Identification, Isolation, and Overexpression of the Gene Encoding the ψ Subunit of DNA Polymerase III Holoenzyme

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The gene encoding the ψ subunit of DNA polymerase III holoenzyme, *holD*, was identified and isolated by an approach in which peptide sequence data were used to obtain a DNA hybridization probe. The gene, which maps to 99.3 centisomes, was sequenced and found to be identical to a previously uncharacterized open reading frame that overlaps the 5' end of *rimI* by 29 bases, contains 411 bp, and is predicted to encode a protein of 15,174 Da. When expressed in a plasmid that also expressed *holC*, *holD* directed expression of the ψ subunit to about 3% of total soluble protein.

DNA polymerase III holoenzyme (referred to here as holoenzyme) is the 10-subunit replicative enzyme of *Escherichia coli*. Several biochemical properties distinguish this polymerase from the nonreplicative polymerases of *E. coli*. These include its requirement for single-stranded DNA-binding protein (15), resistance to physiological concentrations of salt (2, 8, 17) and spermidine (11), and very high processivity (52). In addition, holoenzyme is thought to adopt an asymmetric, dimeric polymerase conformation that allows coordinated leading- and lagging-strand synthesis (19, 21, 33, 51), and to interact with other proteins of the replisome (25), e.g., the primosome (53, 56), allowing additional communication among the various replication enzymes.

Holoenzyme can be divided into three functional components. The core polymerase, polymerase III (31), contains three subunits: α (*dnaE* [50]), the catalytic subunit (28, 29); ϵ (*dnaQ* [9, 44]), the 3'→5' proofreading subunit (9); and θ (*holE* [4, 46]), which has no known role. These three subunits are also isolable as part of a four-subunit complex, polymerase III' (30), which contains the τ subunit (*dnaX* [27, 35]). Polymerase III is distinguished from holoenzyme by its sensitivity to single-stranded DNA-binding protein and spermidine (11) and by its very low processivity (11, 12). Processivity is conferred on the polymerase by the β subunit (*dnaN* [3, 8]), which assembles as a torus-shaped dimer around primed template DNA, forming a sliding clamp that fastens the polymerase to the template (23, 47). The β subunit is loaded onto a primed template in a reaction requiring ATP hydrolysis and catalyzed by the γ complex (23, 47), a DNA-dependent ATPase containing γ (*dnaX* [13, 27]), δ (*holA* [5, 10]), δ' (*holB* [5, 10]), χ (*holC* [6, 54]), and ψ .

In *in vitro* replication reactions, the indispensable activity of the γ complex can be provided by the two-subunit complexes $\gamma\delta$, $\tau\delta$, and $\tau\delta'$ (36). The contribution of the remaining two γ complex subunits, χ and ψ , is more subtle. Together, χ and ψ stabilize reconstituted polymerase ($\alpha\epsilon\beta\gamma\delta$) against higher concentrations of salt (36) and moderately stimulate the DNA-dependent ATPase activity of reconstituted γ complex ($\gamma\delta\delta'$ [37]). To understand fully the

contribution of χ and ψ to holoenzyme requires purification of large quantities of each subunit. In this report, we present a vital step toward this objective: the identification, isolation, and overexpression of the gene encoding ψ .

MATERIALS AND METHODS

Chemicals. Tris-HCl, polyvinylpyrrolidone, dextran sulfate, bovine serum albumin, and Ficoll were purchased from Sigma. Sodium dodecyl sulfate (SDS), acrylamide, *N,N'*-methylenebisacrylamide, ammonium persulfate, and Coomassie brilliant blue R-250 were purchased from Bio-Rad. Urea was purchased from Fisher. SeaKem LE agarose was purchased from FMC BioProducts.

Oligonucleotides. Oligonucleotides were synthesized at the University of Colorado Cancer Center Macromolecular Synthesis Core Facility and purified as described before (5). Oligonucleotide sequences are shown in Fig. 1.

Bacterial strains, plasmids, phages, and media. XL1Blue [*F'* *proAB lacI^qΔM15 Tn10 (Tet^r)/recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lac*; Stratagene] was used for routine plasmid transformation and 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 fermenter (probably bacteriophage T1), was the source of holoenzyme. MAF102, a *lexA3 uvrD* (49) derivative of the wild-type strain MG1655 (18), was the source of *E. coli* chromosomal DNA.

