

# Identification, Isolation, and Characterization of the Structural Gene Encoding the $\delta'$ Subunit of *Escherichia coli* DNA Polymerase III Holoenzyme

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The gene encoding the  $\delta'$  subunit of DNA polymerase III holoenzyme, designated *holB*, was cloned by a strategy in which peptide sequence was used to derive a DNA hybridization probe. The gene maps to 24.95 centisomes of the chromosome. Sequencing of *holB* revealed a 1,002-bp open reading frame predicted to produce a 36,936-Da protein. The gene has a ribosome-binding site and promoter that are highly similar to the consensus sequences and is flanked by two potential open reading frames. Protein sequence analysis of  $\delta'$  revealed a high degree of similarity to the *dnaX* gene products of *Escherichia coli* and *Bacillus subtilis*, including one stretch of 10 identical amino acid residues. A lesser degree of similarity to the gene 44 protein of bacteriophage T4 and the 40-kDa protein of the A1 complex (replication factor C) of HeLa cells was seen. The gene, when placed into a *tac* promoter-based expression plasmid, directed expression of two proteins of similar size. By immunodetection with anti-holoenzyme immunoglobulin G, both proteins are judged to be products of *holB*.

DNA polymerase III (Pol III) holoenzyme, the replicative enzyme of *Escherichia coli*, is composed of at least 10 different subunits. The core enzyme, Pol III (33), contains the catalytic  $\alpha$  subunit (*dnaE* [66]), the 3'→5' proofreading exonuclease  $\epsilon$  (*dnaQ* [11, 42, 51]), and the  $\theta$  subunit. Seven accessory proteins combine with Pol III to form the holoenzyme, an asymmetric, dimeric enzyme complex capable of coordinated leading- and lagging-strand DNA synthesis (26, 32, 69–71). The  $\tau$  subunit, the larger of two alternative *dnaX* gene products (22, 39), promotes dimerization of Pol III (31, 57) and promotes lagging-strand synthesis to prevent formation of short Okazaki fragments in the presence of physiological concentrations of glutamate (69).

The major processivity subunit of holoenzyme is the  $\beta$  subunit (*dnaN* [5, 9]), a dimeric, torus-shaped sliding clamp that tethers holoenzyme to a primed template (24, 58). Transfer of the  $\beta$  subunit in vitro to a primed template to form a preinitiation complex is an ATP-dependent reaction (41, 67, 68) mediated by the  $\gamma$  complex (30, 35, 36, 41). Of the five subunits of the  $\gamma$  complex ( $\gamma$ , *dnaX* [12, 28];  $\delta$ , *holA* [6, 11a];  $\delta'$ ;  $\chi$ , *holC* [5b, 8a]; and  $\psi$ , *holD* [5a, 8a]), the two-subunit complexes  $\gamma\delta$ ,  $\tau\delta$ , and  $\tau\delta'$  can effect transfer of the  $\beta$  subunit (40). Although unable to combine with  $\gamma$  to effect  $\beta$  subunit transfer, the  $\delta'$  subunit stimulates the DNA-dependent ATPase activity of both  $\gamma$  and  $\gamma\delta$  and enhances the activity of polymerase reconstituted with  $\alpha$ ,  $\epsilon$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  (43).

Further study of the contribution of  $\delta'$  to the function of holoenzyme requires a source of large quantities of the protein. In this report, we present a major step toward this goal: identification, isolation, characterization, and overexpression of the structural gene encoding the  $\delta'$  subunit.

## MATERIALS AND METHODS

**Chemicals.** Bovine serum albumin, polyvinylpyrrolidone, dextran sulfate, and Tris-HCl were purchased from Sigma. Sodium dodecyl sulfate (SDS), acrylamide, *N,N'*-bis-acrylamide, and Coomassie brilliant blue R-250 were purchased from Bio-Rad. Polyethylene glycol was from Fisher. Spermidine was from Fluka. SeaKem LE agarose was from FMC BioProducts. [ $\alpha$ -<sup>32</sup>P]dATP and <sup>35</sup>S-dATP were purchased from ICN. Bacteriophage  $\lambda$  DNA digested with *Hind*III was purchased from Promega and a 100-bp ladder was purchased from Bethesda Research Laboratories. All other chemicals were reagent grade.

**Oligonucleotides.** Table 1 lists the sequences of oligonucleotides used in this work. All oligonucleotides were synthesized by the University of Colorado Cancer Center Macromolecular Synthesis Core Facility. Oligonucleotides were incubated in ammonium hydroxide for 16 h at room temperature to cleave the DNA from the support and were incubated for a further 16 h at 65°C to deprotect the 5' end. Oligonucleotides were purified on a DE52 column equilibrated with 40 mM Tris-HCl (pH 8.0)–2 mM EDTA–100 mM NaCl and were developed with 3 M potassium acetate. Oligonucleotides were precipitated with ethanol, dissolved in H<sub>2</sub>O to a final concentration of 20 mM, and stored at –20°C.

**Strains, phages, plasmids, and media.** XL1 Blue [*F'* *proAB lacI<sup>q</sup>Z $\Delta$ M15 Tn10 (Tet<sup>r</sup>)/recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lac*], purchased from Stratagene, was used for routine plasmid transformation and for plasmid purification. MGC100, an isolate of RS320 [*Δlac(IPOZYA)U169 Δlon araD139 strA supF*; gift of R. Sclafani, University of Colorado Health Sciences Center] resistant to a phage that contaminates our fermentor (probably bacteriophage T1), was the source of holoenzyme. MAF102, a *lexA3 wrd* (64) derivative of the wild-type MG1655 (17), was the source of

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TABLE 1. Oligonucleotides used in this study

PCR Primers<sup>a</sup>

|                                   |       |
|-----------------------------------|-------|
| CTCGAATTCTGGTWGCNWSYTAYCARGC      | (1.1) |
| CTCGAATTCGCGYGRSWNGCNAC           | (1.2) |
| CTCGAATTCGARCARTAYGCNGTNAC        | (2.1) |
| CTGCATCTAGAGTNACNGCRTAYTGYTC      | (2.2) |
| CTGCATCTAGACTGCTGACYGAYGCNGCNGCNA | (3.1) |
| CTGCATCTAGACANTGRCTRGCNGCGACGAC   | (3.2) |

Reconstruction of 5' end of gene encoding  $\delta'$ 

GGTCGACTATTTCGAAGTCCGGGCGCAGCCAGGGTACCAGCGCATTATTTATTACCTCTA  
GATCTAGGAGGTAATAAATAATGCGCTGGTACCCGTGGCTGCGCCGGACTTCGAATAGTCGACCTGCA

<sup>a</sup> Oligonucleotides are referred to in the text by the numbers in parentheses. Bases in boldface were chosen on the basis of peptide sequences of  $\delta'$ . Bases in lightface include an *EcoRI* site (underlined) or an *XbaI* site (double underlined). Nucleotide abbreviations: R, A or G; Y, T or C; W, A or T; S, C or G; N=A, C, G, or T.

*E. coli* chromosomal DNA. Bacteriophage  $\lambda$  was propagated on MC1061 [*araD139*  $\Delta$ (*ara leu*)7697 *lacX74 galU galK hsr hsm strA*] (7). HB101 [*supE44 hsdS20* ( $r_B^- m_B^-$ ) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*] (4) was used in the overexpression experiment.

The primary cloning vectors were pBlueScript II SK+ and pBlueScript II SK- (Stratagene). The expression plasmid of the gene encoding  $\delta'$  was derived from pBBMD11, a *tac* promoter-based expression plasmid of *dnaX* (14, 34). The source of the gene encoding  $\delta'$  was the Kohara bacteriophage  $\lambda$  clone E9G1 (23) (gift of D. E. Berg).

