ˚C, then treated for 2 h at 42 ˚C (for those containing pCE30 derivatives) or with 0.5 mM isopropyl-b-D-thiogalactoside (IPTG; the ptac-85 derivative). The RBS-*Hpa*I adaptor was an equimolar mixture of the complementary oligonucleotides 5'–GATCC*TAAGGAGGT*TAAC and 5'–GTTAAC-CTCCTTAG (*Hpa*I site underlined; ribosome-binding site italicized). The self-complementary *Bam*HI linker (Pharmacia) had the sequence 5'–pCCCGGATCCGGG.

A sequence of plasmid constructions (Figure S1) was carried out to provide the *dnaE* gene with an optimized translation initiation region within a restriction fragment that could be transferred easily into various expression vectors. The first steps used pCE30 as vector, and resulted in progressive improvement in the level of overproduction of α (Figure S2).

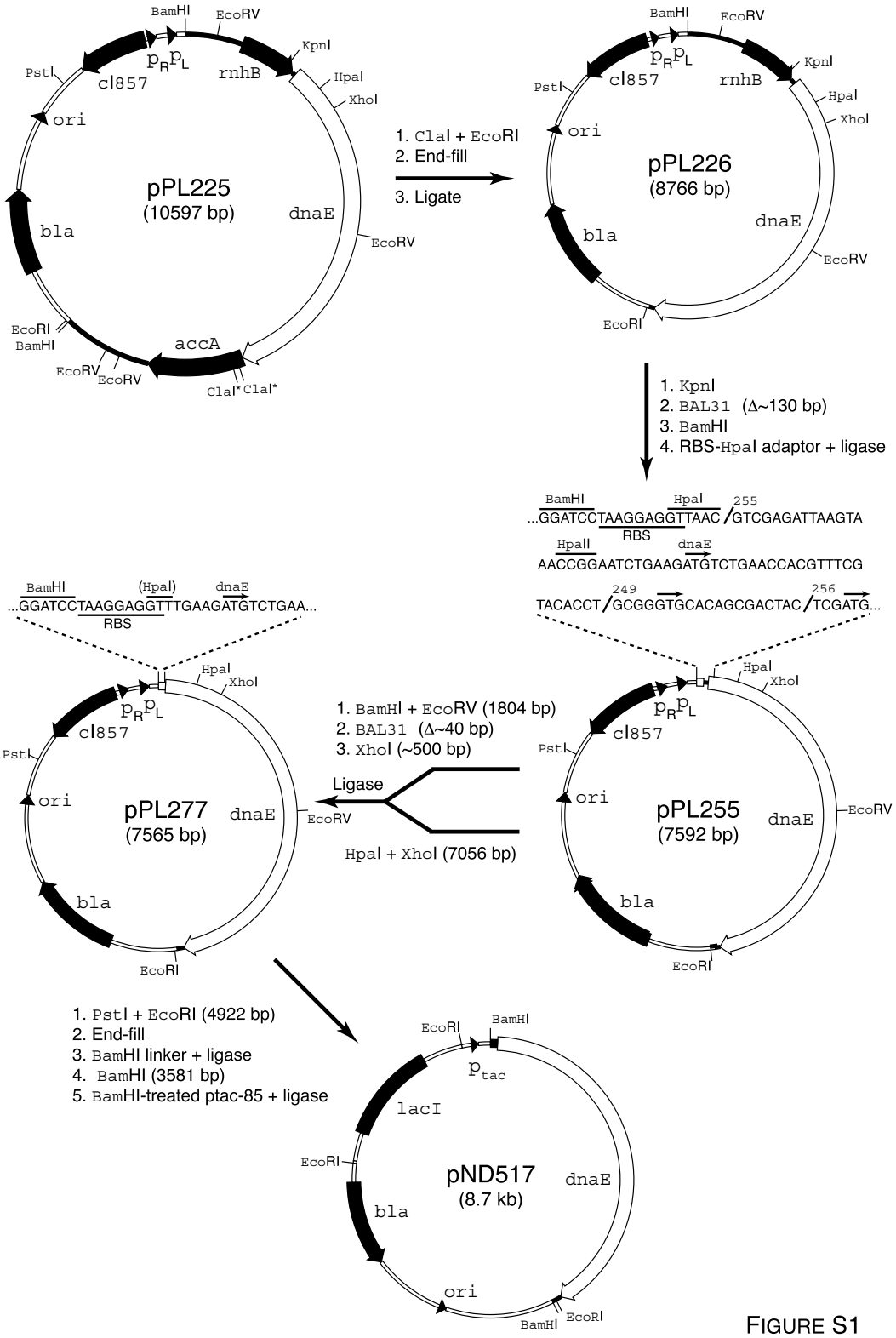
pPL225
(10597 bp)
pPL226
(8766 bp)
pPL277
(7565 bp)
pPL255
(7592 bp)
pND517
(8.7 kb)
1. ClaI + EcoRI
2. End-fill
3. Ligate
1. KpnI
2. BAL31 (Δ~130 bp)
3. BamHI
4. RBS-HpaI adaptor + ligase
1. BamHI + EcoRV (1804 bp)
2. BAL31 (Δ~40 bp)
3. XhoI (~500 bp)
Ligase
HpaI + XhoI (7056 bp)
1. PstI + EcoRI (4922 bp)
2. End-fill
3. BamHI linker + ligase
4. BamHI (3581 bp)
5. BamHI-treated ptac-85 + ligase
...GGATCCTAAGGAGGTTAAC/GTCGAGATTAAGTA
RBS
AACCGGAATCTGAAGATGTCTGAACCACGTTTCG
TACACCT/GCGGGTGCACAGCGACTAC/TCGATG...
...GGATCCTAAGGAGGTTTGAAGATGTCTGAA...
RBS
BamHI
HpaI
XhoI
EcoRV
EcoRI
PstI
ori
bla
dnaE
cI857
rnhB
KpnI
accA
ClaI*
lacI
ptac

