JOURNAL OF BACTERIOLOGY, May 1996, p. 2804–2812 Vol. 178, No. 10
0021-9193/96/$04.00+0
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Escherichia coli Thymidylate Kinase: Molecular Cloning, Nucleotide Sequence, and Genetic Organization of the Corresponding *tmk* Locus

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Received 11 December 1995/Accepted 22 February 1996

Thymidylate kinase (dTMP kinase; EC 2.7.4.9) catalyzes the phosphorylation of dTMP to form dTDP in both de novo and salvage pathways of dTTP synthesis. The nucleotide sequence of the *tmk* gene encoding this essential *Escherichia coli* enzyme is the last one among all the *E. coli* nucleoside and nucleotide kinase genes which has not yet been reported. By subcloning the 24.0-min region where the *tmk* gene has been previously mapped from the λ phage 236 (E9G1) of the Kohara *E. coli* genomic library (Y. Kohara, K. Akiyama, and K. Isono, Cell 50:495–508, 1987), we precisely located *tmk* between *acpP* and *holB* genes. Here we report the nucleotide sequence of *tmk*, including the end portion of an upstream open reading frame (ORF 340) of unknown function that may be cotranscribed with the *pabC* gene. The *tmk* gene was located clockwise of and just upstream of the *holB* gene. Our sequencing data allowed the filling in of the unsequenced gap between the *acpP* and *holB* genes within the 24-min region of the *E. coli* chromosome. Identification of this region as the *E. coli tmk* gene was confirmed by functional complementation of a yeast dTMP kinase temperature-sensitive mutant and by in vitro enzyme assay of the thymidylate kinase activity in cell extracts of *E. coli* by use of *tmk*-overproducing plasmids. The deduced amino acid sequence of the *E. coli tmk* gene showed significant similarity to the sequences of the thymidylate kinases of vertebrates, yeasts, and viruses as well as two uncharacterized proteins of bacteria belonging to *Bacillus* and *Haemophilus* species.

With the exception of (deoxy)thymidylate kinase (dTMP kinase or TMK), which catalyzes the phosphorylation of dTMP to form dTDP in the dTTP synthesis pathway, all of the *Escherichia coli* enzymes of the metabolic pathways for the de novo synthesis of deoxynucleotide precursors of DNA have been well characterized genetically and biochemically (34). dATP, dCTP, and dGTP are derived from the corresponding deoxyribonucleoside diphosphates by phosphorylation catalyzed by the nonspecific nucleoside diphosphate kinase. Because thymine deoxyribonucleotides have no ribonucleotide counterpart, additional reactions are required for dTTP synthesis involving successively dCTP deaminase, dUTPase, thymidylate synthase, dTMP kinase, and nucleoside diphosphate kinase to convert dCTP to dTTP. Since conversion of dTDP to dTTP is catalyzed by the nonspecific nucleoside diphosphate kinase, the dTMP kinase is the last specific enzyme of both de novo and salvage pathways of dTTP synthesis. Because the overall control of DNA synthesis is regulated in part by the finely adjusted pool of dTTP, it would be crucial to explore the expression and the regulation of the *E. coli* dTMP kinase gene.

For *Saccharomyces cerevisiae*, knowledge of the function of the dTMP kinase gene (*cdc8*) comes from the studies of a *cdc8* temperature-sensitive cell cycle mutant. The transcription of the yeast dTMP kinase gene has been shown to be cell cycle regulated (peaking at the S phase) and coexpressed with DNA ligase and thymidylate synthase genes, suggesting a coordination between processes of DNA replication and biosynthesis of nucleotide precursors. It has been proposed that multienzyme complexes of DNA precursor-synthesizing enzymes or functional compartments, including dTMP kinase, are involved in the replication process of *S. cerevisiae* (50), human lymphoblastoid cell line (53), and T4 phage-infected *E. coli* (32). By functional complementation of the *S. cerevisiae cdc8* mutant or by systematic sequencing, other dTMP kinase genes from human (24, 50), mouse (30), nematode (54), *Schizosaccharomyces pombe* (1), and large DNA viruses (25, 46, 55) were further isolated and studied.

For *E. coli*, conditional lethal mutants defective in dTMP kinase have been isolated and characterized (10). The genetic location of *tmk*, thought to encode the dTMP kinase, has been mapped by cotransduction to approximately 24 min on the *E. coli* chromosome, between the *pyrC* and *ptsG* loci (3). Despite the genetic work that has been done on *tmk*, neither has the dTMP kinase-encoding gene been cloned nor has the 24-min region been systematically sequenced in international genome programs aimed to sequence entirely the *E. coli* chromosome.