L broth and agar (37) were used for routine bacterial growth. The liquid medium for phage growth was NZCYM (49). F medium (1.4% yeast extract, 0.8% peptone, and 1.2% potassium phosphate [pH 7.5]) was used for expression of  $\delta'$ . When required, ampicillin and tetracycline were used at 50 and 10  $\mu$ g/ml, respectively.

**Enzymes.** Restriction enzymes and T4 DNA ligase were purchased from Promega or New England Biolabs. *Taq* polymerase was purchased from Perkin-Elmer Cetus. Lysozyme was purchased from Worthington. Enzymes were used according to manufacturers' instructions.

**DNA purification.** Plasmid DNA was extracted as described previously (2) and was purified by two rounds of CsCl-ethidium bromide equilibrium density gradient centrifugation (49). Chromosomal DNA was extracted as described previously (6) and was processed through two 55% (wt/vol) CsCl equilibrium density gradients. Bacteriophage  $\lambda$  DNA was extracted from phage particles purified from a 2-liter liquid lysate by using standard bacteriophage  $\lambda$  methods (49).

**$\delta'$  peptide sequences.** The  $\delta'$  subunit was isolated from holoenzyme purified as described previously (6). Briefly, 500  $\mu$ g of holoenzyme was purified, concentrated 35-fold by vacuum dialysis, and subjected to polyacrylamide gel electrophoresis (PAGE) to resolve the subunits. The sample was transferred to nitrocellulose (Schleicher & Schuell; 0.45- $\mu$ m pore size) in preparation for sequencing of tryptic peptides of the  $\delta'$  subunit. Densitometric analysis of the stained blot indicated that about 20  $\mu$ g of  $\delta'$  was transferred. The  $\delta'$  subunit was excised from the blot and was digested with trypsin (1). Tryptic fragments of  $\delta'$  were purified by using a narrow-bore Brownlee Aquapore Bu-300 column reversed-phase high-performance liquid chromatography (HPLC) system and were sequenced on an Applied Biosystems 477A protein sequencer (1, 18).

**PCR.** Polymerase chain reaction (PCR) was performed

with a Perkin-Elmer Cetus model 480 PCR machine. Reactions were performed by using the reaction components in the Perkin-Elmer GeneAmp PCR Reagent Kit that included *AmpliTaq* DNA polymerase. Each 100- $\mu$ l reaction mixture included 1 ng of *E. coli* chromosomal DNA and two oligonucleotide primers, each at 1 mM. Reactions were begun by incubating tubes lacking polymerase and deoxynucleoside triphosphates (dNTPs) in the PCR machine at 94°C for 7 min to denature template. Tubes were brought to 85°C for 4 min to allow addition of polymerase and dNTPs, and reactions were cycled 35 times through a 1-min incubation at 94°C, a 5-min ramp from 50°C to 65°C, and a rapid return to 94°C. Reaction products were precipitated with ethanol, dissolved in 10 mM Tris-HCl (pH 8.0)-1 mM EDTA, digested with restriction enzymes, extracted with phenol and chloroform, and purified by agarose gel electrophoresis in preparation for cloning.

**Agarose gel electrophoresis.** DNA restriction fragments were separated by using standard agarose gel electrophoresis techniques (49). When required for DNA purification, restriction fragments were excised from the gel by the method of Carter et al. (6), which avoids UV irradiation of the restriction fragment. DNA was then purified from agarose by using the GeneClean II DNA purification kit from Bio 101 or an Elutrap apparatus (Schleicher & Schuell).

**Preparation of radiolabeled DNA.** Restriction fragments were purified by agarose gel electrophoresis and were used in a random hexamer-primed labeling reaction with the Random Primed DNA Labeling Kit from Boehringer Mannheim Biochemicals. The reaction mixture, which included 50 ng of heat-denatured DNA, 1 U of Klenow enzyme, and 50  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]dATP (3,000 Ci/mmol, 10 mCi/ml), was incubated at 37°C for 30 min. Labeled DNA was purified by chromatography on a G-25 gel filtration column (Pharmacia) equilibrated and developed with 20 mM Tris-HCl (pH 8.0)-1 mM EDTA-50 mM NaCl and was heat-denatured before use in hybridization experiments.

**DNA sequencing.** Dideoxy chain termination DNA sequencing (50) of PCR products was performed with the Sequenase version 2.0 DNA sequencing kit from United States Biochemical Corp. DNA was labeled with <sup>35</sup>S-dATP (12.5 mCi/ml). Sequencing reactions were subjected to electrophoresis on a 6% polyacrylamide-8 M urea gel (49), which was then dried and autoradiographed for 24 to 48 h with Kodak X-Omat AR X-ray film. Dideoxy chain termination sequencing of the gene encoding  $\delta'$  was performed by Lark

Sequencing Technologies, Inc. (Houston, Tex.). The entire gene was sequenced in both directions.

**DNA blotting and hybridization.** DNA blotting and hybridization were performed as follows. Two micrograms of restriction enzyme-digested MAF102 chromosomal DNA or 200 ng of bacteriophage DNA was size-fractionated on a 0.7% agarose gel, denatured, neutralized, and transferred to GeneScreen nylon membrane (New England Nuclear) for 18 h in 1.5 M NaCl–0.15 M Na citrate · 2H<sub>2</sub>O as described previously (6) by using a conventional DNA transfer assembly (53). The blot was irradiated with 1.6 kJ of UV light m<sup>-2</sup> from a germicidal lamp, following the instructions provided with the membrane to cross-link DNA to the membrane.

The blot was prehybridized, hybridized, and washed according to the instructions provided by New England Nuclear and was autoradiographed for 8 to 24 h with a Molecular Dynamics Phosphorimager screen. The screen was scanned on a Molecular Dynamics Phosphorimager model 400E, and the data were analyzed with ImageQuant version 3.0. Phosphorimager images were printed on a Hewlett-Packard Laserjet III modified to permit printing of 256 shades of gray.

A blot of the mini-set of Kohara bacteriophage clones was obtained from Takara Shuzo, Inc. Hybridization of radiolabeled probe DNA to this blot was performed according to the instructions provided with the blot.

**SDS-PAGE.** Proteins were separated by electrophoresis (27) in an SDS–7.5 to 17.5% polyacrylamide gel run in a Hoefer vertical gel electrophoresis apparatus for 16 h at 7 mA. Gels were stained with a 0.25% solution of Coomassie brilliant blue R-250 in 45% methanol and 10% acetic acid and were destained in a solution of 7.5% methanol–10% acetic acid.

**Expression of the  $\delta'$  subunit.** Fresh overnight cultures were diluted 100-fold into 250 ml of F medium supplemented with glucose to 1% and were incubated with rapid shaking at 37°C. Growth was monitored by  $A_{600}$  measurements. At an  $A_{600}$  of 0.5, IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) was added to a final concentration of 1 mM, and the incubation was continued. After 3 h, cells were pelleted by centrifugation, suspended in 2.5 ml of Tris-sucrose buffer (50 mM Tris [pH 7.5], 10% [wt/vol] sucrose, 5 mM dithiothreitol), and frozen at –80°C. Cells were thawed and diluted with an additional 8.5 ml of Tris-sucrose buffer and 600  $\mu$ l of lysis buffer (50 mM Tris [pH 7.5], 10% [wt/vol] sucrose, 2 M NaCl, 0.3 M spermidine). The pH of the sample was adjusted to 7.5  $\pm$  0.4 by addition of 2 M Tris base. Addition of lysozyme to 0.2 mg/ml was followed by a 1-h incubation on ice and a 4-min incubation at 37°C. Lysates were centrifuged at 14,000  $\times$  g for 1 h to remove cell debris, and saturated ammonium sulfate was added to the supernatants to a final concentration of 40%. A 30-min incubation on ice was followed by centrifugation for 30 min at 14,000  $\times$  g. The resulting protein pellets were dissolved in 140  $\mu$ l of 50 mM Tris (pH 7.5)–20% glycerol–1 mM EDTA–5 mM dithiothreitol. The protein concentration of each sample was determined by using the Bio-Rad protein determination reagent, and about 350  $\mu$ g of each sample was loaded onto an SDS–7.5 to 17.5% polyacrylamide gel.