The primary cloning vector was pBlueScript II SK+ (Stratagene). All expression plasmids (pMAF51, pMAF300, pMAF310, and pRT581) were derivatives of pBBMD11, the original laboratory *tac* promoter-based expression plasmid (14, 32, 48). pRT581 expresses the 51-kDa subunit of human immunodeficiency virus reverse transcriptase (48). The χ subunit expression plasmid pMAF51 (6) was the positive-control plasmid in the overexpression experiment.

L broth and agar (34) were used for routine bacterial growth. F medium (1.4% yeast extract, 0.8% peptone, 1% glucose, 1.2% potassium phosphate [pH 7.5]) was used in the *holD* expression experiment. When required, ampicillin, streptomycin, and tetracycline were used at 150, 25, and 10 μ g/ml, respectively.

Enzymes. Restriction enzymes and T4 DNA ligase were

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PCR to obtain a DNA probe

CTCGAATTCARCARYTNGGNATHAC	#1.1
CTCGAATTCGCNATGYTNCCNCARGG	#2.1
CTGCATCTAGACCYTGNGGNARCATNGC	#2.2
CTCGAATTCGARGGNGCNCARGTNGC	#3.1
CTGCATCTAGAGCNACYTGNGCNCCYTC	#3.2

PCR to clone the entire gene

CTGCATCTAGACGCCCTGGTTGCTGGCAAACG
CTCGAATTCCTGGCGCGGTATCGACGAATT

Construction of *holD* overexpression plasmid

CCATAGATCTGATATCAGGAGGTAATAAATA-
ATGACTTCCCGTCGCGACTGGCAG
GGACAGTCGACGGTAAGCCGGCGGTAAATCAGTCG

FIG. 1. Oligonucleotides. Abbreviations: H, A, C, or T; R, A or G; Y, C or T; N, A, C, G, or T.

purchased from Promega or New England Biolabs. Vent polymerase was purchased from New England Biolabs. Calf intestinal alkaline phosphatase was purchased from Boehringer Mannheim Biochemicals. Commercial proteins were used according to instructions provided by the manufacturers. Holoenzyme was purified as described before (7).

DNA purification. Plasmid DNA was isolated by the alkaline-SDS lysis procedure (1a), and purified by two CsCl-ethidium bromide equilibrium density gradient centrifugations (42). Alternatively, plasmid DNA was purified with the Promega Magic Mini Prep or Magic Maxi Prep kit. Chromosomal DNA was extracted and purified by two 55% (wt/vol) CsCl equilibrium density gradient centrifugations as described before (7).

DNA restriction fragments were separated by agarose gel electrophoresis, excised from the gel without UV irradiation of the DNA (7), and purified with the GeneClean DNA purification kit from Bio 101.

Agarose gel electrophoresis. Horizontal agarose gel electrophoresis was performed as described before (42). To separate chromosomal DNA restriction fragments, gels were run at 4°C.

Preparation of radiolabeled DNA. Restriction fragments were purified by agarose gel electrophoresis and radiolabeled with the Random Primed DNA Labeling Kit from Boehringer Mannheim Biochemicals. Each labeling reaction mix included 50 ng of heat-denatured DNA, 1 U of Klenow enzyme, and 50 μ Ci of [α - 32 P]dATP (3,000 Ci/mmol, 10 mCi/ml) and was incubated at 37°C for 30 min. Labeled DNA was heat denatured before use in hybridization experiments.

Southern hybridization. Two micrograms of MAF102 chromosomal DNA was restriction enzyme digested, size fractionated on a 0.7% agarose gel, denatured and neutralized as described before (7), and transferred to a GeneScreen nylon membrane (New England Nuclear) for 18 h in 1.5 M NaCl-0.15 M sodium citrate $\cdot$ 2H₂O with a conventional DNA transfer assembly (45). The membrane was irradiated with 1.6 kJ of UV light per m² from a germicidal lamp, prehybridized, hybridized, and washed according to the instructions provided with the membrane, and 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.

A blot of the miniset of Kohara bacteriophage clones was

obtained from Takara Shuzo, Inc. Hybridization of radiolabeled probe DNA to this blot was performed according to the procedures provided with the blot.