FIGURE S1

FIGURE S1: Plasmids that direct overproduction of the α subunit of DNA polymerase III.

Methods for DNA manipulation were essentially as described (*4*, *5*), using enzymes from Roche Molecular Biochemicals. DNA fragments isolated following separation by agarose gel electrophoresis (sizes indicated in parentheses) were used for ligations with bacteriophage T4 DNA ligase. **pPL225:** This plasmid was constructed by ligation of the *dnaE*$^{+}$ *Bam*HI fragment from pRW203 into the *Bam*HI site of the bacteriophage λ-promoter vector pCE30 (*3*). **pPL226:** This plasmid was prepared by digestion of pPL225 with *Cla*I and *Eco*RI, filling of the ends with the large fragment of DNA polymerase I, and religation of the blunt ends. This manipulation resulted in restoration of the *Eco*RI site. **pPL249, pPL255 and pPL256:** Plasmid pPL226 was lineraized with *Kpn*I, then treated with sufficient exonuclease *BAL31* to remove about 130 bp from each end. The products were digested with *Bam*HI to completely remove the *rnhB* gene, then the ends were religated in the presence of excess of the (5'-unphosphorylated) RBS-*Hpa*I adapter. The end points of *BAL31* deletion in each of the three plasmids retained for further study is indicated by the slashes and plasmid numbers. **pPL277:** The smaller *Bam*HI–*Eco*RV fragment from pPL255 was isolated and treated sufficiently with *BAL31* to remove about 40 bp of DNA from each end. The product was then cleaved with *Xho*I and fragments ~500 bp in length were isolated and inserted between the *Hpa*I and *Xho*I sites of pPL255 to yield a second library of plasmids. After screening and DNA sequence determination, one plasmid was retained as pPL277. **pND517:** The largest *Pst*I–*Eco*RI fragment from pPL277 was treated with the large fragment of DNA polymerase I to fill in the *Eco*RI cohesive end, then isolated and ligated to 5'-phosphorylated *Bam*HI linkers. The larger fragment obtained after digestion with *Bam*HI was then inserted at the *Bam*HI site of the *tac*-promoter vector ptac-85 (*2*). This operation regenerates the *Eco*RI site in the region following *dnaE*.

The chromosomal *Bam*HI fragment from pRW203 was first inserted at the *Bam*HI site of pCE30 (*3*) in the orientation required to place the expression of *dnaE* under control of the tandem λ promoters, giving plasmid pPL225 (Figure S1). Two (unmethylated) *Cla*I sites are present in pPL225, one being 67 bp beyond the *dnaE* stop codon (Figure S1). Digestion of pPL225 with *Cla*I and *Eco*RI, filling of the overhanging ends with the large fragment of DNA polymerase I, and recircularization of the plasmid with ligase resulted in removal of 1831 bp of DNA from, and creation of a new *Eco*RI site in, the region just following *dnaE*. This resulted in construction of the plasmid pPL226 (Figure S1).

