Biotin Tagging Deletion Analysis of Domain Limits Involved in Protein-Macromolecular Interactions

MAPPING THE $\gamma$ BINDING DOMAIN OF THE DNA POLYMERASE III $\alpha$ SUBUNIT*

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Deok Ryong Kim and Charles S. McHenry
From the Department of Biochemistry, Biophysics, and Genetics, University of Colorado Health Sciences Center, Denver, Colorado 80262

The $\gamma$ subunit dimerizes DNA polymerase III via interaction with the $\alpha$ subunit, allowing DNA polymerase III holoenzyme to synthesize both leading and lagging strands simultaneously at the DNA replication fork. Here, we report a general method to map the limits of domains required for heterologous protein-protein interactions using surface plasmon resonance. The method employs fusion of a short biotinylation sequence at either the NH$_2$ or COOH terminus of the protein to be immobilized on streptavidin-derivatized biosensor chips. Inclusion of a hexahistidine sequence permits one to indicate this fact.

DNA polymerase III holoenzyme (holoenzyme)$^1$ is the major DNA polymerase responsible for Escherichia coli chromosomal DNA synthesis. Holoenzyme consists of 10 individual subunits: $\alpha$, $\beta$, $\theta$, $\gamma$, $\delta$, $\delta'$, $\chi$, $\psi$, and $\beta$ (McHenry, 1988; Kornberg, 1988), all of which act cooperatively in the coordinate and processive synthesis of leading and lagging strands (Wu et al., 1992a, 1992b; Fay et al., 1981). Each subunit is encoded by a separate gene on the chromosome, except for $\tau$ and $\gamma$, which are both expressed from dnaX. The $\tau$ subunit (71 kDa) is the full-length product of the dnaX gene; the $\gamma$ subunit (47 kDa) is synthesized by a -1 translational frameshift and comprises the NH$_2$-terminal two-thirds of the $\tau$ subunit (McHenry et al., 1989; 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The abbreviations used are: holoenzyme, DNA polymerase III holoenzyme; pol III, DNA polymerase III core; pol III', DNA polymerase III' (the BIAcore chip, and the

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Development of vectors that permit expression of deletion-con

aries required for

loenzymereplicative complex. Here, we report the development

derivatized BIAcore biosensor chip. Immobilized

nylated

of a short 13-nucleotide consensus sequence that can be bioti-

rify, and immobilize the fusion proteins on a streptavidin-

boxyl carrier protein of acetyl-CoA carboxylase in terminating

of a relatively short unstructured biotinylated peptide rather

were analyzed for

plasmid pET-11c was obtained from Novagen.

Buffers

Buffer N is 50 mM sodium phosphate (pH 7.8), 500 mM NaCl, 10% glycerol (w/v), 0.5 mM DTT, 0.1 mM PMSF, and 1 mM imidazole. Buffer S is 50 mM sodium phosphate (pH 7.8), 20% glycerol (w/v), 500 mM NaCl, 0.5 mM DTT, and 0.1 mM PMSF. Buffer H is 10 mM HEPES (pH 7.4), 3.4 mM EDTA, 150 mM NaCl, 5 mM DTT, and 20% glycerol (w/v). HBS buffer is 10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005% P-20 surfactant. HKBG buffer is 10 mM HEPES (pH 7.4), potassium glutamate, 10 mM magnesium acetate, 200 µM ATP, and 0.005% P-20 surfactant. Buffer E is 50 mM HEPES (pH 7.5), 1 mM EDTA, 5 mM DTT, 100 mM potassium glutamate, and 15% glycerol. TBS buffer is 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl.

Chemicals and Reagents

d-Biotin was purchased from Sigma. Nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt were obtained from Life Technologies, Inc. Ni-NTA (nitrilotriacetic acid) resin, the QIAquick Gel extraction kit, and the plasmid preparation kit were from Qiagen. Immobilized avidin AffinityPak column and SoftLink SoftRelease avidin resin were purchased from Pierce and Promega, respectively. CM5 sensor chips (research grade), P-20 surfactant, N-hydroxysuccinimide, N-ethyl-N-‘-(3-dithiylamino-propyl)carbodiimide and ethylenediamine hydrochloride were obtained from Pharmacia Biosensor.

Enzymes

Alkaline phosphatase-conjugated streptavidin and immunopure streptavidin were purchased from Pierce. PuF DNA polymerase was purchased from Stratagene. The α subunit was purified to homogeneity from an overproducing E. coli strain and had a specific activity of 5.9 × 106 units/mg on activated calf thymus DNA (Kim and McHenry, 1996). The α subunit was purified as described (Dallmann et al., 1995).