DNA sequencing. Dideoxy chain termination DNA sequencing (43) of polymerase chain reaction (PCR) products cloned into pBlueScript II SK+ was performed with the Sequenase version 2.0 DNA sequencing kit from United States Biochemical Corp. DNA was labeled with [35 S]dATP (12.5 mCi/ml). Sequencing reactions were subjected to electrophoresis on a 6% polyacrylamide-8 M urea gel as described before (42). Gels were dried and autoradiographed for 24 to 48 h with Kodak X-Omat AR X-ray film. Dideoxy chain termination sequencing of two independent isolates of the gene encoding ψ was performed by Lark Sequencing Technologies, Inc. (Houston, Tex.). The entire gene was sequenced in both directions.

PCR. PCR was performed in a Perkin Elmer Cetus model 480 PCR machine. Reactions designed to amplify fragments of the gene encoding ψ were performed with the Perkin Elmer GeneAmp PCR reagent kit that included AmpliTaq DNA polymerase. Each 100- μ l reaction mix contained 1 ng of *E. coli* chromosomal DNA and two oligonucleotide primers, each at 1 μ M. Reaction mixes were incubated without polymerase or deoxynucleoside triphosphates (dNTPs) at 94°C for 7 min to denature template DNA and shifted to 85°C for 4 min to allow addition of polymerase and dNTPs. Reaction mixes were then cycled 35 times through a 1-min incubation at 94°C, a 5-min ramp from 50 to 65°C, and a rapid return to 94°C.

PCRs used to amplify the entire gene encoding ψ were performed with Vent polymerase and the reaction buffer supplied by New England Biolabs. Each 60- μ l reaction mix contained 100 ng of template DNA, two oligonucleotide primers, each at 1 μ M, and bovine serum albumin at 100 μ g/ml. Tubes containing template DNA and primers were incubated at 94°C for 7 min to denature the template and shifted to 85°C. One unit of Vent polymerase and the four dNTPs, each at a final concentration of 0.2 mM, were added to each reaction mixture. The reaction mixes were cycled 25 times through a 1-min incubation at 94°C, a 2-min incubation at 50°C, a 3-min incubation at 72°C, and a rapid return to 94°C. All PCR products were purified by agarose gel electrophoresis.

SDS-polyacrylamide gel electrophoresis. Holoenzyme subunits were separated by electrophoresis (26) in an SDS-7.5 to 17.5% polyacrylamide gel to obtain purified ψ subunit. Protein from total cell lysates was separated on an SDS-12.5 to 20% polyacrylamide gel to analyze cells for overexpression of the ψ subunit. Gels were run in a Hoefer vertical gel electrophoresis apparatus for 16 h at 7 mA. Protein was visualized by staining with a 0.25% solution of Coomassie brilliant blue R-250 in 45% methanol and 10% acetic acid and destaining in a solution of 7.5% methanol and 10% acetic acid.

Overexpression of the ψ subunit. Overnight cultures were diluted 1:100 into 25 ml of fresh medium containing ampicillin. The 25-ml cultures were incubated at 37°C in a shaking waterbath. At an A_{600} of 0.5, 10 ml of each culture was induced by addition of IPTG (isopropylthiogalactopyranoside) to a final concentration of 1.0 mM, and growth of induced and noninduced cultures was continued for 5 h. Cells were pelleted by centrifugation, suspended in lysis buffer (100 mM Tris-HCl [pH 7.5], 100 mM NaCl, 5% SDS, 100 mM β -mercaptoethanol, 15% glycerol, 0.02% bromophenol blue), and boiled for 10 min. Lysed cells were centrifuged for 20 min to remove debris and boiled for 5 min, and

FIG. 2. Sequences of internal tryptic peptides of ψ . Subunits of holoenzyme were resolved by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The ψ subunit was excised from the membrane and digested on the membrane with trypsin, the tryptic fragments were separated by reversed-phase HPLC, and the amino-terminal sequences of three peptides were determined. Oligonucleotide primers, represented by arrows, were designed on the basis of the peptide sequences immediately above or below the arrows. Arrowheads indicate the 3' ends of the primers.

material corresponding to 0.2 OD₆₀₀ units of cells was loaded onto an SDS-12.5 to 20% polyacrylamide gel.