In pPL226, a unique *Kpn*I site occurs 139 bp before the start codon of *dnaE*. A sequence of steps beginning with *Kpn*I-digested pPL226 was followed to provide the *dnaE* gene with an appropriately spaced ribosome-binding site (RBS) perfectly complementary to the 3' end of the 16 *S* ribosomal RNA (Figure S1). A unidirectional deletion library of plasmids derived from pPL226

was generated, whose members had various amounts of DNA between the *Bam*HI site and the *dnaE* start codon replaced by the RBS and a new *Hpa*I site (using the RBS-*Hpa*I adaptor; see Figure S1). Plasmids in 68 transformants were screened individually by restriction fragment size analysis (using digestion with *Bam*HI and *Hae*III, and end-labeling of fragments with a-[^{32}P]dATP) to determine the positions of insertion of the new RBS. Nucleotide sequences of the region preceding *dnaE* in five of them were then determined, using vector primers as described (*3*). Several plasmids were obtained that directed overproduction of truncated α subunits missing the first 10 residues (using the GTG that encodes Val11 as start codon; see Figure S1 and pPL249 in Figure S2), or the first 16 residues (using the Met17 codon as a new start codon; see Figure S1 and pPL256 in Figure S2). The plasmid which had the new RBS in closest proximity to the natural start codon was retained as pPL255 (Figure S1). The RBS in pPL255 was 33 bp upstream of the start codon, and the plasmid directed little or no overproduction of native α subunit (Figure S2). (This is believed to be due to the presence of the new, stronger but unproductive RBS being placed close enough to interfere with translational initiation from the natural RBS.)

A modified strategy (Figure S1) was then used to generate a second, more extensive, library of transformants containing derivatives of pPL255 with more finely-spaced deletions between the RBS and start codon (Figure S1). Reasoning that high-level overproduction of α would be toxic to the host cells, as observed for other DNA replication proteins (*5*, *6*), 288 members of the library were first screened to identify 60 that grew poorly on plates at 41 ˚C. There is an *Hpa*II site 12 bp before the *dnaE* start codon in pPL255 (Figure S1). Digestion of plasmids with *Hpa*II identified 54 in which the deletion of DNA from this region had removed or disrupted this site. Restriction fragment size analysis (as above, or with *Hpa*II) led to the isolation of several further plasmids that directed overproduction of N-terminally truncated versions of α (as before), along with seven where the RBS was variously spaced 3–12 nucleotides before the natural start codon. One of these, which had a 6 nucleotide spacing, was retained as pPL277 (Figure S1).

Although treatment of strain AN1459/pPL277 at 42 ˚C led to acceptable overproduction of α (Figure S2), the protein was recovered in soluble fractions in very low yields (not shown). Similar behavior had been observed previously when α was overproduced (to lower levels) using a similar expression system (*7*). Several different lysis procedures were used to prepare cell-free extracts, with poor results in each case. This problem was overcome by using an IPTG-inducible system, so that overexpression of α could be carried out at lower temperatures. The *dnaE* gene with its optimized RBS was isolated from pPL277 and inserted in the required orientation at the

*Bam*HI site of the *tac* promoter vector ptac-85, to yield plasmid pND517 (Figure S1). This plasmid directed overproduction of α to high levels (Figure S2), and most of the protein could be obtained in a soluble fraction provided cells were grown at 30 ˚C or lower temperatures (not shown).