Oligonucleotides

Oligonucleotides (Table 1) were synthesized on automated synthesizers. All were purified by DE52 column chromatography (Hagerman, 1985). Some oligonucleotides were purified on a 10% polyacrylamide, 8 M urea gel, followed by DE52 column chromatography (Hagerman, 1985).

### TABLE 1

| Oligonucleotide | Use | Sequence |
|---------------|-----|---------|
| 114 αNΔ1, αNΔ60, αNΔ240 deletion | GATGTAACCTGAGATTT | |
| 48 αNΔ360, αNΔ542 deletion | ATACACGATATAGCGCT | |
| 47 αΔC0, αΔC48 deletion | AGCGGCTGAAATCCTGC | |
| 2276 αNΔ1 deletion | CCCCCCTGCAAGGCGGAGCGAGCTGTT | |
| 2277 αNΔ60 deletion | CCCCCCTGCAAGGCGGAGCGAGCTGTT | |
| 2279 αNΔ240 deletion | CCCCCCTGCAAGGCGGAGCGAGCTGTT | |
| 2280 αNΔ360 deletion | CCCCCCTGCAAGGCGGAGCGAGCTGTT | |
| 2281 αNΔ542 deletion | CCCCCCTGCAAGGCGGAGCGAGCTGTT | |
| 2286 αNΔ812 deletion | CCCCCCTGCAAGGCGGAGCGAGCTGTT | |
| 2287 αΔC0 deletion | AAAAAACTAGTCACAATCGTGTCAC | |
| 2288 αΔC48 deletion | AAAAAACTAGTCACAATCGTGTCAC | |
| 2752 αNΔ812, αNΔ705, αNΔ859 deletion | TGGTATAGGGTCCTGGCGT | |
| 600 αNΔ705 deletion | CCCCCCTGCAAGGCGGAGCGAGCTGTT | |
| 700 αNΔ859 deletion | CCCCCCTGCAAGGCGGAGCGAGCTGTT | |
| 2253 N-terminal fusion vector | TCTAGAGGAAGTTAAGCAATGACGCTGTCAGCAAGTCTCCAAGAATCTGCAG | |
| 2254 N-terminal fusion vector | CACCCACCAACACACCTGGTCCTCGCTCGTGTGCAGCTGCAACCGATCACTTCGCGGTCGACTGCTCAG | |
| 2255 N-terminal fusion vector | GATGGTGGTGCTGCAATCTGATTTTCTGAGCAGCTGCAACCGATCACTTCGCGGTCGACTGCTCAG | |
| 2256 N-terminal fusion vector | CACCCACCAACACACCTGGTCCTCGCTCGTGTGCAGCTGCAACCGATCACTTCGCGGTCGACTGCTCAG | |
| 2257 C-terminal fusion vector | CATGGGCGCCGCACACTGGGTAGCTGTGTTGTTGCTCTGGCTTGGCTTGTCCTCAG | |
| 2258 C-terminal fusion vector | CACCCACCAACACACCTGGTCCTCGCTCGTGTGCAGCTGCAACCGATCACTTCGCGGTCGACTGCTCAG | |
| 2259 C-terminal fusion vector | GTGTTGCGGGGACACACCGGAAACGCCACCGACACACAGGATGTCGCTGTCCTGCGGTGGCTCGTTGGGTGGTG | |
| 2260 C-terminal fusion vector | CTATCAAGGAGTCTGGCTCGCTCGTGTGCAGCTGCAACCGATCACTTCGCGGTCGACTGCTCAG | |
| 2261 C-terminal fusion vector | CGAGGCTCAGAGTGAGGGATGCTGATGAACTCGCT | |
| 2262 C-terminal fusion vector | GGGCGGCGGCTGCA | |
| 2263 N-terminal fusion vector repair | GGCACCCCGCGCGCCAGACGCGAAGGAGGTAATGTCGTTCAGGCAACCGATCACTTCGCGGTCGACTGCTCAG | |
| 2264 N-terminal fusion vector repair | GCACCACTTGACTGGCGCCGCTGACACTGGGTGGCATGGAATGGCA | |
| 2265 N-terminal fusion vector | GTGTTGCGGGGACACACCGGAAACGCCACCGACACACAGGATGTCGCTGTCCTGCGGTGGCTCGTTGGGTGGTG | |
| 2266 N-terminal fusion vector repair | CTATCAAGGAGTCTGGCTCGCTCGTGTGCAGCTGCAACCGATCACTTCGCGGTCGACTGCTCAG | |
| 2267 N-terminal fusion vector repair | CGAGGCTCAGAGTGAGGGATGCTGATGAACTCGCT | |
| 2268 N-terminal fusion vector repair | GGGCGGCGGCTGCA | |
| 100 N-terminal fusion (T7) | CTAGCACTATGGGCGCCAGACTGGGTGGTGACCC | |
| 200 N-terminal fusion (T7) | GATCGCGTGACCACTCCGCTGACCATTCGCGGTCGACTGCTCAG | |