**Preparation of polyclonal rabbit anti-holoenzyme IgG.** One New Zealand White rabbit was injected with 100  $\mu$ g of purified holoenzyme, followed by four 50- $\mu$ g boosters at 2-week intervals. Immunoglobulin G (IgG) was purified from serum as described previously (65) and titers were determined by using 4  $\mu$ g of holoenzyme in an Ouchterlony assay in which a positive response produced an optical density of

$\geq 0.5$  at 600 nm after 1 h. Purification increased the titer of the IgG 2,000-fold. The final concentration of IgG was 3.5 mg/ml.

**Immunological detection of  $\delta'$ .** Protein samples were split in half and were resolved on two identical SDS-polyacrylamide gels run under identical conditions. After electrophoresis, one gel was stained with Coomassie brilliant blue R-250 and destained. Proteins in the second gel were transferred to a nitrocellulose membrane (Schleicher & Schuell; BA85, 0.45- $\mu$ m pore size) (60) for 2 h at 0.5 A in transfer buffer (25 mM Tris-HCl [pH 8.2], 192 mM glycine, 20% methanol) by using a Hoefer Transblot apparatus. Immunological detection of holoenzyme subunits was performed as described previously (21), with the following exceptions: the urea treatment of the gel was omitted, the transfer was in a Hoefer transfer apparatus for 2.5 h at 0.5 A, the transfer buffer contained 20% methanol, the membrane was blocked with a 1-h incubation in 2.5% powdered milk in 10 mM Tris (pH 7.5)–0.9% NaCl, washes to remove the rabbit anti-holoenzyme IgG contained 0.05% Tween 20, and the terminal detection reagent was peroxidase-conjugated goat IgG directed against rabbit IgG. About 35  $\mu$ g of primary antibody, polyclonal rabbit anti-holoenzyme IgG, was used per membrane. This antibody reacts with  $\alpha$ ,  $\tau$ ,  $\beta$ ,  $\gamma$ ,  $\epsilon$ , and  $\delta$  and/or  $\delta'$  (59). The stock peroxidase-conjugated goat IgG (0.5 mg/ml; KPL) was used at a dilution of 1/500.

**Sequence analysis.** GenBank DNA sequences were translated in all six reading frames by the TFASTA program and were searched for similarity to the predicted amino acid sequence of  $\delta'$  by using the method of Pearson and Lipman (44). Codon usage analysis, translation of the gene encoding  $\delta'$ , and sequence alignments were done with PC/GENE version 6.5 (Intelligenetics).

**Nucleotide sequence accession number.** The GenBank nucleotide accession number of the sequence reported in this paper is L01483.

## RESULTS

To clone the gene encoding the  $\delta'$  subunit, we first obtained a gene-specific DNA hybridization probe by using a “reverse genetic” approach in which degenerate oligonucleotides were designed on the basis of  $\delta'$  peptide sequences and were used in PCR experiments to amplify a region of the gene encoding  $\delta'$ . The PCR-generated DNA fragment obtained was used to map the gene and to identify a restriction fragment that contained the entire gene.

**Sequences of tryptic peptides of  $\delta'$ .** The sequences of the tryptic peptides of  $\delta'$  were determined as follows. Approximately 20  $\mu$ g of the  $\delta'$  subunit was isolated from purified holoenzyme and digested with trypsin. Tryptic fragments were separated by using reversed-phase HPLC, and four well-resolved peptides (Fig. 1A) were subjected to amino-terminal sequencing (Fig. 1B) (see Materials and Methods).

**PCRs.** Each peptide sequence was used in a search of the DNA sequence in GenBank translated in all six reading frames. No identities between the first three peptides and previously reported proteins were revealed. The first six residues of the fourth peptide sequence were identical to a segment of the  $\gamma$  subunit of holoenzyme, which caused concern regarding the authenticity of the sequence. To obtain a fragment of the gene encoding  $\delta'$ , degenerate primers were designed on the basis of peptide sequences 1, 2, and 3 (Fig. 1B). Because the order of the peptides within  $\delta'$  was not known, two primers were required for each peptide sequence to allow priming in both directions. One

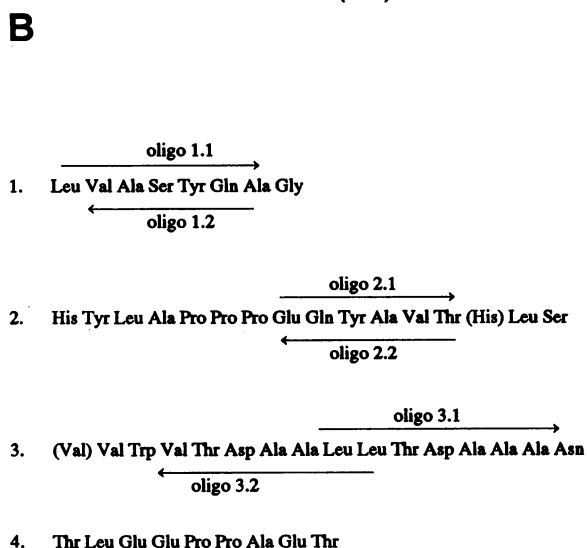
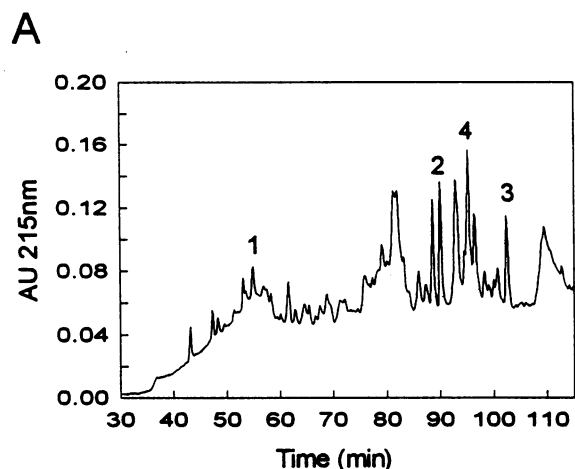


FIG. 1. Peptide sequencing of the  $\delta'$  subunit. The  $\delta'$  subunit was resolved from the other holoenzyme subunits by SDS-PAGE and was transferred to a nitrocellulose filter. The  $\delta'$  subunit was excised from the filter and digested with trypsin directly on the filter. Tryptic fragments of  $\delta'$  were separated by reversed-phase HPLC (A). Peptides corresponding to the labeled peaks were subjected to amino-terminal sequencing (B). Residues in parentheses were not definitively identified. Oligonucleotides used for PCR experiments were designed on the basis of the amino acid sequences immediately above or below the arrows.

primer was designed directly from a reverse translation of peptide sequence, and the second primer was from the complement of the reverse translation. Several features were included in the design of the primers. (i) To minimize degeneracy, primers were based on regions of peptide sequence that contained the fewest amino acids encoded by six codons, and in some cases, only preferred codons (10) were used. In addition, the degeneracy and size of each primer were kept as close as possible to favor equal priming during PCR. (ii) When possible, the carboxy- and amino-terminal amino acid residues of a peptide were not used to design the primers, so that each PCR product could be checked by DNA sequencing to determine whether it encoded predicted sequence not contained in the primer. (iii) To facilitate cloning of PCR products, tails including restriction enzyme sites were appended to the 5' end of each primer.