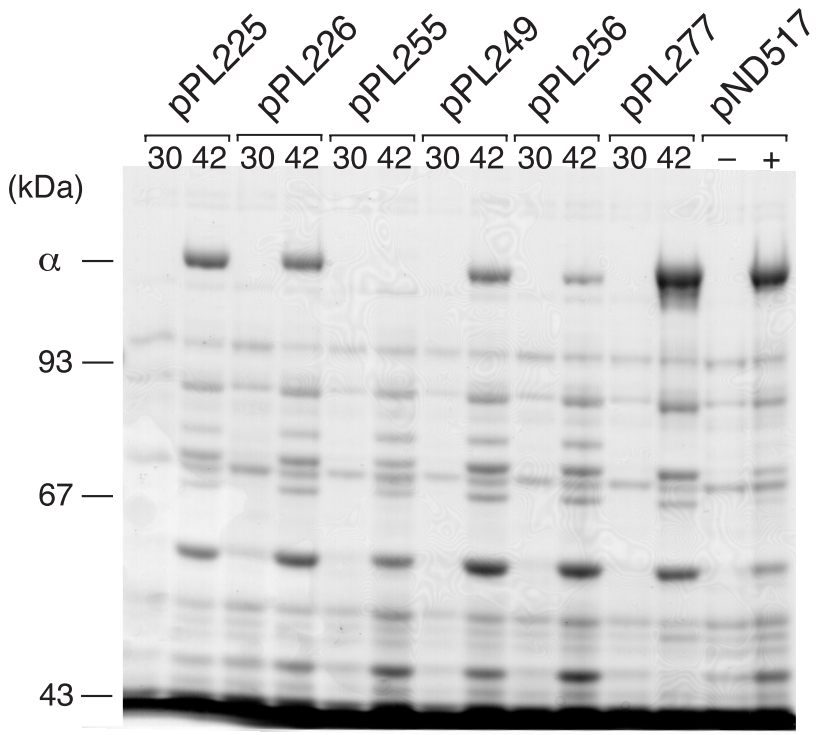

FIGURE S2: Overproduction of the α subunit of DNA polymerase III.

Cultures (50 mL) of strain AN1459 containing the λ-promoter plasmids pPL225, pPL226, pPL255, pPL249, pPL256, and pPL277 (as indicated) were grown at 30 ˚C to A_{595} = 0.5–0.6, then incubated at 42 ˚C. Cells were harvested from portions removed before (lanes marked '30') and 2 h after (lanes marked '42') the temperature shift. Similarly, a culture (50 mL) of strain AN1459 containing the *tac*-promoter plasmid pND517 was grown at 30 ˚C to A_{595} = 0.5–0.6 (lane marked '–'), then treated for 2 h with 0.5 mM IPTG (lane marked '+'). Harvested cells were resuspended to A_{595} = 10 in an gel loading buffer containing SDS and heated for 2 min at 95 ˚C, before proteins in samples (20 μL each) were resolved by SDS-PAGE on a 7% polyacrylamide gel, as described (*8*); proteins were visualized with Coomassie brilliant blue. The mobilities of molecular weight markers (sizes in kDa) were as indicated. Plasmids pPL249 and pPL256 direct production of α subunits with ten and sixteen residues, respectively, deleted from their N termini (see Figure S1). The other plasmids direct overproduction of wild-type α.

PROTEIN PURIFICATION

The bacteriophage T7 promoter plasmids pET-δ and pET-δ' (*9*), used for overproduction of the δ and δ' subunits, respectively, of DNA polymerase III holoenzyme in *E. coli* strain BL21(DE3)/pLysS (*10*), were generously supplied by Mike O'Donnell. Proteins were purified as described below. All steps following cell growth were carried out at 0–5 ˚C. FPLC or Äkta Explorer systems (Amersham Pharmacia Biotech) were used for chromatographic steps. Heparin-agarose was prepared as described (*11*), with activation of Sepharose 4B (Amersham Pharmacia Biotech) by cyanogen bromide. Molecular weights of purified proteins were determined by ESI-MS using a VG Quattro II mass spectrometer with samples that had been dialyzed extensively into 0.1% formic acid, 1 mM in β-mercaptoethanol.

The α subunit of DNA polymerase III

Several attempts at purification of the α subunit using strain AN1459/pND517 gave variable amounts of impurities that appeared to be proteolytic fragments of α. Suspecting that this might be due to cleavage of the protein by the OmpT protease (*12*), we subsequently used the *ompT* strain BL21(DE3) (*10*) as host. The α subunit was purified (Figure S3) by a procedure that involved cell lysis with egg-white lysozyme, ammonium sulfate precipitation, and three chromatographic separations. In the first of these, the ammonium sulfate fraction was passed through a column of a DEAE resin in buffer containing 200 mM NaCl, which efficiently removed nucleic acids that otherwise interfered with separation of a from other proteins. After dialysis to remove salt, α was then separated from other proteins by chromatography on a column of the same resin, and finally purified using a column of phosphocellulose (Whatman P-11).

Buffers were: lysis buffer (50 mM Tris.HCl, pH 7.6, 1 mM EDTA, 2 mM dithiothreitol, 20 mM spermidine.3HCl); buffer A (50 mM Tris.HCl, pH 7.6, 4 mM EDTA, 1 mM dithiothreitol, 30% v/v glycerol); buffer B (20 mM sodium phosphate, pH 6.5, 4 mM EDTA, 1 mM dithiothreitol, 20% v/v glycerol).

Strain BL21(DE3)/pND517 was grown at 30 ˚C in LB medium containing thymine (50 mg/L) and ampicillin (50 mg/L) to A_{595} = 0.5–0.7. To induce overproduction of α, IPTG was added

to 0.5 mM and the 1-L cultures were shaken for a further 4 h. After being chilled in ice, cells were harvested by centrifugation (11000 × *g*; 5 min), frozen in liquid nitrogen and stored at –70 ˚C.