EXPERIMENTAL PROCEDURES

Strains and Plasmids

E. coli strains HMS174/F−, recA, HB101(F−, recA13, ara 14, proA2, lacY1, galK2, rpsL20, xyl 5) and BL21(DE3)(F−, hsdS, gal, ompT, lambdA(DE3)) were used for plasmid propagation and protein expression. Plasmid pET-11c was obtained from Novagen.

Buffers

Buffer N is 50 mM sodium phosphate (pH 7.8), 500 mM NaCl, 10% glycerol (w/v), 0.5 mM DTT, 0.1 mM PMSF, and 1 mM imidazole. Buffer S is 50 mM sodium phosphate (pH 7.8), 20% glycerol (w/v), 500 mM NaCl, 0.5 mM DTT, and 0.1 mM PMSF. Buffer H is 10 mM HEPES (pH 7.4), 3.4 mM EDTA, 150 mM NaCl, 5 mM DTT, and 20% glycerol (w/v). HBS buffer is 10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005% P-20 surfactant. HKBG buffer is 10 mM HEPES (pH 7.4), potassium glutamate, 10 mM magnesium acetate, 200 µM ATP, and 0.005% P-20 surfactant. Buffer E is 50 mM HEPES (pH 7.5), 1 mM EDTA, 5 mM DTT, 100 mM potassium glutamate, and 15% glycerol. TBS buffer is 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl.

Promoters

The P404/3 T7 promoter/operator (referred to as P404) was constructed by the combination of an early E. coli RNA polymerase-dependent T7 promoter A1 and two lac operators (Laner, 1988; Laner and Bujard, 1988); O4 sequences carry a 17-bp core region (TGG TGA CCG GAT AAC AA) of the E. coli lac operator downstream of the −10 hexamer of the promoter. P404 is 136-fold more tightly repressible than the tac promoter (Ptac), but upon induction with isopropyl-β-D-thiogalactoside, is 2-fold more active than

Construction of Fusion Vectors with the PA1 Promoter

Vectors were constructed to enable NH2- or COOH-terminal fusions with a peptide containing a biotinylation site, a thrombin deavage site, and four oligonucleotides were used to generate a double-stranded DNA fragment encoding the fusion peptide. The T7 promoter (Studier, 1985) was derived from pET-11c vector (Novagen).

Construction of Fusion Vectors with the T7 Promoter

To make NH2- and COOH-terminal fusion vectors containing a T7 promoter, the NdeI-BamHI fragment of pET-11c was replaced by a DNA fragment with annealed oligonucleotides 2250 and 2257, resulting in plasmid pDRK-N(M). This replaced fragment encoded the same amino acid sequence as the original sequence, although some bases were changed to avoid a potential hairpin structure. The DNA sequence at the oligonucleotide-generated region of pDRK-N(M) was verified.

For the COOH-terminal fusion vector, oligonucleotides 2258 and 2259 were phosphorylated and ligated to oligonucleotides 2257 and 2260 to generate a DNA duplex. This DNA fragment was ligated to the NcoI-KpnI fragment of pD’DSP.4 to generate pDRK-C (Fig. 1B).

Construction of Fusion Vectors with the T7 Promoter

To make NH2- and COOH-terminal fusion vectors containing a T7 promoter, the NdeI-BamHI fragment of pET-11c was replaced by a DNA fragment with annealed oligonucleotides 100 and 200, containing restriction enzyme sites for NdeI, NheI, NotI, DraII, KpnI, and BamHI in order to provide restriction enzyme sites after the T7 promoter to facilitate subsequent cloning steps (Fig. 1A). The resulting plasmid was called pET11c(M). The NdeI-DraII fragment containing a fusion peptide region of pDRK-N(M), was inserted into the NdeI-DraII sites of pET11c(M) to generate pET11c(M)-N. The PsiI site at the β-lactamase gene of pET11c(M)-N was removed by replacing the Psul-AlwNI fragment with one derived from phn1 (Kim and McHenry, 1996) because the other PsiI was required to make the fusion protein of the desired gene. This plasmid was derived from pJF118EH (Fürste et al., 1986) containing a β-lactamase gene in which the PsiI site was modified but still displayed an AmpR phenotype. The resulting NH2-terminal fusion vector containing a T7 promoter, was pET11c-N. The COOH-terminal fusion vector, pET11c-KC, was created by ligation of the XbaI-KpnI fragments of pDRK-C and pET11c(M). Fig. 2 depicts the final fusion vectors and their fusion peptide regions. The biotin tag in the fusion protein was used for detection, purification, and immobilization through a specific interaction with streptavidin or avidin. A hexahistidine sequence was introduced for protein purification using Ni2+-NTA chelating chromatography. Four glycines provided a hinge region between the fusion peptide and the protein of interest.