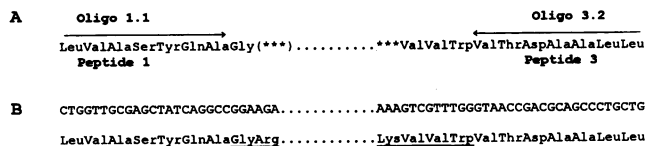


FIG. 2. Confirmation by DNA sequencing that a PCR product is part of the gene encoding  $\delta'$ . (A) Oligonucleotides 1.1 and 3.2 (arrows) were designed on the basis of peptides 1 and 3, respectively. Basic amino acid residues, predicted to exist on the basis of the specificity of trypsin, are indicated by three asterisks. Parentheses indicate that this basic residue was not necessarily expected to occur immediately after the last sequenced amino acid residue of the peptide. (B) The 350-bp PCR fragment was sequenced and translated. Amino acid residues expected if the PCR product corresponded to a fragment of the gene encoding  $\delta'$  are double underlined.

PCRs were run with all possible pairs of primers to amplify portions of the gene encoding  $\delta'$  from the chromosomal DNA template. Although several pairs of primers directed production of small amounts of DNA fragments, only three primer pairs directed production of single DNA fragments in large quantity: a 510-bp fragment from primers 1.1 and 2.2, a 350-bp fragment from primers 1.1 and 3.2, and a 180-bp fragment from primers 3.1 and 2.2. The sum of the sizes of the two smaller products (530 bp) approximately equaled the size of the largest fragment (510 bp), permitting the relative order of three of the  $\delta'$  peptides (NH<sub>2</sub>-peptide 1, peptide 3, peptide 2) and providing additional evidence that the three fragments were part of the structural gene for  $\delta'$ .

The 350- and 510-bp fragments were cloned into pBlue-Script II SK+. The termini of the 350-bp fragment produced by using primers 1.1 and 3.2 were sequenced (Fig. 2). The sequence of each terminus of the fragment was identical to the sequence of the PCR primer used to generate it. In addition, peptide sequence obtained experimentally but not used to design the PCR primers was encoded by DNA found in the expected position adjacent to the primer sequence. These sequences were followed by the codon for a basic amino acid residue (arginine or lysine). (Trypsin, the enzyme used to produce the peptides, cleaves to the carboxy-terminal side of basic amino acid residues.) Thus, six residues were found in the 350-bp fragment in positions predicted from protein sequence data. Moreover, the sequences after primer 1.1 were identical in both the 510- and the 350-bp fragments for at least 100 bp (data not shown). These results indicated that the PCR fragments were part of the gene encoding  $\delta'$ . The 350-bp fragment was chosen as a gene-specific DNA hybridization probe.

**Mapping the gene encoding  $\delta'$ .** Restriction fragments of chromosomal DNA digested with all one- and two-enzyme combinations of *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hind*III, and *Pst*I were resolved by agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with a radiolabeled 350-bp PCR fragment (Fig. 3A). Single restriction fragments complementary to the probe were identified for each restriction digestion, indicating that only one copy of the gene exists in the chromosome. The restriction fragment sizes were used to construct a restriction map of the region of the chromosome containing the gene (Fig. 3B). This map was compared with the restriction map of the entire *E. coli* chromosome (23, 46, 47) to determine the chromosomal location of the gene. A single region of the reported map (46, 47), located at 24.95 centisomes, corresponded to the map in Fig. 3B. The gene required to encode the ca. 32-kDa  $\delta'$

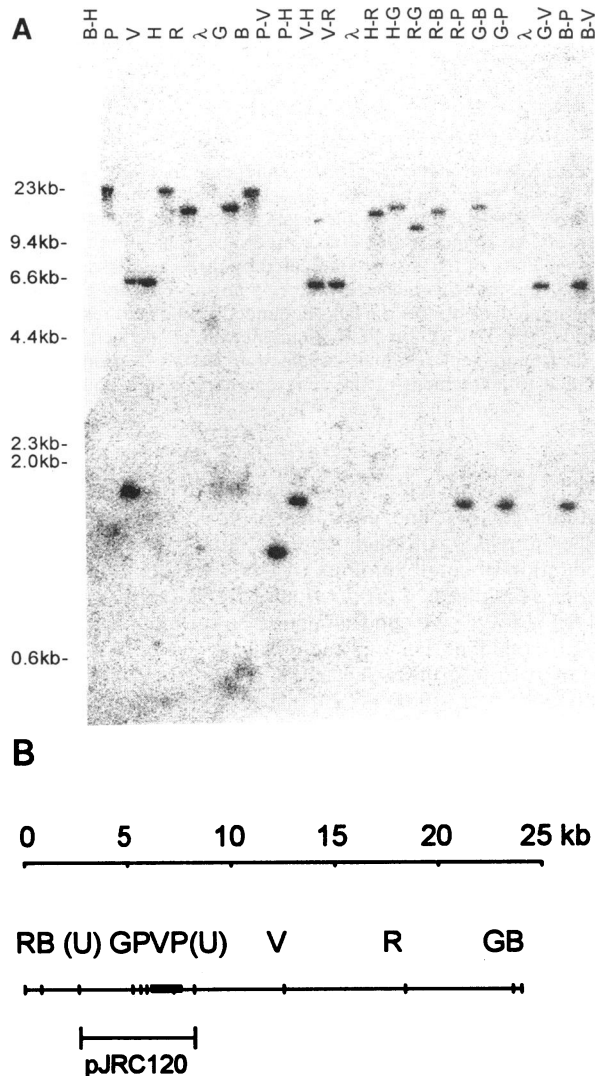


FIG. 3. Restriction map of the region of the chromosome including the gene encoding  $\delta'$ . (A) MAF102 chromosomal DNA was transferred onto a GeneScreen nylon membrane and was hybridized with the radiolabeled 350-bp PCR fragment. Restriction enzyme abbreviations: B, *Bam*HI; G, *Bgl*II; R, *Eco*RI; V, *Eco*RV; H, *Hind*III; P, *Pst*I. Kilobase size standards are indicated to the left of the blot. (B) A restriction map was constructed on the basis of the sizes of chromosomal restriction fragments that hybridized to the probe. The 6-kb *Pvu*II fragment (U), predicted in the *E. coli* restriction map (23) and verified by hybridization of the 350-bp fragment to *Pvu*II-digested Kohara clone E9G1 (data not shown) to contain the gene encoding  $\delta'$ , is contained in pJRC120.

subunit would be about 1 kb. The 350-bp probe hybridized to a single *Pst*I fragment of 1.8 kb (Fig. 3A), indicating that the probe is complementary to DNA within this fragment. This localizes the gene to a 2.8-kb region of DNA between 1,164 and 1,168 kb of the reported chromosome map (46).