DNA and protein sequence analysis. GenBank DNA sequences were translated in all six reading frames with the TFasta program and compared with the predicted sequence of ψ by the method of Pearson and Lipman (38).

RESULTS

Cloning of the gene that encodes ψ required that we first obtain a DNA probe for the gene. To achieve this, we designed degenerate oligonucleotides based on peptide sequences of ψ and used these oligonucleotides in PCR to amplify a DNA fragment representing a portion of the gene. This fragment was used in hybridization experiments to map the gene, and the DNA sequence of the fragment was compared with sequences in GenBank to allow identification of the gene.

Peptide sequences of ψ . The ψ subunit was separated from purified holoenzyme by SDS-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and digested with trypsin. Tryptic fragments were separated by reversed-phase high-performance liquid chromatography (HPLC) (data not shown), and the amino-terminal sequences of three well-resolved peptides were determined, (1) (Fig. 2). Amino-terminal sequence analysis of the full-length protein (data not shown) revealed overlap between peptide 1 and the amino terminus of the full-length protein, identifying the first

glutamine residue in peptide 1 as the seventh or eighth residue of the full-length protein. Oligonucleotides were designed from reverse translation of the three peptide sequences (Fig. 2). Two oligonucleotides, made to prime DNA synthesis in opposite directions, were derived from each of peptides 2 and 3. Because peptide 1 was close to the amino terminus, only one oligonucleotide, designed to prime synthesis extending into the gene, was based on peptide 1.

Amplification of fragments of the gene encoding ψ . PCR was performed with all pairs of oligonucleotides, and three products were obtained: a 300-bp fragment from oligonucleotides 1.1 and 3.2, a 250-bp product from oligonucleotides 1.1 and 2.2, and an 80-bp fragment from oligonucleotides 2.1 and 3.2 (data not shown). That the sum of the sizes of the two smaller products was approximately equal to the size of the largest fragment indicated that all three fragments originated from the same region of DNA and allowed prediction of the relative order of three of the ψ peptides: peptide 1, peptide 2, and peptide 3.

To further analyze the PCR fragments, the 250- and 300-bp products were radiolabeled and hybridized to the Kohara miniset of chromosomal DNA clones. Both products hybridized to clones 672 and 673 (not shown), which both contain the DNA from kb coordinates 4634 to 4640 (about 99.2 centisomes) of the chromosome (22, 40). That both PCR products hybridized to the same two chromosomal clones further indicated that these products originated from the same region of DNA.

Sequencing of the 300-bp fragment confirmed that the fragment represented an authentic portion of the gene encoding ψ (Fig. 3). The sequence following primer 3.2 encoded nine consecutive amino acids located immediately after those residues used to design the primer. The sequence following primer 1 encoded the final amino acid of peptide 1, which was not used to design the primer (Fig. 3B). In addition, the 300-bp fragment contained DNA that encoded all of peptide 2 (with the exception of the two ambiguous residues of this peptide). In total, the 300-bp fragment contained sequence encoding 19 experimentally derived amino acid residues that were not used in design of the PCR primers used to amplify the fragment.

Mapping the gene encoding ψ . Chromosomal DNA, digested with a battery of restriction enzymes, was analyzed by Southern hybridization with the 300-bp fragment as a gene-specific probe. A single restriction fragment was identified for each digestion, indicating a single locus complementary to the probe. Data from the Southern blot (not shown) were used to construct a restriction map of the region of the chromosome complementary to the probe (Fig. 4). This restriction map aligned with the 99-min region of the *E. coli* chromosomal restriction map (22), in agreement with the map position of the two phage clones, 672 and 673, that

FIG. 3. DNA sequence of a PCR product representing part of the gene encoding ψ . (A) Oligonucleotide primers 1.1 and 3.2, the sequences of which were based on the underlined residues of peptides 1 and 3, respectively, primed synthesis of a 300-bp PCR product. (B) The fragment was sequenced and the DNA was translated. The double-underlined residues matched exactly to the experimentally derived residues in the regions of the two peptides not used in primer design.