Growth and Induction of Overexpressing E. coli Strains

E. coli strains containing overexpressing plasmids were grown in 1 liter of F-medium (1.5% yeast extract, 1% peptone, 1.2% K2HPO4, 0.02% KH2PO4, and 1% glucose) plus 50 μg/ml ampicillin at 37°C unless otherwise stated. Cells were induced with isopropyl-β-D-thiogalactoside (1 mM final concentration), followed by addition of d-biotin and ampicillin to 10 μg/ml and 50 μg/ml, respectively. After 3 h of induction, cells were harvested by centrifugation at 5,860 × g for 10 min at 4°C and resuspended in 1 ml of Tris-sucrose buffer (50 mM Tris-HCl [pH 7.5] and 10% sucrose) of cells. Cells were quickly frozen in liquid N2 and stored at −80°C. Overproduction of αNab12 was performed at 15°C in a 200-L fermentor using similar conditions, except the pH was maintained at 7.5 and cells were rapidly harvested with chilling as described (Cull and McHenry, 1995).

Purification of the α Deletion Proteins

Ni2+-NTA Ion Chelating Chromatography—Cells were lysed as described (Cull and McHenry, 1995) in the presence of 2 mg of lysozyme of cells, 5 mM benzamidine, and 1 mM PMSF. Cells containing αNab12 deletion mutant were lysed in 1 mg/ml lysozyme and 0.1% Triton X-100

H. Bujard, personal communication.
Monomeric Avidin Affinity Chromatography—When additional purification was required, the pooled material from the Ni²⁺-NTA column was loaded directly (~20 nmol of biotinylated protein/ml of resin) onto a pre-equilibrated SoftLink-SoftRelease avidin column and recirculated three times at a flow rate of 5 column volumes/h. The column load was 100 mg of activated calf thymus cellulose membrane at 500 A for 3 h in 25 mM Tris-HCl, 192 mM glycine (pH 8.3), 20% methanol and blocked in TBS containing 3% nonfat milk for 1 h, washed in TBS plus 0.3% nonfat milk three times, developed in 10 ml of a solution containing nitro blue tetrazolium chloride (1.65 mg) and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (2.2 mg) in 10 ml diethanolamine (pH 9.5) and 10 mM MgCl₂ and washed in water to stop the reaction.

Surface Plasmon Resonance—A Pharmacia Biosensor BIAcore instrument was used for protein-protein binding analysis. All buffers were filtered before use. Streptavidin (30 μl, 200 μg/ml) in coupling buffer (10 mM sodium acetate (pH 4.6) and 0.1–1 M ethanolamine-HCl (pH 8.0)) was injected over the sensor chip at 5 ml/min to capture streptavidin to the carboxymethyl dextran matrix of the chip by NHS/EDC coupling reaction (30 μl of mix) as described (Olson et al., 1995). Unreacted N-hydroxysuccinimide ester groups were inactivated using 1 mM ethanamine-HCl (pH 8.0). Typically, this reaction immobilizes about 5000 response units (RU) of streptavidin. The biotinylated α deletions were then injected over the immobilized streptavidin in HBS buffer. Typically, 500-1000 RU of each α deletion bound to the immobilized streptavidin. Binding analysis of the α subunit to a solution (25 μl) containing 32 mM HEPES (pH 7.5), 6.4 mM DTT, four dNTPs (100 μM each dNTPs), 10 mM MgCl₂ and 1 μg of activated calf thymus DNA. One unit is defined as the amount of enzyme catalyzing the incorporation of 1 pmol of dNTPs/min at 30°C.

Other Methods—Protein concentrations were determined by the method of Bradford (1976). SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (1970).