**Cloning and sequencing the structural gene of the  $\delta'$  subunit.** A 6-kb *Pvu*II restriction fragment in the *E. coli* chromosomal map (23, 46) is reported to contain the entire 1.8-kb *Pst*I fragment flanked by 1 and 3 kb of DNA. On the basis of the size of the probe and the predicted size of the gene, the 6-kb *Pvu*II restriction fragment should contain the entire gene. The presence of the 6-kb *Pvu*II fragment in  $\lambda$  clone

E9G1 (23), one of two  $\lambda$  clones complementary to the 350-bp probe, was confirmed by Southern hybridization analysis (data not shown). The 6-kb *Pvu*II fragment was isolated from clone E9G1 and was ligated into the *Eco*RV site of pBlue-Script II SK- to create pJRC120.

To initiate sequencing of the gene, five primers were synthesized on the basis of the DNA sequences of the PCR products (data not shown). Sequences obtained with these primers were used to design a second set of primers and to derive additional sequence data. This strategy was used to gain double-stranded sequence data for 1,401 bp of DNA (Fig. 4). The 1,401 bp were analyzed and coding regions for the four tryptic peptides of  $\delta'$  were identified and were found to all be in the same reading frame (Fig. 4). This confirmed the order of the three  $\delta'$  peptides predicted from the fragment sizes generated by the corresponding PCR primers. From the sequence containing the peptides, a 1,002-bp open reading frame that encoded a 36,936-Da protein was identified. Because all four experimentally derived peptide sequences are encoded by this open reading frame and the estimated size of  $\delta'$  (32,000 Da) is in approximate agreement with the calculated protein size, we tentatively identified the open reading frame as the gene that encodes  $\delta'$ . The ATG initiation codon of this open reading frame is 7 bp downstream of a strong consensus ribosome-binding site (52 [Fig. 4]), which fulfills the spacing requirement for a ribosome-binding site (55). A TTG codon spaced 5 bp upstream of the predicted ATG initiation codon cannot be formally ruled out as a potential initiation codon, but TTG represents only 1% of the *E. coli* initiation codons (16). Furthermore, there is no correctly positioned ribosome-binding site upstream of the TTG codon. Potential -35 and -10 promoter elements (45) are 144 bp from the ATG codon (Fig. 4). The 170 bp upstream of the ATG is part of a reading frame with a single termination codon that overlaps the ATG initiation codon of the putative gene encoding  $\delta'$ . An ATG codon located 10 bp after the termination codon of the putative gene encoding  $\delta'$  could initiate an open reading frame that extends 209 bp to the end of the sequence reported in Fig. 4. These observations suggest that the putative gene encoding  $\delta'$  is in an operon with one or both of the identified open reading frames that flank the gene.

**Codon usage.** Eight rarely used codons, AUA, UCG, CCU, CCC, ACG, CAA, AAU, and AGG, have been identified from a survey of 25 nonregulatory *E. coli* genes (25). These codons occur at a combined frequency of 4.2% in the 25 genes and at a frequency of 1.7% in a subset of 10 ribosomal protein-encoding genes that are very highly expressed in *E. coli*. The codon usage of the gene encoding  $\delta'$  was examined to determine whether the gene contains a high percentage of rare codons, possibly contributing to regulation of gene expression, as has been suggested for *dnaG* (25). The eight rare codons occur in the putative gene encoding  $\delta'$  at a frequency of 6.3%, 1.5-fold higher than the percentage of rare codons in the 25 nonregulatory genes and 3.7-fold higher than the 10 very highly expressed ribosomal protein genes. The codon usage of the gene encoding  $\delta'$  is typical of that seen in several other *E. coli* replication genes (Table 2).

Codon usage analysis of the two flanking open reading frames indicated 3.4% rare codons in the 170-bp open reading frame, versus an average of 9.5% rare codons in the two stop-codon-interrupted reading frames. The downstream 209-bp open reading frame contains 0% rare codons, versus an average of 11.5% rare codons in the two interrupted reading frames. These numbers favor the possibility that the open reading frames encode proteins.

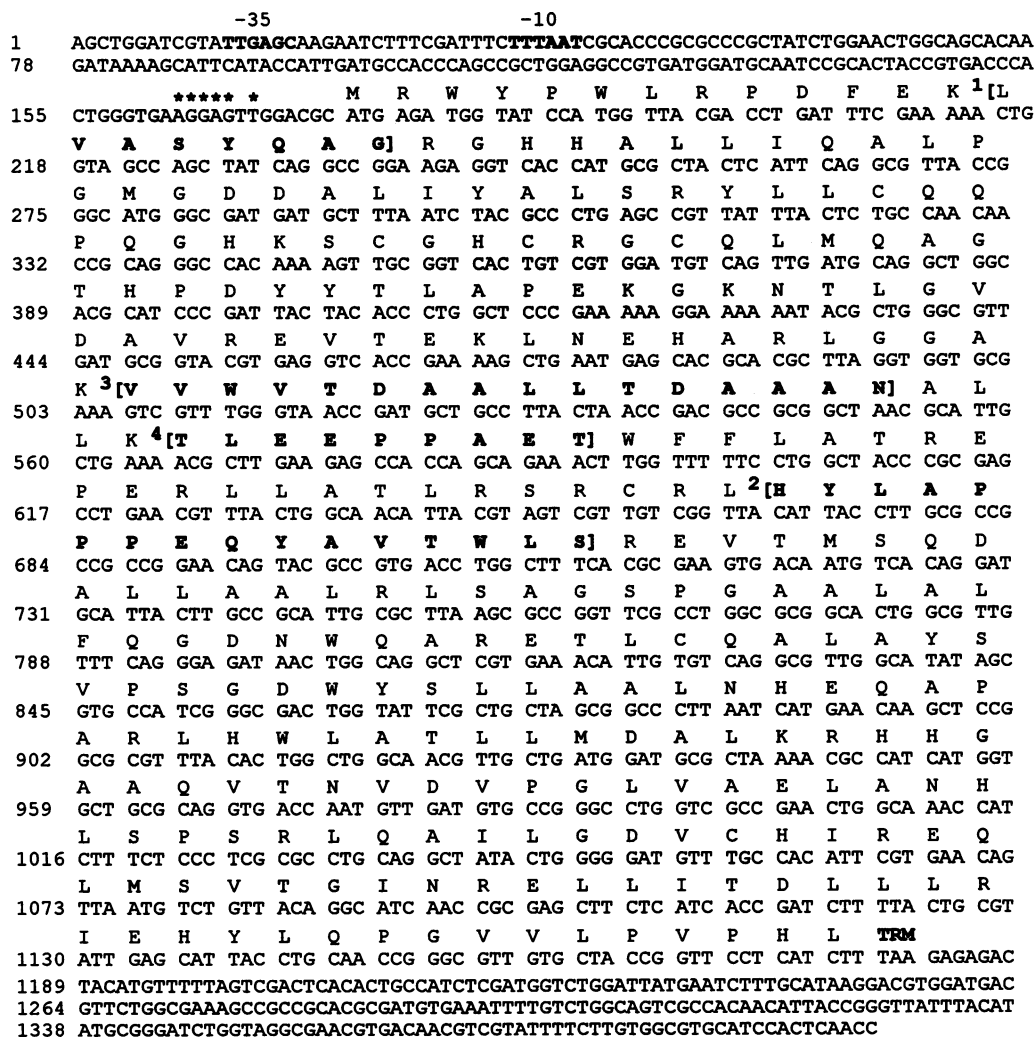


FIG. 4. DNA sequence of the gene encoding δ'. The gene is predicted to begin at base 176 and to end at base 1177. TRM indicates the termination codon, and asterisks indicate a potential ribosome-binding site. Potential -35 and -10 promoter elements are in boldface and are labeled. Amino acid residues encoded by the gene are indicated by single-letter abbreviations above the first letter of each codon. The four experimentally derived δ' peptides are in boldface, are bracketed, and are numbered.