FIG. 4. Restriction map of the region of the chromosome encoding ψ . Chromosomal DNA was digested with *Bam*HI (B), *Bgl*II (G), *Eco*RI (R), *Eco*RV (V), *Hind*III (H), and *Pst*I (P) used singly and in all two-enzyme combinations. The DNA was size-fractionated by agarose gel electrophoresis, transferred to a nylon membrane, hybridized to the 300-bp radiolabeled PCR product, and autoradiographed. The restriction map of the chromosomal region containing the gene encoding ψ was constructed from the autoradiographic data. The thick bar indicates the smallest restriction fragment that hybridized to the 300-bp probe.

hybridized to the 300-bp probe. The gene was mapped more precisely to 99.3 centisomes by comparing the restriction map shown in Fig. 4 with the most recent map of the *E. coli* chromosome, which correlates the physical and genetic maps (40, 41).

Cloning and sequencing the gene encoding ψ . The sequence of the 300-bp fragment was compared with the DNA sequences in GenBank and found to be identical to an open reading frame upstream of *rimI*, which encodes an enzyme that acetylates the amino-terminal alanine of the ribosomal protein S18 (54). (*rimI* and the open reading frame are reported to be on a 2.1-kb *Pst*I chromosomal fragment [40, 55], whereas we map this same DNA to a 15-kb *Pst*I chromosomal DNA fragment immediately adjacent to a 2-kb chromosomal fragment [40]. The reason for this discrepancy is not known.) Potential initiation and termination codons were identified for this open reading frame, and two oligonucleotide primers complementary to DNA flanking either side of the open reading frame were synthesized. The open reading frame was amplified independently from Kohara phage 673 in two separate PCRs. Each PCR product was digested with *Xba*I and *Eco*RI and ligated into pBlueScript II SK+. The sequences of both PCR products (Fig. 5) were found to be identical to each other and to the published

sequence of the DNA upstream of *rimI* (55). Within this sequence were three regions of DNA that encoded all three of the experimentally determined peptide sequences in the same reading frame. Moreover, the spacing and order of the three peptide-encoding regions of DNA (Fig. 5) were consistent with the sizes of the initial PCR products obtained. From this information, we tentatively identified the open reading frame as the gene encoding ψ .

The putative gene encoding ψ contains 441 bp and is predicted to encode a 147-amino-acid protein of 15,174 Da, in agreement with the size of 16,000 Da estimated by SDS-polyacrylamide gel electrophoresis. The gene has an active promoter and a potential ribosome-binding site 10 bases upstream of the initiation codon (55) (Fig. 5). The 3' end of the open reading frame predicted to encode ψ overlaps the 5' end of *rimI* by 29 bp. Since there is no apparent promoter or ribosome-binding site dedicated to *rimI*, it is possible that *rimI* expression is partially dependent on expression of the gene encoding ψ . Analysis of the predicted amino acid sequence of ψ revealed no similarity to other proteins or to consensus functional protein motifs.

Expression of the gene encoding ψ . Final proof that we had correctly identified the gene encoding ψ required demonstration that the open reading frame directs expression of a protein that comigrates with ψ found in purified holoenzyme. The open reading frame predicted to encode ψ was amplified from Kohara phage 673 (22) by PCR with two oligonucleotide primers. One primer was complementary to the predicted 3' end of the open reading frame. The second primer was complementary to the predicted 5' end of the open reading frame except for two base changes that created preferred codons synonymous with the poorly used codons found in the wild-type sequence (24) and contained a consensus ribosome-binding site (39) separated from the ATG initiation codon by 9 bp. Both primers also contained restriction endonuclease recognition sites to aid in cloning of the PCR fragment. The modified open reading frame predicted to encode ψ was inserted downstream of the strong *tac* promoter of pRT581 (Fig. 6A) to create pMAF300. Sequence