RESULTS

Deletion of the α Subunit—To identify the NH₂-terminal limit of sequences required to form the τ-binding domain of α, we constructed a series of deletions of the α subunit using the polymerase chain reaction (PCR). All deletion proteins were

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**Fig. 2. Fusion peptide region of vectors.** A, NH₂-terminal fusion vector with either T7 promoter (pETT11-N) or PaI (pDRK-N(M)). The DNA sequence represents the fusion peptide region encoding a short sequence that is biotinylated in vivo (indicated as bold amino acids) (Schatz, 1993), a hexahistidine sequence, and a thrombin cleavage site. Fusions to the NH₂ terminus of proteins can be achieved using cloning sites: PstI, AvrII, DraIII, SphI (PaI vector only), Kpnl, or BamHI (PaI vector only). The vector with PaI contains lac as instead lac. S/D and T indicate a Shine-Dalgarno site and transcriptional terminator, respectively. The vertical arrow indicates the thrombin cleavage site. B, COOH-terminal fusion vector. Using restriction endonucleases of XbaI, Ncol, NotI, DraIII, or Spel, the COOH terminus of a protein can be fused to the fusion peptide. Two tandem stop codons were introduced at the end of the fusion peptide.

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2 The natural form (tetramer) of avidin binds biotin very tightly with a KD of 10⁻¹⁵, so that the interaction can be disrupted only in denaturing conditions. However, the monomeric form of avidin binds biotin with a weaker affinity (KD of 10⁻⁷), which allows elution of biotin under mild conditions (5 mM biotin).
CdnaE, which does not produce a fragment of pHN4 (Kim and McHenry, 1996) was cloned into a vector at the same sites. The resulting plasmid was named pET11-N(dnaE) or pET11-N(dnaE). The fusion region immediately preceding pHN4 was Thr-Ser, followed by the 4 glycines shown in Fig. 1A. This plasmid was digested with SpeI and Stul, and the large fragment was ligated to PCR products digested with SpeI and Stul to produce plasmids pA1-C0 and pA1-C1, which express proteins αCD0 and αCD48 (Fig. 3B).

All α deletion plasmids with a T7 promoter were constructed as described above using the two fusion vectors pET11-N and pET11-KC. Two intermediate constructs, pET11-NdnaE and pET11-CdnaE, were used to make NH2- and COOH-terminal α deletion mutants, respectively. pET11-NdnaE was constructed using pET11-N and pHN3 at the same restriction sites as described in construction of pDRK-NdnaE, and pET11-CdnaE was constructed as described in construction of pDRK-CdnaE using pET11-KC and pHN4. The resulting plasmids pET11-N0, pET11-N1, pET11-N2, pET11-N3, pET11-N4, pET11-N6, pET11-N5, pET11-N7, pET11-C0, and pET11-C1 express αN11, αN542, αN812, αN859, αCΔ0, and αCΔ48, respectively.

Expression of the α Deletion Proteins—Based on expression and solubility of proteins, we selected a panel of α deletion constructs for expression in E. coli: αN11, αN60, αN240, αN360, αN705, and αN859 from the PA1, and αN542, αN812, αCΔ0, and αCΔ48 from the T7 promoter. E. coli strain HMS174 was used for the PA1-based plasmids, and BL21(DE3) for T7 promoter-based plasmids to express the deletion proteins. Cells expressing the αN11, αN60, and αN240 deletion proteins were grown and induced at 37°C, but other deletion strains were grown and induced at room temperature except cells expressing αN812 (15°C) because they were insoluble when expressed at 37°C. The expressed fusion proteins were detected by a biotin blot as described under "Experimental Procedures." Fig. 4 shows the expression pattern of the αN11 deletion protein. Based on a densitometric scan of the gel, αN11 represented ~5% of the total protein. Biotin blotting (lane 3) revealed a high-molecular-weight protein corresponding to the αN11 deletion protein and the biotin carboxyl carrier protein of acetyl-CoA carboxylase (BCCP), the only other biotinylated protein in E. coli (Samols et al., 1988).

Purification of the α Deletion Proteins—Two special properties of the fusion peptide enabled purification of the α deletion proteins to near homogeneity in one or two chromatographic steps. The hexahistidine sequence fused to the α sequences specifically interacts with Ni2+ ions chelated to column resins, and biotinylated deletion proteins were further purified by exploiting the high affinity interaction between biotin and avidin. The αN11, αN60, αN240, and αN542 proteins were purified to near homogeneity by Ni2+-NTA ion chelating chromatography alone as determined by SDS-polyacrylamide gel analysis (Fig. 5). αN11, αN60, αN240, αN812, αCΔ0, and αCΔ48 proteins required further purification by monomeric avidin chromatography, resulting in nearly homogeneous preparations (Fig. 5).