TABLE 2. Percentage of rare codons in the three reading frames of seven *E. coli* genes<sup>a</sup>

| Gene <sup>b</sup> | % Rare codons in open reading frame: |      |      |
|-------------------|--------------------------------------|------|------|
|                   | 1 <sup>c</sup>                       | 2    | 3    |
| Gene encoding δ'  | 6.3                                  | 8.7  | 9.3  |
| <i>dnaN</i>       | 4.1                                  | 12.5 | 11.4 |
| <i>dnaE</i>       | 4.9                                  | 13.4 | 10.8 |
| <i>dnaX</i>       | 6.8                                  | 15.6 | 6.7  |
| <i>dnaQ</i>       | 7.0                                  | 13.2 | 11.5 |
| <i>holA</i>       | 8.7                                  | 11.4 | 8.7  |
| <i>dnaG</i>       | 11.3                                 | 12.4 | 12.9 |

<sup>a</sup> Rare codons include AUA (Ile), UCG (Ser), CCU and CCC (Pro), ACG (Thr), CAA (Gln), AAU (Asn), and AGG (Arg) (25).

<sup>b</sup> Sequence data were derived from the following references: *dnaN*, 42; *dnaE*, 59; *dnaX*, 12; *dnaQ*, 29; *holA*, 6; *dnaG*, 25.

<sup>c</sup> Frame 1 is the coding frame.

**Protein sequence analysis.** The predicted amino acid sequence of the δ' subunit was compared with that of DNA in GenBank translated in all six reading frames by using the Pearson and Lipman search method (44). Amino acid sequence similarity to two proteins was found: the *dnaX* gene products of *E. coli* (12) and *Bacillus subtilis* (56). Comparison of the δ' amino acid sequence individually to other DNA replication proteins revealed similarity to the 40-kDa subunit of the A1 complex (replication factor C) (8) of HeLa cells and the gene 44 protein of bacteriophage T4 (54). All four proteins are involved in DNA replication as subunits of complexes that load processivity factors onto primed DNA. The δ' subunit contains the highest degree of similarity to the *dnaX* gene products of *E. coli* and *B. subtilis* (34 and 32%, respectively [Fig. 5]). This similarity includes a stretch of 10 identical amino acid residues. The 40-kDa A1 subunit is 25% similar to δ', and the gene 44 protein is 20% similar over the entire lengths of the proteins. Similarities between the 40-kDa A1 subunit and gene 44 protein (8) and between the *dnaX* genes of *E. coli* and *B. subtilis* (56) have been reported.

|           |  |     |
|-----------|--|-----|
| $\delta'$ | MRW---Y-----PWLRPD-FEKL                              | 14  |
| ec-dnax   | MSYQVLA-----RKWRPQTFADV                              | 18  |
| bs-dnax   | VSYQALY-----RVFRPQRFADV                              | 18  |
| 40-kDa A1 | MEVEAVCGGAGEVEAQSDPAPAFSKAPGSAGHYELPWVEKYRFVKLNEI    | 50  |
| gene44    | M-----ITVNEKEHILE-----QKYRPESTIDEK                   | 23  |
| $\delta'$ | VASYQA-----GRGHALLIQALPGMDDALIYALSRVLLCQQQPQ         | 54  |
| ec-dnax   | VGOEHLVLTALANGLSLGRHHAYLFSGTRGVKETSARLLARGINCETGI    | 68  |
| bs-dnax   | VGOEHITKTLQNALLOKRFSHAYLFSGPRGTGKTSAAKIFAKAVNCEHAP   | 68  |
| 40-kDa A1 | VGNEDTMSRLEVFAREGNVFNIIAGP-PGTGKTSILCLARAL-----      | 93  |
| gene 44   | ILPAFDKETFKSITSKGKIPHIIHSPSPGTGKTT----YAKAL-----     | 63  |
| $\delta'$ | GHKSCGHRGRCQIMQAGTHPDYYTLAPERGENTLQVDAVREVTEKLNHA    | 104 |
| ec-dnax   | TATPCGVCDNCREIEQGRFVDLIEIDAASRT---KVEDTRDLLDNVQYAP   | 115 |
| bs-dnax   | VDEPCNECAACKGITNGSISDVIEIDAASNN---GVDEIRDIRDKVKFAP   | 115 |
| 40-kDa A1 | -----LGALKD-----AMLEINASNDRGIDVVRNKIKMFAQQKVTL       | 129 |
| gene44    | -----CHDVNA-----TMMFYNGSDCK-IDFVRGPLTNFASAASF-       | 97  |
| $\delta'$ | RLGGARVVVVDAAALIT-DAANALLKTLLEPPAETWFFIATREPERLLA    | 153 |
| ec-dnax   | ARGRFVYLIIDEVHMLR-RHSFALLKTLLEPPFHVKFLATTDPOKLPV     | 164 |
| bs-dnax   | SAVTYKVYIIDEVHMLR-IGAFHALLKTLLEPPFHCIFILATTEPHIPL    | 164 |
| 40-kDa A1 | PKGRHKIILDEADSMF-DGAQALRRIMEIYSKTRFALACNASDKIEK      | 178 |
| gene44    | -DGRQKVIIVIDEFDRSGLAESQRHRSFMEAYSSNCSIITANNIDGIEK    | 146 |
| $\delta'$ | TLRSRCLHYLAPPPEQY-----AVFWLSR-----EVTMSQ-DALLAA      | 190 |
| ec-dnax   | TILSRQLQPHLKALDVEQ-----IRHQLEHILNEEHIAHEP-RALQLL     | 206 |
| bs-dnax   | TLISRCQRFDFKRITSAQ-----IVGRMKNIVDAEQVVEE-GSLEII      | 206 |
| 40-kDa A1 | PIQSRCAVLRVYTKLTDA-----QILTRIAMNVIEKERVYPTD-DGLEAI   | 220 |
| gene44    | PLQSRKRVITFGQPTDEDKIEMMKQIRRLTEICKHEGIAIADMKVVAAL    | 196 |
| $\delta'$ | LELSAGSPGAALALFQ-----GDNWQARETI-----CQALAYSU         | 224 |
| ec-dnax   | ABAAEGSLRDALSETDQAIASGDGQVSTQAVSAMLGTLDDDDQALSVEAM   | 256 |
| bs-dnax   | ASAADGMRDALSLLDQAI SFSGIILKVEDALLITGAVSOLYIGKLAKSL   | 256 |
| 40-kDa A1 | IFTAQGDMRQALNNLQSTFS-GFGFINSENVFKVCDEPHLLVKEMIQHC    | 269 |
| gene44    | VKKNFPDFRKTIGELDS-YS-SKGVLDAGILSLVTNDRGAI---DDVLES   | 241 |
| $\delta'$ | PSGDWYSLAALNHEQAPAR-----LEWLATLMDALKRHHGAAQVTHVD     | 269 |
| ec-dnax   | VEANGERVMA LINEAAARGIEWEALLVEMLGILLERIAMVQLSPAALGN-D | 305 |
| bs-dnax   | HDKNVSDALETLELLQCGKDPAKLIEDMI FYFRDMLLYKTAPGLEGVLE   | 306 |
| 40-kDa A1 | VNANIDEAYKILAHLWHLGYS-----PEDI-----                  | 294 |
| gene44    | LKNKDVQLRALAPKYAADYSW-----FVGKI-----                 | 268 |
| $\delta'$ | VPGL---VAELANHSRRLQALIGDVCHIEQIMSVTGINRELLITDIL      | 316 |
| ec-dnax   | MAIIELRMRELARTIPPTDIQLYQTLIGKELPYAP---DPRMGVEMTI     | 353 |
| bs-dnax   | KVKVDETFRELSEQIPAAALYEMEDIINKSHQEMKWTN--HPRIFFEVAV   | 354 |
| 40-kDa A1 | ---IGNIF-RVCKTFQMAEYLKLEFIKEIGYTEMKIAEGVNSLLOMAGLI   | 340 |
| gene44    | ---AEEIYSRVTPQSIIRMY-----EIVGENNOYHGIAANTEHLAYLF     | 309 |
| $\delta'$ | LRI--EHYLQGVVLEVEHL-----                             | 334 |
| ec-dnax   | LR---ALAFHFRMPLPEPEVPROSFAPVAPTAV-MTPTQVPPQPSAPQQ    | 399 |
| bs-dnax   | VKI-CQTSHQSAADLPEVDMIMKKIQOLEQEVERLKTGIAKAAEAPKKE    | 403 |
| 40-kDa A1 | ARL-CQRTMAPVAS-----                                  | 353 |
| gene44    | IQLACEMQW---K-----                                   | 319 |
| $\delta'$ | -----  | 334 |
| ec-dnax   | APTVPPLPETTSQVLAARQQLQRVQGATKAKKE                    | 431 |
| bs-dnax   | APRVPRGGKSN---YKAPVGRV-----                          | 422 |
| 40-kDa A1 | -----  | 353 |
| gene44    | -----  | 319 |