In this report, we describe the isolation, characterization, and overexpression of the structural gene encoding the TMK which constitute the prerequisite steps for further studies of the mechanism of action and regulation of this key enzyme in *E. coli*. Furthermore, our sequencing data revealed the gene organization around the *tmk* gene and allowed us to fill in the unsequenced gap between the *acpP* and *holB* genes within the 24-min region of the *E. coli* chromosome.

MATERIALS AND METHODS

Microbial strains, plasmids, and media. The genotypes of *E. coli* and *S. cerevisiae* strains and characteristics of plasmids used in this study are given in Table 1. LB broth and agar were used for routine bacterial growth. When required, ampicillin, kanamycin, and zeocin (CAYLA, Toulouse, France) were used at 100, 50, and 20 μg/ml, respectively.

Oligonucleotides. Figure 1 lists the sequences of PCR primers used in this

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference or source
E. coli		
BL21(DE3)	F$^-$ *dcm ompT* r_B^- m_B^- *lon* λ(DE3)	48
DH5α	F$^-$ φ80*dlacZ*ΔM15 Δ(*lacZYA-argF*) *U169 deoR recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44* λ$^-$ *thi-1 gyrA96 relA1*	GIBCO-BRL, Inc.
TD205	F$^-$ *metB leu hisA lacY malA xyl mtl rpsL dcd-1 tpp-75 cdd-50 argG*$^+$ (P1) *zcj-297*::Tn*10 tmk-1*	J. A. Fuchs (10)
S. cerevisiae CMY616	*MAT*α *cdc8 ura3 leu2 his7 can1-100*	J. M. François
Plasmids		
pAPT110	p15A replication origin vector carrying the *lacI*^{q} gene and Kn^r and Sp^r Sm^r determinants	P. Polard (9)
pET-His	pET3a carrying a double-stranded oligonucleotide encoding a His-6 tag inserted in the polylinker	B. Chen (7)
pZEO SV1	Mammalian cell expression vector carrying a multiple-cloning-site region and the Zeo^r determinant; pUC19 replication origin	CAYLA
pUT 58	pUC19 derivative carrying the *Sh ble* gene (Zeo^r determinant)	12; CAYLA
pUT 106	Mammalian cell promoter probe vector carrying the Zeo^r determinant; pUC19 replication origin	CAYLA
pUT 377	Yeast expression vector of the Zeo^r determinant; pUC19 replication origin	CAYLA

[a] Abbreviations: Kn^r, kanamycin resistance; Sp^r, streptomycin resistance; Sm^r, spectinomycin resistance; Zeo^r, phleomycin or zeocin resistance.

work. All oligonucleotides were synthesized by Isoprim SA (Toulouse, France). Lyophilized purified oligonucleotides were dissolved in H_2O to a final concentration of 1 μg/μl and stored at −20°C.

DNA cloning and sequencing. The source of the gene encoding TMK was bacteriophage λ clone E9G1 (27) DNA (gift of J. M. Louarn). General techniques for plasmid DNA preparations, restriction enzyme mapping, molecular cloning, and agarose gel electrophoresis were performed by use of standard procedures (40). Restriction enzymes and DNA modification enzymes were purchased from New England Biolabs and Boehringer Mannheim. The *E. coli* DH5α strain was used as the host for all of the plasmid constructions. PCR was performed with a Thermojet machine (Eurogentec, Seraing, Belgium). PCRs were cycled 30 times (one cycle consisting of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C) with Tfl DNA polymerase (Epicentre, Madison, Wis.) and PCR primers shown in Fig. 1. When required for DNA purification, DNA restriction fragments or PCR products were separated and purified by use of low-melting-temperature agarose gel electrophoresis (FMC, Rockland, Maine) as described in the recommendations of the commercial supplier. The double-stranded DNA sequences of plasmid inserts as well as DNA fragments from λ phage 236 were determined with a Sequenase 2.0 kit (U.S. Biochemical Corp., Cleveland, Ohio) and α-^{35}S-dATP in accordance with the manufacturer's instructions by use of the sequencing primers indicated below (see Fig. 2B). Synthetic primers were synthesized on the basis of the DNA sequences of the cloning vectors pUT 106 and pAPT 110 to initiate sequencing of the inserts from *Pst*I to *Asp*718 in pUT 125 and from *Pst*I to *Bgl*II in pUT 126. Sequences obtained with these primers were used to design a second set of primers and to derive additional sequence data.