Protein αCΔ48 was expressed at a low level requiring a 270-fold purification. For αN812, only 30 μg was obtained from 21 g of lysate protein. The protein was catalytically inactive, precluding accurate determination of yields, but if yields of the soluble fraction of protein were the same as αCD0 and αCΔ48, a 30,000-fold purification was achieved, illustrating the power of two affinity tags in one fusion peptide in facilitating difficult purifications. Table II summarizes the purification for αN11, αN812, αCΔ0, and αCΔ48 deletion proteins. The gap-filling polymerase assay indicated that αN11 and αCΔ0 were fully active, whereas the αCΔ48 deletion retained ~85% of the gap filling activity of wild-type α. Purification of the other deletion proteins was monitored by gel electrophoresis because they showed no detectable polymerase activity in the gap-filling assay. Twelve mg of αN160, 15 mg of αN240, 21 μg of αN360, and 6 mg of αN542 were purified from 1500, 2000, 960, and 390 mg of total proteins from cell lysates, respectively. αN705 (3.2 mg) and αN859 (1.4 mg) were partially purified from 470 and 530 mg of cell lysate proteins, respectively.

A N-terminal Deletion

B C-terminal deletion

Fig. 3. A strategy used to construct deletions in NH2-terminal fusion proteins of the α subunit. PCR primers (2276, 2278, 2279, 2280, 2281, 2286, 600, or 700) containing a 12-nucleotide noncomplementary sequence with a PstI site are followed by an 18-nucleotide sequence complementary to the first 6 dnaE codons of the intended fusion protein. A second primer (114, 48, or 2752) was used to generate the desired PCR product. PCR was carried out in a Minicycler (MJ Research) for 35 cycles of denaturation at 94°C, annealing at 52°C and extension at 72°C in a buffer (20 mM Tris-HCl (pH 8.75), 10 mM KCl, 10 mM (NH4)2SO4, 2 mM MgCl2, 0.1% Triton X-100, and 100 μM/dNTPs) containing two primers (each 1 μM), DNA template (200 ng), four dNTPs (each 200 μM), and Pfu DNA polymerase (2.5 units). The resulting PCR product was cleaved with PstI and either AffI for αN11, αN60, αN240, BglII for αN360 and αN542, or Stul for αN705, αN812, and αN859, and ligated to the corresponding fragment of pDRK-NdnaE or pET11-NdnaE. The fusion region immediately preceding α was Leu-Gln preceded by the 4 glycines shown in Fig. 2. B, strategy used to construct deletions in COOH-terminal fusion proteins. PCR primers (2287 and 2288) containing a noncomplementary sequence with a SpeI site are followed by an 18-nucleotide sequence complementary to the last 6 codons of the intended fusion protein. A second primer (47) was used to generate the desired PCR product. The resulting PCR product was cleaved with SpeI and Stul and cloned into the corresponding sites of pDRK-CdnaE or pET11-CdnaE. The fusion region immediately following α was Thr-Ser, followed by the 4 glycines shown in Fig. 2.
purity of all purified proteins was determined by 10% SDS-polyacrylamide gel electrophoresis (Fig. 5). The degree of biotinyl- 
tination of αN11 was determined to be 80% using immobilized avidin AffinityPak column analysis.

Fusion Peptide Does Not Block Interactions with Other Sub- 
units—Holoenzyme reconstitution assays of two full-length α fusion proteins were performed as described (Dallmann et al., 1995) to determine whether the fusion peptide interferes with the interaction of α and other accessory subunits required for 
full holoenzyme activity. αN11 and αΔC04 had a specific activity of 
2.2 × 10⁶ and 2.4 × 10⁶ units/mg, respectively, comparable to 
the activity of wild-type α (2.9 × 10⁶ units/mg). Thus, the 
fusion peptide did not block interactions between full-length α and other subunits required in the holoenzyme reconstitution 
assay.

α-τ Binding Analysis Using Surface Plasmon Resonance— 
Kinetic parameters for α-τ binding were determined using the 
αN11 deletion protein immobilized to a sensor chip in a BIA- 
core. Streptavidin was chemically coupled to the chip and αN11 
was immobilized via the biotin-streptavidin interaction. Three 
different concentrations (34 nM, 68 nM, and 170 nM) of the τ 
subunit were used for binding analysis. The off-rate (Koff) was 
calculated by injection of saturating amounts of τ over the 
αN11 fusion to minimize reassociation and was used in calcula-
tions of the on-rates (kon) at all three concentrations of τ 
(Table III). τ dissociated very slowly from α with a half-life of 
−3.4 h (Fig. 6). The dissociation constant (Kd) of α-τ binding 
was determined to be approximately 70 pm. BIAcore analysis of 
γ-τ or γ-complex (γδγδψγψ)-α binding revealed no detectable 
interaction between α and γ (data not shown).