FIG. 5. Alignment of  $\delta'$  amino acid sequence to four replication proteins. Abbreviations: ec-dnax, *E. coli dnaX* gene product; bs-dnax, *B. subtilis dnaX* gene product; 40-kDa A1, 40-kDa subunit of the A1 replication complex of HeLa cells; gene 44, gene product of bacteriophage T4. Amino acids are represented in the single-letter code. Numbers to the right indicate the amino acid residue numbers of the respective proteins. Dashes indicate breaks in amino acid sequence required to align the sequences. Similar and identical residues are indicated by shading. Similar residues are E and D; I, L, and V; S and T; R and K; Q and N; and G and A.

One region of  $\delta'$  that lacks similarity to other proteins occurs between residues 30 and 38, which in the other four proteins contains a consensus ATP-binding site (19, 38, 63).

**Construction of a plasmid to express the  $\delta'$  subunit.** To verify that the gene tentatively identified as the gene encoding  $\delta'$  directs expression of a protein and to verify that this protein is the size of authentic  $\delta'$  contained in holoenzyme, we constructed a plasmid to overexpress this gene. The expression vector used was pBBMD11, a *tac* promoter-based *dnaX* expression plasmid. The *dnaX* gene was replaced with a double-stranded oligonucleotide containing a strong ribosome-binding site, an ATG initiation codon separated from the ribosome-binding site by 9 A/T bp, and the next nine codons of the gene encoding  $\delta'$  to create pMAF199. Seven of these codons were changed to high-usage codons synonymous with wild-type codons to elevate expression of the gene. The remainder of the gene was inserted in frame with the beginning of the gene contained in the oligonucleotide to generate pMAF200, the  $\delta'$  expression plasmid (Fig. 6).

**Overexpression of the  $\delta'$  subunit.** Plasmids pMAF199 and pMAF200 were transformed into HB101 to create MAF199 and MAF200, respectively. HB101, MAF199, and MAF200 were induced with IPTG to test for expression of the putative gene encoding  $\delta'$  by MAF200. HB101 and MAF199 were negative control strains used to demonstrate that  $\delta'$  is not overexpressed in plasmidless cells or in cells containing only the parent *tac* expression plasmid.

Initial attempts to visualize an IPTG-dependent protein the size of  $\delta'$  by SDS-PAGE of total soluble protein from cell lysates revealed an abundant protein in *E. coli* that comigrated with  $\delta'$  in all lanes of the gel (data not shown). To determine whether this protein obscured  $\delta'$ , we fractionated the cell lysate by precipitating the protein with ammonium sulfate to 40% saturation. Protein not soluble in ammonium sulfate was dissolved and subjected to electrophoresis; the abundant protein that comigrated with  $\delta'$  did not precipitate. Elimination of this protein allowed visualization of two proteins produced by MAF200 in the presence of IPTG (Fig. 7A, lane 5). One protein comigrated with the protein identified as authentic  $\delta'$ , and the second migrated slightly more slowly. Western blot (immunoblot) analysis with a polyclonal antibody raised against holoenzyme revealed the reactivity of both proteins (Fig. 7B, lane 5). The presence of two  $\delta'$  bands in purified holoenzyme (Fig. 7B, lane 6), argues against the possibility that the production of two proteins represents an artifact of overexpression. Moreover, the larger protein is not the  $\delta$  subunit, because this subunit does not react with the anti-holoenzyme polyclonal antibody (Fig. 7B, lane 7). On the basis of densitometry (Fig. 7C) of lanes 4 and 5 of Fig. 7A, we calculate that the two  $\delta'$  proteins comprise about 2% of the protein precipitated with ammonium sulfate.

## DISCUSSION

The structural gene encoding the  $\delta'$  subunit of DNA Pol III holoenzyme was identified and isolated by using a reverse genetic approach in which degenerate oligonucleotide primers were derived from the peptide sequences of tryptic fragments of  $\delta'$  and were used in PCRs to amplify a fragment of the gene from total chromosomal DNA. The fragment was used in hybridizations that identified the map location of the gene and the phage  $\lambda$  clone of the Kohara collection that contained the gene.

A fragment of DNA predicted to contain the gene was

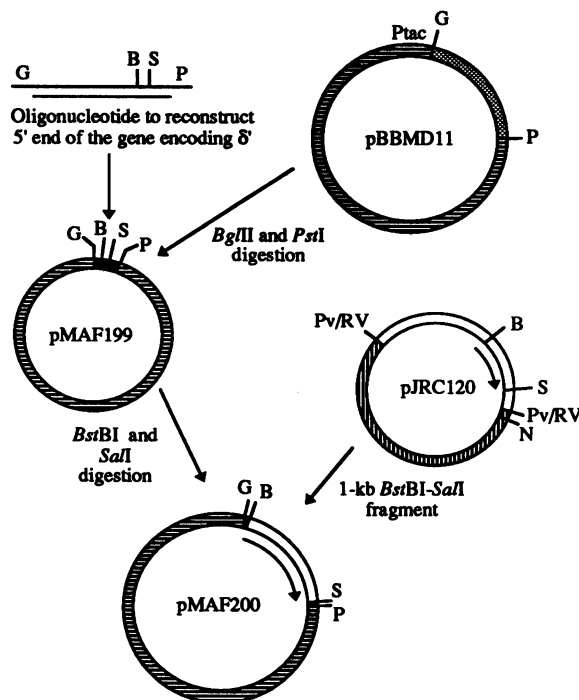


FIG. 6. Construction of a plasmid to express the  $\delta'$  subunit. pBBMD11 is a *dnaX* overexpression plasmid (*dnaX* is contained in the stippled region). The entire *dnaX* was removed from pBBMD11 by digestion with *Bgl*II and *Pst*I and replaced with a double-strand oligonucleotide to create pMAF199. The oligonucleotide contained a consensus ribosome-binding site and an ATG initiation codon separated by 9 A/T base pairs. An adenosine residue is 3 bases 5' to the ATG, and after the ATG are the next nine codons of the gene encoding  $\delta'$  followed by *Bst*BI and *Sal*I restriction sites. The coding strand of the double-strand oligonucleotide is 5'-GATCTAGGAG GTAATAAATAATGCGCTGGTACCCGTGGCTGCGCCCGGACTTCGAATAGTTCGACCTGCA-3'. pMAF199 was digested with *Bst*BI and *Sal*I. A 1-kb *Bst*BI-*Sal*I fragment from pJRC120, containing all except the first nine codons of the gene encoding  $\delta'$ , was inserted into pMAF199 to create pMAF200, the  $\delta'$  expression plasmid. Restriction site abbreviations: G, *Bgl*II; B, *Bst*BI; RV, *Eco*RV; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I. The curved arrow indicates the gene encoding  $\delta'$ .