Frozen cells (29 g, from 6 L of culture) were resuspended in 147 mL of lysis buffer. A solution of lysozyme (3 mL of 10 mg/mL) in lysis buffer was added, with stirring. After the suspension had been agitated gently at 4 ˚C for 30 min, it was warmed in a bath at 37 ˚C for 4 min (with gentle inversion each minute), then cooled in ice (30 min). Cell debris was removed by centrifugation (43000 × *g*; 1 h). Solid ammonium sulfate (0.30 g/mL) was added to the supernatant (Fraction I), with stirring. After 1 h, the pellet was harvested by centrifugation (38000 × *g*; 30 min), dissolved in 40 mL of buffer A+200mM NaCl, and dialyzed against three changes of 2 L of the same buffer, to yield Fraction II.

The dialysate was diluted to 90 mL with buffer A+200 mM NaCl, and divided into two portions which were loaded separately at 1 mL/min onto columns (2.5 × 16 cm) of Toyopearl DEAE-650M anion-exchange resin that had been equilibrated in the same buffer. Columns were washed with the same buffer. Fractions containing α, which did not bind to the resin, were pooled and dialyzed against three changes of 2 L of buffer A. The dialysate (Fraction III, 130 mL) was divided into three equal proportions that were separately loaded at 1 mL/min onto columns (2.5 × 16 cm) of the same resin, now equilibrated in buffer A (without NaCl). The columns were washed with 100 ml of buffer A, after which a linear gradient (300 mL) of 0–0.5 M NaCl in buffer A was applied. Fractions containing α, which eluted in a single peak at ~180 mM NaCl, were pooled and dialyzed against buffer B. The dialysate (Fraction IV, 135 mL) was again divided into three portions that were loaded separately at a flow rate of 1 mL/min onto columns (2.5 × 10 cm) of phosphocelullose that had been equilibrated with buffer B. After the columns had each been washed with 100 mL of buffer B, α was eluted in a linear gradient (300 mL) of 0–0.5 M NaCl in buffer B. Fractions containing α, which eluted in a single peak, were pooled and dialyzed against three changes of 2 L of buffer A, to yield Fraction V (88 mL).

To concentrate the purified protein, Fraction V was loaded (at 1 mL/min) onto a column (2.5 × 16 cm) of Toyopearl DEAE-650M resin that had been equilibrated in buffer A. The α subunit was then eluted with buffer A containing 500 mM NaCl. Fractions containing α were pooled (18 mL), frozen in liquid nitrogen and stored at –70 ˚C. A typical preparation yielded 49 mg of highly-purified α (see Figure S3).

ESI-MS gave a value of ~129820 for the molecular weight of α, which may be compared to the calculated value of 129774 (assuming that the N-terminal methionine had been removed).

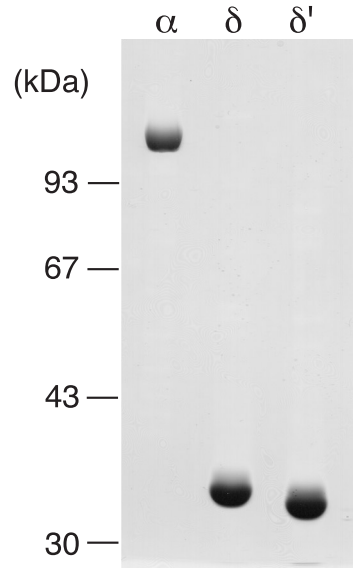

FIGURE S3: Purification of the α, δ and δ' subunits of DNA polymerase III holoenzyme.

SDS-PAGE (9%) analysis of samples (10 μg) of the purified proteins. The gel was stained with Coomassie brilliant blue. Concentrations of the proteins were determined spectrophotometrically using values of ε_{280} calculated as described by Gill and von Hippel (*13*): 95440 $M^{-1}cm^{-1}$ for α; 46230 $M^{-1}cm^{-1}$ for δ; 59600 $M^{-1}cm^{-1}$ for δ'.

The δ subunit of DNA polymerase III

The methods described by Dong *et al.* (*9*) were followed, with some modifications, for purification of the δ subunit.

Buffers were: lysis buffer (50 mM Tris.HCl, pH 7.6, 1 mM EDTA, 2 mM dithiothreitol, 20 mM spermidine.3HCl); buffer C (50 mM Tris.HCl, pH 7.6, 1 mM EDTA, 2 mM dithiothreitol); buffer D (50 mM Tris.HCl, pH 7.6, 1 mM EDTA, 2 mM dithiothreitol, 150 mM NaCl, 20% v/v glycerol).