Mapping the τ Binding Domain of the α Subunit—The τ 
binding domain of the α subunit was mapped by BIAcore bind-
ing analysis using a series of deletions from the NH₂ or COOH 
terminus. All α deletion proteins were individually immobilized 
to the streptavidin chip, and the binding with the τ subunit was 
analyzed. α fusions with 60, 240, 360 and 542 amino acids 
deleted from the NH₂ terminus all bound tightly to the τ subunit 
(Table IV), with Kd values in the range of 0.19–0.39 nM. A 
Further α deletion of 812 amino acids from the NH₂ terminus 
yielded a protein that interacted weakly with τ with a 5% 
binding stoichiometry. It is possible that most of the αN3812 
deletion protein has an unfolded structure that is unable to 
binding to τ and a low level of properly folded proteins results in 
substoichiometric binding to τ. However, we are unable to 
distinguish this possibility from a low-level nonspecific inter-
action with an unfolded α COOH-terminal domain. Two other 
plasmids pA1-N6 and pA1-N7 were constructed to express 
larger and smaller α deletion proteins (αN3859 and αN705) 
in hope of obtaining better behaved proteins. Both αN705 and 
αN3859 deletion proteins were partially purified from Ni²⁺-
NTA column chromatography and final purification was car-
ried out on the BIAcore sensor chip by a specific interaction 
between biotinylated protein and immobilized streptavidin. 
Neither of the two deletions bound to the τ subunit on BIAcore.

The COOH-terminal fusion, αC10, bound almost as tightly 
(Kd = 0.37 nM) as the other NH₂-terminal deletion proteins 
(Table IV). The αC348 deletion protein lost binding activity 
to the τ subunit, but the polymerase domain was functionally 
intact as indicated by its nearly full polymerase activity in the 
gap-filling assay (Table II). Further deletions of α from the
The COOH terminus showed no interaction with the subunit (data not shown). The stoichiometric ratio of the α-τ interaction was about 1:1 (Table IV), consistent with the assigned stoichiometry of pol III D in solution (α2τ2) (McHenry, 1982; Studwell-Vaughan and O'Donnell, 1991). Presumably, two immobilized αs come together to bind a τ dimer.

Superox 12 Gel Filtration of αNΔ705 and τ—To examine the possibility that αNΔ705 and τ interact at levels too low or with such slow binding kinetics that no binding is detected on the BIAcore, we tested the binding of αNΔ705 to τ by Superose 12 gel filtration chromatography. αNΔ542, the last deletion protein that still had τ binding activity, was used as a positive control. αNΔ542 and τ4 alone were detected at fractions 21–27 when the mix of αNΔ542 and τ was applied to the gel filtration column (Fig. 7C), indicating that αNΔ542 bound tightly to τ. When αNΔ705 alone was injected into the column, it eluted in a broad range (fractions 16 to 28), giving two separate peaks (Fig. 7D). The first peak might be an unfolded or aggregated form of αNΔ705 eluting in the excluded volume, while the second peak may represent the properly folded form. The mix of αNΔ705 and τ also eluted broadly, but two separate peaks were not detected and the low molecular weight αNΔ705 was shifted to a higher molecular weight (Fig. 7E). These data suggest that αNΔ705 could be shifted by the interaction with τ, although the partial unfolding or aggregation of αNΔ705 complicates interpretation of the data.

48-Amino Acid Deletion from the COOH Terminus Eliminates τ Binding Activity of α—αCΔ48 was unable to interact

![Graph of αNΔ1-τ binding on BIAcore.](image-url)
with the $\tau$ subunit (Table IV). Thus, $\alpha C\Delta 48$ could not be dimerized by $\tau$ for the coordinated DNA synthesis of both leading and lagging strands. When this protein was analyzed in holoenzyme reconstitution assays using $\tau$ complex ($\gamma_{\delta\omega}^\gamma\psi$) (Dallmann et al., 1995), which requires interactions with other subunits, $\alpha C\Delta 48$ possessed only 7% of the activity of wild-type $\alpha$. Interestingly, when $\gamma$-complex ($\gamma_{\delta\omega}^\gamma\psi$) replaced $\tau$ complex in this assay, $\alpha C\Delta 48$ retained about 40% activity of wild-type $\alpha$. Nevertheless, $\alpha C\Delta 48$ showed nearly full polymerase activity in gap-filling assay (Table II). Thus, the COOH terminus of the $\alpha$ subunit is required for full reconstitution of an active replication complex.