cloned, and 1,401 bp of this DNA was sequenced. Within the sequence, four regions of DNA encoding the four tryptic peptides of  $\delta'$  were identified. The four peptide-encoding regions were found in one reading frame, and the spacing between them corresponded to the sizes of the PCR products generated with primers based on three of the four peptides. Further analysis of the sequence revealed a single pair of initiation and termination codons that flanked the four regions of tryptic fragment-encoding DNA and defined an open reading frame of 1,002 bp. The predicted size of protein encoded by this open reading frame was 36,936 Da, which is in reasonable agreement with the estimated size of  $\delta'$  of 32,000 Da. As a final test of the authenticity of the isolated gene, we constructed a plasmid to express this gene to determine the size of protein expressed from it. The gene directed expression of two proteins, one of which comigrates with the protein identified as the  $\delta'$  subunit of purified holoenzyme. Both proteins react with antibody directed against holoenzyme and are also detected in purified holoenzyme, indicating that both are products of the gene encoding



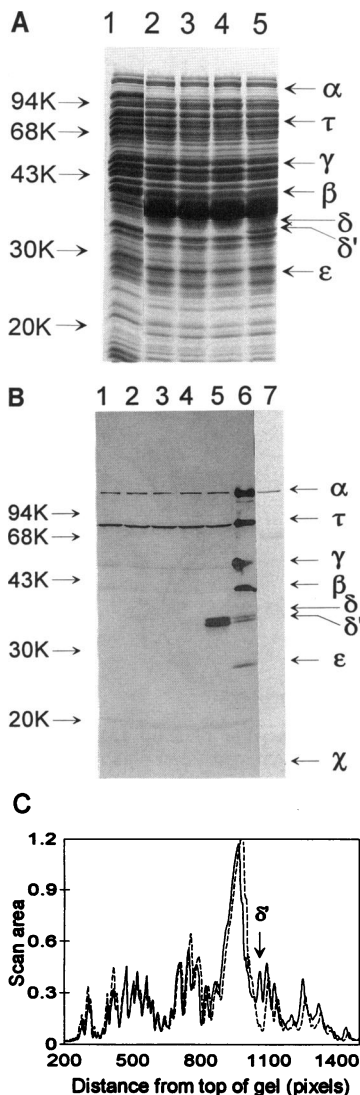


FIG. 7. Overexpression of the  $\delta'$  subunit. Cells were grown, induced with IPTG, and lysed, and lysates were fractionated with ammonium sulfate as described in Materials and Methods. Proteins insoluble in ammonium sulfate were resolved by SDS-PAGE on two identical gels. (A) One gel was stained with Coomassie brilliant blue. (B) Proteins in the second gel were blotted onto nitrocellulose and reacted with rabbit anti-holoenzyme IgG. Lanes: 1, HB101, IPTG induced; 2, MAF199; 3, MAF199, IPTG induced; 4, MAF200; 5, MAF200, IPTG induced; 6, holoenzyme; 7, ammonium sulfate precipitation of lysate of cells that overexpress the  $\delta$  subunit. In (A) and (B), the positions of molecular mass markers (kilodaltons) and holoenzyme subunits are indicated. (C) Densitometric scans of lanes 6 (dotted line) and 7 (solid line) of panel A. The origin of the gel is to the left, and the location of  $\delta'$  is indicated.

$\delta'$  and that one is not an artifact of overexpression of the subunit. This result is consistent with those of O'Donnell and Studwell (40), who isolated subunits from purified  $\gamma$  complex and found that  $\delta'$  migrated as a doublet on SDS-polyacrylamide gels. Taken together, these results strongly support the conclusion that we have isolated the gene encoding the  $\delta'$  subunit of the DNA Pol III holoenzyme. This gene has also been isolated by the O'Donnell laboratory (11a). Following the suggestion of Ken Mariani (Sloan

Kettering), we and the O'Donnell laboratory have agreed to name the gene *holB*.

The basis for expression of the two  $\delta'$  proteins from one gene is not known. We found no DNA sequence in *holB* with characteristics typical of translational frameshifting, as occurs in *dnaX* to produce  $\gamma$  and  $\tau$  (3, 13, 62). Examination of the DNA sequence of *holB* identified a potential ribosome-binding site and a consensus promoter element 7 and 144 bp from the predicted ATG initiation codon, respectively. The degrees of similarity of the predicted ribosome-binding site and promoter to their consensus sequences suggest that the strength of these sites is greater than would be expected of a gene whose product probably exists at a copy number of 10 to 20 per cell. Analysis of the codon usage of the gene indicated that the percentage of rare codons in *holB* is 1.5 times the overall percentage of rare codons in 25 nonregulatory genes and 3.7 times the percentage for 10 very highly expressed ribosome protein genes (25). It has been proposed that a preponderance of codons whose cognate tRNAs are underrepresented in the cell contributes to a low level of gene expression (25), and it is possible that rare codon usage contributes to low expression of *holB*.

Flanking *holB* are segments of potential open reading frames of 170 and 209 bp. These open reading frames are likely to be unidentified genes, as suggested by the position of their predicted ends. The predicted termination codon of the open reading frame upstream of *holB* overlaps the proposed initiation codon of *holB*, and the termination codon of *holB* is 10 bp from the putative initiation codon of the open reading frame downstream of *holB*. This relative positioning of initiation and termination codons is characteristic of genes that are translationally coupled (15) and that exist in an operon. The low percentage of rare codons compared with that of the two interrupted reading frames also supports the hypothesis that these open reading frames are genes.

The protein sequence of the  $\delta'$  subunit was used in a data base search to identify proteins with similar primary structure. We found that  $\delta'$  is similar to four other DNA replication proteins which, like  $\delta'$ , are subunits of complexes that load their cognate polymerase clamps onto primed DNA templates. One of these four proteins is the  $\gamma$  subunit, which is part of the same complex in which  $\delta'$  is found. One region of similarity shared by the four proteins but not by  $\delta'$  is an ATP-binding site. The *E. coli dnaX* gene products  $\tau$  and  $\gamma$  (26, 43, 61), the T4 gene 44 gene product (20, 48), and the 40-kDa subunit of the A1 complex (9) have been shown experimentally to bind or hydrolyze ATP. It is likely that the consensus ATP-binding site in these proteins is the basis for their ATP-binding or ATP-hydrolyzing activity and that  $\delta'$  does not perform a similar function.

The role of  $\delta'$  in holoenzyme is only partially elucidated. Although rapid and processive polymerases can be reconstituted without  $\delta'$ , the subunit has stimulatory effects on polymerase reconstituted with  $\alpha$ ,  $\epsilon$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  and on the ATPase activity of subassemblies of the  $\gamma$  complex (43). Having successfully isolated and overexpressed *holB*, we are now in a position to purify large quantities of  $\delta'$  and examine the contribution of  $\delta'$  to the structure and function of DNA Pol III holoenzyme.

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