E. coli strain BL21(DE3)/pLysS/pET-δ was grown at 37 ˚C in LB medium containing thymine (50 mg/L) and ampicillin (200 mg/L) to A_{595} = 1.5. The temperature was then reduced to 25 ˚C, and IPTG was added to 0.5 mM. Cultures were shaken at 25 ˚C for a further 4 h, then

chilled in ice. Cells were harvested by centrifugation (11000 × *g*; 5 min), frozen in liquid nitrogen and stored at –70 ˚C.

After thawing, cells (11.5 g, from 3 L of culture) were resuspended in lysis buffer (170 mL) and lysed by being passed twice through a French press (12000 psi). The lysate was clarified by centrifugation (43000 × *g*; 60 min), to yield the soluble Fraction I. Proteins that were precipitated from Fraction I by addition of solid ammonium sulfate (0.2 g/mL), and stirring for 60 min, were collected by centrifugation (38000 × *g*; 30 min) and dissolved in buffer C+150 mM NaCl (30 mL). The solution was dialyzed against three changes of 2 L of the same buffer and clarified by centrifugation, yielding Fraction II.

Fraction II was diluted with buffer C+150 mM NaCl to 45 mL and applied at 1 mL/min to a column (2.5 × 16 cm) of Toyopearl DEAE-650M resin that had been equilibrated in the same buffer. Fractions containing the δ subunit, which passed unretarded through the column, were collected and dialyzed against two changes of 2 L of buffer C+90 mM NaCl. The dialysate was clarified by centrifugation, giving Fraction III (80 mL). This fraction was diluted 3-fold with buffer C (in small portions because δ tends to precipitate at concentrations of NaCl less than 70 mM; see Ref. 9) just prior to being loaded at 1 mL/min onto a column (2.5 × 16 cm) of DEAE-650M resin that had been equilibrated in buffer C+50 mM NaCl. After the column had been washed with 100 mL of the same buffer, δ was eluted in a linear gradient (240 mL) of 50–400 mM NaCl in buffer C. Fractions containing δ were pooled and dialyzed against two changes of 2 L of buffer C+90 mM NaCl (Fraction IV; 65 mL).

Fraction IV was diluted with 18 mL of buffer C just prior to being loaded at a flow rate of 0.5 mL/min onto a column (2.5 × 10 cm) of heparin-agarose that had been equilibrated in buffer C+50 mM NaCl. The column was washed with 100 mL of the same buffer and δ was eluted (at ~200 mM NaCl) in a linear gradient (600 mL) of 50–400 mM NaCl in buffer C. Fractions containing highly-purified δ (Figure S3) were pooled and dialyzed against 5 L of buffer D (Fraction V; 27 mL containing 56 mg of protein). Aliquots were frozen in liquid nitrogen and stored at –70 ˚C. The measured molecular weight of 38710 ± 2 for δ (by ESI-MS) may be compared with the calculated value of 38704, and indicated that the N-terminal methionine had not been removed.

The δ' subunit of DNA polymerase III

The δ' subunit was purified by a similar procedure to that described above for δ, again based on methods described by Dong *et al.* (9).

Buffers were: lysis buffer (50 mM Tris.HCl, pH 7.6, 1 mM EDTA, 2 mM dithiothreitol, 20 mM spermidine.3HCl); buffer C (50 mM Tris.HCl, pH 7.6, 1 mM EDTA, 2 mM dithiothreitol); buffer E (30 mM Na.HEPES, pH 7.2, 1 mM EDTA, 2 mM dithiothreitol); buffer F (50 mM Tris.HCl, pH 7.6, 1 mM EDTA, 2 mM dithiothreitol, 150 mM NaCl, 30% v/v glycerol).

E. coli strain BL21(DE3)/pLysS/pET-δ' was grown at 37 ˚C in LB medium supplemented with glucose (5 g/L), thymine (50 mg/L), thiamine (10 mg/L), ampicillin (200 mg/L) and chloramphenicol (50 mg/L). Upon growth to an A_{595} = 0.5, IPTG was added to 0.5 mM and the 1-L cultures were shaken at 37 ˚C for further 4 h, after which they were chilled in ice. Cells were harvested by centrifugation (11000 × *g*; 5 min), frozen in liquid nitrogen and stored at –70 ˚C.