**DISCUSSION**

We report here a novel method to map the terminal limits of the binding domains of a protein in the BIAcore using a series of deletion proteins fused to a 13-amino acid consensus sequence (Schatz, 1993) that is efficiently biotinylated in vivo with the biotin carboxyl carrier protein, flows through Ni$^{2+}$-NTA columns, providing preparations that can be further purified, when necessary, on reduced affinity monomeric avidin columns. Use of monomeric instead of native avidin permits bound protein to be eluted with 5 mM biotin under mild conditions. For some types of analyses, the avidin chromatography step can be skipped, since the streptavidin-BIAcore chip also provides a purification, as non-biotinylated proteins wash off during the chip preparation stage and should not be present during the binding analysis. In this report, we exploited this technique for two $\alpha$ derivatives, $\alpha N\Delta 705$ and $\alpha N\Delta 859$, that were expressed at very low levels.

We mapped the $\tau$ binding domain of the 1160-amino acid $\alpha$ subunit using eight proteins containing deletions from the NH$_2$ terminus and two proteins containing deletions from the COOH terminus. $\alpha$ proteins containing amino-terminal deletions up to 542 amino acids showed high-affinity binding to the $\tau$ subunit and displayed very similar off-rates ($\sim$3.4 h half-life). Deletion of 705 or more residues from the amino terminus yielded proteins that showed no or limited interactions with $\tau$. Limited interaction, with only a 5% stoichiometric interaction, was observed between $\tau$ and $\alpha N\Delta 812$. Two additional fusions, $\alpha N\Delta 705$ and $\alpha N\Delta 859$, designed with deletions that flank either side of the 812 residue deletion, did not show interaction in the

The presence of a fusion peptide that interacts with two affinity matrices provides a powerful and rapid purification method, even for proteins that are expressed at very low levels. For most proteins, Ni$^{2+}$-NTA chromatography provides homogeneous protein. The only naturally biotinylated protein in E. coli, the biotin carboxyl carrier protein, flows through Ni$^{2+}$-NTA columns, providing preparations that can be further purified, when necessary, on reduced affinity monomeric avidin columns. Use of monomeric instead of native avidin permits bound protein to be eluted with 5 mM biotin under mild conditions.

**Fig. 7. Superose 12 gel filtration.** A 24-ml Superose 12 FPLC gel filtration column was equilibrated with buffer E. A 200-μl volume of each sample was injected into the column at 0.1 ml/min. Forty fractions of 0.5 ml were collected. $\alpha N\Delta 542$ (0.35 nmol) and $\tau$ (1.4 nmol as monomer) were mixed in buffer E and incubated at room temperature for 15 min before injection. Fractions (70 μl) were separated by 10% SDS-polyacrylamide electrophoresis, and the gel was either stained with Coomassie Brilliant Blue (A) or subjected to a biotin blot (B–E) as described under “Experimental Procedures.” $\tau$ only (A), $\alpha N\Delta 542$ only (B), $\tau$–$\alpha N\Delta 542$ (C), $\alpha N\Delta 705$ only (D), and $\tau$–$\alpha N\Delta 705$ (E) were gel-filtered.

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BIACore, either due to deletion of part of the \( \tau \)-binding site or due to deletions of sequences required for stable folding of the \( \tau \)-binding domain. Further analysis of the \( \alpha N\Delta 705 \) deletion protein by Superose 12 gel filtration indicated that a significant portion of \( \alpha N\Delta 705 \) was excluded, consistent with an unfolded and/or aggregated conformation. Addition of \( \tau \) to \( \alpha N\Delta 705 \) shifted the lower Stokes' radius peak, presumably properly folded \( \alpha N\Delta 705 \), to a higher molecular weight complex, suggesting a limited, weak interaction. These results suggest that residues beyond 542 in the 705–812 range stabilize a domain in a limited, weak interaction. These results suggest that abrogation of \( \tau \) binding by deletion of only 48 residues from the carboxyl-terminal end of the subunit is involved in \( \tau \) binding. This result is consistent with the retention of auxiliary subunit independent gap-filling polymerase activity by \( \alpha N\Delta 48 \) butbloss of \( \tau \)-complex-dependent holoenzyme activity. Photocrosslinking experiments (Reems et al., 1995) demonstrated that holoenzyme in initiation complexes involved interactions with the polymerase between 13 nucleotides at the 3′-primer terminus and additional contacts at position –18 and –22 for the DnaX and the \( \beta \) subunits, respectively. Because of the evidence for interactions between \( \alpha \) and DnaX and \( \beta \) that do not map to DNA contacts, we proposed a model where an appendage of \( \alpha \), distal from the polymerase active site, contacts these auxiliary subunits (Reems et al., 1995). The present studies would suggest that the appendage is the carboxyl-terminal end of \( \alpha \). The special features of a replicative polymerase derive, in part, from its ability to interact with other replication proteins. The biotin tagging deletion analysis approach should be valuable for analyzing other important interactions between DNA polymerase III holoenzyme subunits and should be generally applicable for analyzing other multiprotein assemblies as well.

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