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-35          -10          SD          begin  $\psi$  gene
TTGGCGCGGTATCGACGAATTTGCTATATTTGCGCCCTGACAACAGGAGCGATTGCTATGACATCCCGACGAGAC
M T S R R D
TGGCAGTTACAGCAACTGGGCATTACCCAGTGGTCGCTGCGTCGCCCTGGCGCGTTGCAGGGGGAGATTGCCATTGCG
W [Q L Q Q L G I T Q]1 W S L R R P G A L Q G E I A I A
ATCCCGGCACACGTCCGTCTGGTGATGGTGGCAAACGATCTTCCCGCCCTGACTGATCCTTTAGTGAGCGATGTTCTG
I P A H V R L V M V A N D L P A L T D P L V S D V L
CGCGCATTAACCGTCAGCCCCGACCAGGTGCTGCAACTGACGCCAGAAAAATCGCGATGCTGCCGAAGGCAGTCAC
R A L T V S P D Q V L Q L T P E K I [A M L P Q G S H
TGCAACAGTTGGCGGTTGGGTACTGACGAACCGCTATCACTGGAAGGCGCTCAGGTGGCATCACCGGCGCTCACCGAT
C N S]2 W R [L G T D E P L S L E G A Q V A S P A L T D]3
begin rimI
TTACGGGGCAAACCCAAACGGGCACGCGCCGCGTTATGGCAACAAATTTGCACATATGAACACGATTTCTTCCCTCGAAAC
L R A N P T A R A A L W Q Q I C T Y E H D F F P R N
end  $\psi$  gene
GACTGATTACCGGCGGCTTACCACATTGAACAACGCGCCACGCCTTTCCGTGGAGTGAAAAACGTTTGCCAGCAA
D
CCAGGGCGTCTAGA

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FIG. 5. DNA sequence of the PCR products containing the putative gene encoding ψ . Two PCR products, obtained in independent reactions, were sequenced and found to be identical to each other and to the previously uncharacterized open reading frame upstream of *rimI* (55). The promoter is double underlined, and the potential ribosome-binding site (SD) is single underlined. Initiation and termination codons are in boldface. The three experimentally derived peptide sequences are in boldface, bracketed and numbered.

FIG. 6. Construction of plasmids to overexpress the gene encoding ψ . (A) The PCR product containing the modified open reading frame encoding ψ was inserted into pBlueScript II SK+ to create pMAF290. The open reading frame was removed from pMAF290 and inserted into pRT581 in place of an open reading frame encoding the 51-kDa reverse transcriptase subunit of human immunodeficiency virus to create pMAF300. To create pMAF310, the gene encoding ψ was removed from pMAF290 and inserted downstream of *holC*. Restriction sites: G, *Bgl*II; V, *Eco*RV; S, *Sa*II; X, *Xba*I. SD indicates the consensus ribosome-binding site, Ptac indicates the *tac* promoter, and curved arrows indicate the direction of transcription of the indicated genes. (B) Insertion of the gene encoding ψ into pMAF51 interrupted *valS* after the ninth codon and created a 12-codon open reading frame that terminates between a consensus ribosome-binding site and the initiation codon of the gene encoding ψ . Initiation codons are in boldface, termination codons are underlined, and a consensus ribosome-binding site is overlined.

analysis of the open reading frame in pMAF300 indicated that no base changes had occurred during PCR. However, when tested in several strains and under several induction conditions, pMAF300 failed to promote overexpression of ψ (data not shown).

At least two possibilities might explain this result: (i) the ψ subunit is very labile, or (ii) translation is inefficient despite the modifications designed to enhance expression. To circumvent the latter problem, we moved the open reading frame into a plasmid that directs high-level expression of *holC*, the gene encoding the χ subunit of holoenzyme (6, 54), to create pMAF310 (Fig. 6A). The possibility of poor translation initiation was addressed with this plasmid by coupling translation of the putative open reading frame encoding ψ to expression of *holC* through translation of a 12-amino-acid

FIG. 7. Overexpression of the ψ subunit. Cultures were grown and induced with IPTG as described under Materials and Methods. Protein from equal cell masses was separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. Lane 1, MC1061, noninduced; lane 2, MC1061, induced; lane 3, MAF151 (contains pMAF51, the χ subunit expression plasmid), noninduced; lane 4, MAF151, induced; lane 5, MAF310 (contains the χ and ψ overexpression plasmid), noninduced; lane 6, MAF310, induced. Positions of molecular mass standards (in kilodaltons) are shown to the left of the gel, and the positions of purified holoenzyme subunits are indicated to the right of the gel.

peptide containing the amino-terminal nine residues of *valS* fused to three additional residues (Fig. 6B). (*valS* is immediately downstream of *holC*. Sequence analysis indicates that *valS* is probably translationally coupled to *holC* [6].) The critical component of the coupling was the placement of the termination codon for the 12-residue peptide immediately after a consensus ribosome-binding site and six bases before the initiation codon of the ψ open reading frame. Translational reinitiation of a downstream gene typically approaches 100% when the upstream gene terminates between a ribosome-binding site and an initiation codon (16).