After thawing, cells (9.3 g, from 2 L of culture) were resuspended in 140 mL of lysis buffer and lysed by two passages through a French press (operated at 12000 psi). The cell-free extract obtained after centrifugation (43000 × *g*; 60 min), was retained as Fraction I. The δ' subunit in Fraction I was precipitated by addition of ammonium sulfate (0.21 g/mL), followed by stirring for 60 min, and then recovered by centrifugation (38000 × *g*; 30 min). The pellet was dissolved in buffer C+150mM NaCl (40 mL) and dialyzed against three changes of 2 L of the same buffer. The solution was then clarified by centrifugation (43000 × *g*; 20 min), yielding Fraction II.

Fraction II (45 mL) was applied at 1 mL/min to a column (2.5 × 16 cm) of Toyopearl DEAE-650M resin, as described for the δ subunit, above. Fractions containing proteins that did not bind to the column were pooled and dialyzed against three changes of 2 L of buffer E+40 mM NaCl. After clarification by centrifugation, the solution (Fraction III; 50 mL) was loaded directly at a flow rate of 0.5 mL/min onto a column (2.5 × 10 cm) of heparin-agarose that had been equilibrated in buffer E. The δ' subunit was eluted using a linear gradient (250 mL) of 20–300 mM NaCl in buffer. It eluted in a single peak at about 140 mM NaCl. Fractions containing the highly-purified δ' subunit (Figure S3) were pooled and dialyzed against 5 L of buffer F, to give Fraction

IV (14 mL, containing 57 mg of protein). Aliquots were frozen in liquid nitrogen and stored at –70 ˚C. The molecular weight of δ' determined by ESI-MS (36944 ± 3) may be compared with the calculated value of 36937, and indicated that the N-terminal methionine was still present in the pure protein.

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SUPPLEMENTARY BIACORE DATA

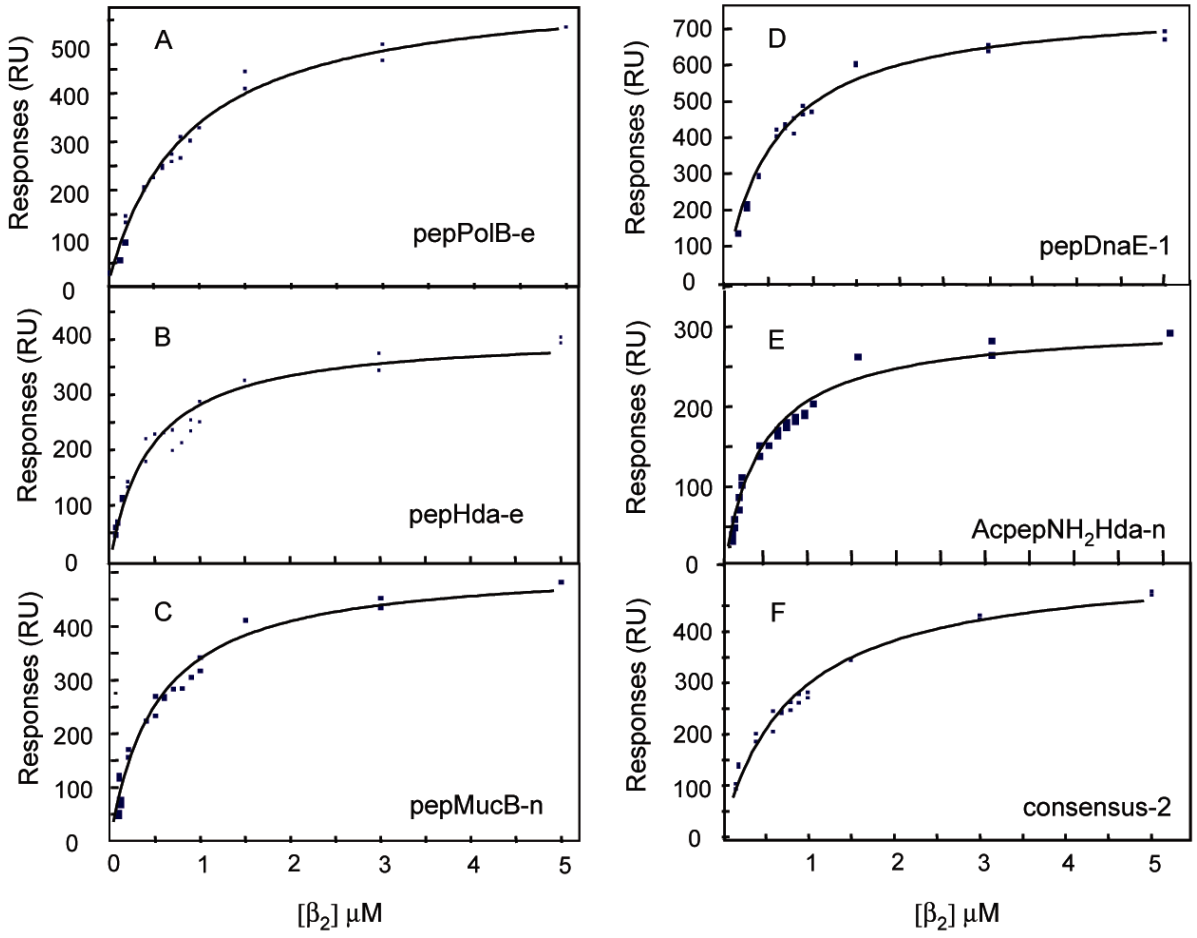

FIGURE S4

FIGURE S4: Binding isotherms for binding of β_2 to immobilized peptides.

Peptide sequences are given in Table 1. (A) pepPolB-e. (B) pepHda-e. (C) pepMuC-n. (D) pepDnaE-1. (E) AcpepNH_2pepHda-n. (F) consensus-2. Fifteen concentrations of β_2 between 0.01 and 5 μM were injected in duplicate (at random) over flow cell surfaces at a rate of 10 μL/min, at 25 ˚C. The instrument response (RU) were measured at equilibrium, and then corrected for the response obtained with the underivatized control surface. Data were fit to a 1:1 Langmuir binding model using the BIAEvaluation software package (Biacore). Solid lines were calculated using values of K_D given in Table 2.

SUPPLEMENTARY MOLECULAR MODELLING DATA

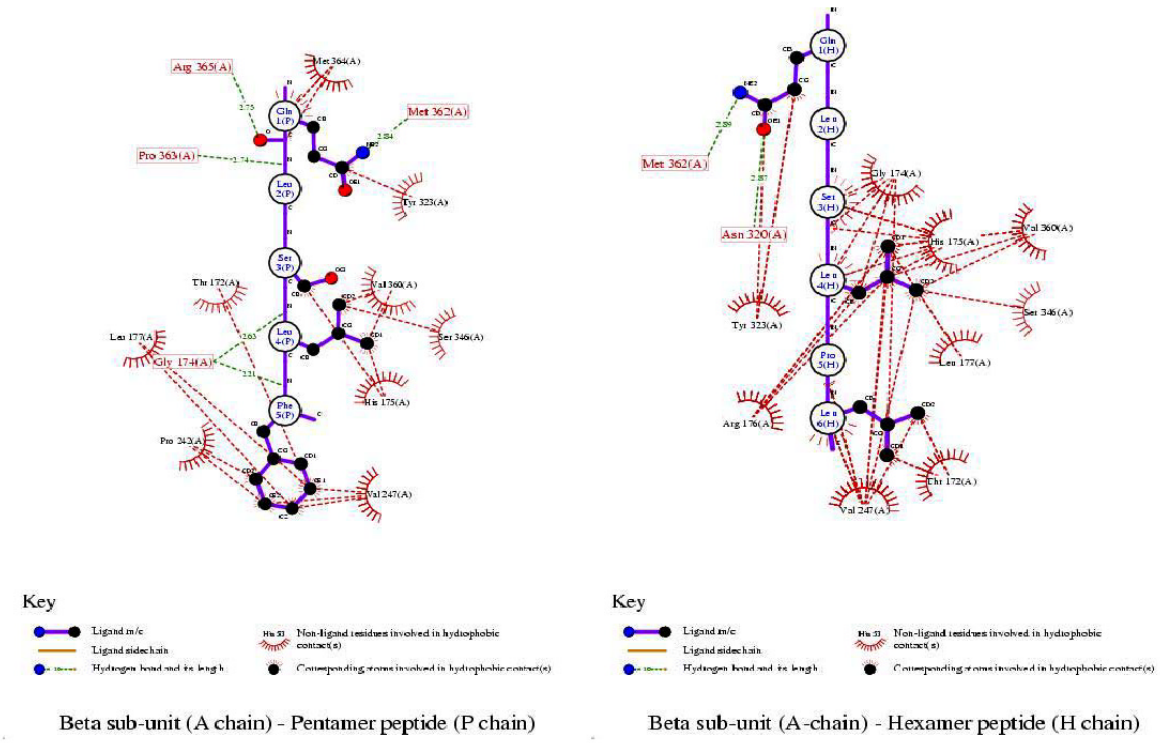

FIGURE S5

FIGURE S5: Interactions for the modelled complexes between peptides and β. These were computed with LIGPLOT (*1*).

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