Plasmid pMAF310 was introduced into MC1061 to create MAF310, and this strain was tested for the ability to produce χ and ψ after IPTG induction (Fig. 7). Also included in this induction experiment were MAF151, containing the *holC* overexpression plasmid pMAF51, and MC1061 with no plasmid. MC1061 overproduces neither subunit when induced with IPTG (Fig. 7, compare lanes 1 and 2). MAF151 overproduces the χ subunit when induced with IPTG (Fig. 7, compare lanes 3 and 4), as reported previously (6). MAF310 overproduces two proteins that comigrate with the χ subunit and the ψ subunit of purified holoenzyme when induced with IPTG (Fig. 7, compare lanes 5 and 6). Densitometric analysis of lanes 5 and 6 of Fig. 7 indicated that the proteins comigrating with χ and ψ represent about 7 and 3% of total soluble protein, respectively (not shown).

DISCUSSION

To identify the gene encoding ψ , we used a reverse genetic approach in which degenerate oligonucleotides were designed from the experimentally derived sequences of three tryptic peptides of ψ . The oligonucleotides were used to prime chromosomal DNA in PCRs that produced DNA fragments which were then used in hybridization experiments to map the gene. Comparison of the DNA sequence of one PCR product to DNA sequences in GenBank allowed tentative identification of a previously reported open reading

frame upstream of *rimI*, the gene encoding an enzyme that acetylates the amino-terminal alanine of the S18 ribosomal protein (55), as the gene encoding ψ . This preliminary identification was based on the open reading frame encoding all three experimentally derived peptides, including all residues of the peptides not used to design the PCR primers, and on the spacing of the peptide-encoding DNA, which was consistent with the sizes of the PCR products initially obtained. The open reading frame, when placed downstream of the strong, inducible *tac* promoter, directed expression of a protein that comigrated with authentic ψ from purified holoenzyme. From these data, we concluded that we had isolated the gene encoding ψ . This gene has also been isolated by others (54). As suggested by Ken Mariani (Sloan-Kettering), we and Xiao et al. (54) agree to call the gene *holD*.

holD is 441 bp long and is predicted to encode a protein of 147 amino acid residues and 15,174 Da. The gene lies 10 bp downstream of a consensus ribosome-binding site and presumably is expressed from the promoter, 30 bp upstream of the ribosome-binding site, that directs transcription of *rimI* (55). The 3' end of *holD* overlaps the 5' end of *rimI* by 29 bp. Given the apparent lack of a correctly positioned ribosome-binding site upstream of *rimI*, it is possible that translation of *rimI* mRNA is at least partially coupled to expression of ψ . However, the 29 bp between the *rimI* initiation codon and the *holD* termination codon is longer than the usual distance of not more than 10 bases (16) required for translational coupling. Moreover, a protein of the molecular mass of ψ was not detected in maxicells that expressed RimI from a plasmid containing both *holD* and *rimI* (55).

We observed similar results with a plasmid designed to overproduce ψ . Regardless of the strain or the induction conditions used, we were not able to detect expression of ψ by SDS-polyacrylamide gel electrophoresis. However, when coexpressed from a plasmid that also produces the χ subunit of holoenzyme, ψ was produced to about 3% of total soluble protein. Since χ and ψ are components of the γ complex (γ , δ , δ' , χ , and ψ) and have activity in vitro, it is possible that χ and ψ form a complex in which ψ becomes resistant to proteolytic degradation or which is more soluble than ψ when the subunit is expressed alone at high levels. Alternatively, the high level of ψ expression from cells containing the plasmid with *holC* and *holD* could be due to efficient translation initiation of *holD* through translational coupling of *holC* to a *valS* peptide and of the *valS* peptide to *holD*. However, Heck and Hatfield (20) have shown that *valS* is expressed from two promoters about 100 bp upstream of its initiation codon, i.e., within *holC*. Thus, *valS* expression is at least partially independent of *holC* expression.

The structural and functional contribution of ψ to holoenzyme is poorly understood. However, having successfully isolated and overexpressed *holD*, we are now in a position to purify large quantities of the protein and to determine the interactions of the ψ subunit with χ , with the remainder of the γ complex, and with the remaining complement of holoenzyme subunits.

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