Complementation of *S. cerevisiae cdc8* mutation. Transformations of *S. cerevisiae* CMY616 were performed on whole cells (18), and recombinant clones were selected on uracil-deficient YNB medium supplemented with 0.2 M Casamino Acids at 23°C. Some of the colonies were streaked on yeast extract-peptone-dextrose (YEPD) (22) plates and then incubated at both 23 and 32°C and screened for a temperature-resistant phenotype after 48 h of incubation.

Assay of TMK activity. Preparation of crude *E. coli* cell extracts and enzyme assays were performed as described by Hughes et al. (25). The protein concentration of the extracts was determined by the Bradford method. Briefly, 0.1 to 1 μg of purified protein or proteins from sonic extracts of growing cells were incubated in 40 μl of reaction buffer containing 15 μCi of [^{3}H]dTMP (Moravek Biochemicals Inc., Brea, Calif.) per ml at 37°C for 10 min. The reaction was terminated by heating at 75°C for 10 min. Samples of 5 μl were spotted on polyethyleneimine-cellulose thin-layer plates (Schleicher & Schuell, Dassel, Germany) with thymidine nucleotide markers from Sigma (4 μg each of dTMP, dTDP, and dTTP). The thin-layer chromatography plate was developed with a solution composed of 0.5 M LiCl and 2 N acetic acid to a distance of 15 cm. Spots of the dTDP and dTTP reaction products were scraped from the chromatogram, and the radioactivity was determined by liquid scintillation counting in a Packard 1900 TR liquid scintillation analyzer.

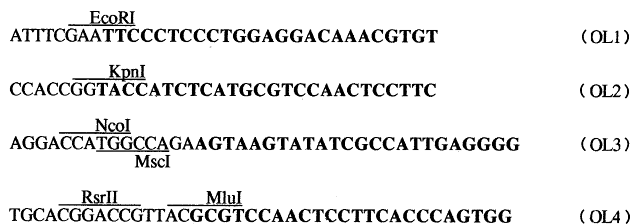

FIG. 1. PCR primers (5′→3′) used. Oligonucleotides are referred to elsewhere in the article by the designations shown in parentheses. Bases in boldface are homologous to the DNA template (λ E9G1 for OL1 and OL2; pUT 125 for OL3 and OL4). Restriction sites generated in extra bases are indicated.

Purification of TMK. *E. coli* BL21(DE3) organisms transformed with the pET-HisTmk vector were grown to an A_{600} of 0.75 in ampicillin-supplemented 2 TY medium (200 ml). The expression of the *tmk* gene was induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the culture was incubated at 37°C, under agitation, for 3 additional hours. Cells were recovered by centrifugation, resuspended in 15 ml of lysis buffer (50 mM Tris buffer [pH 7.8], 1 mM EDTA, 100 mM NaCl, 0.5 mg of lysozyme per ml) and left at room temperature for 15 min. After the addition of 1 mM phenylmethylsulfonyl fluoride, the cells were lysed by two freeze-thaw cycles and the viscosity of the lysate was reduced by two sonication cycles (30 s each) at 4°C. The homogenate was centrifuged at 8,000 × *g* for 10 min to remove insoluble material. The supernatant containing soluble proteins (54 mg) was loaded onto a G25 column (XK 50; Pharmacia) equilibrated in 50 mM Tris buffer (pH 7.8)–100 mM NaCl and run at a flow rate of 15 ml/min by fast protein liquid chromatography (Pharmacia). The eluted fraction containing proteins was then loaded in the same buffer on nickel-charged chelating Sepharose in an HR 5/5 column (Pharmacia) at a flow rate of 2 ml/min. The column was washed with 20 mM phosphate buffer (pH 7.8)–200 mM NaCl until an A_{280} of 0 was reached, and fixed proteins were eluted in 20 mM phosphate buffer (pH 4)–1 M NaCl. Since TMK protein was not pure at this stage, a second round of chromatography on Ni-charged chelating Sepharose was performed. For this, fractions containing the TMK recombinant protein were pooled and diluted twofold in 20 mM phosphate buffer (pH 7.8) to decrease the NaCl concentration to 0.5 M, and the pH was adjusted to 7.8. The solution was loaded onto the Ni-chelating Sepharose as described above, and the TMK protein was eluted in the 1 M NaCl phosphate buffer. The fractions containing TMK were pooled (5.5 mg of protein), concentrated, and desalted in a Macrosep 3-kDa system (Filtron) and analyzed on Coomassie blue-stained sodium dodecyl sulfate (SDS)-polyacrylamide gels.

PAGE of proteins. Polyacrylamide gel electrophoresis (PAGE) was performed on SDS–12% polyacrylamide slab gels by the method of Laemmli (29). Samples of 10 to 25 μl were applied, and protein bands were detected by Coomassie blue staining.

Sequence analysis. Analysis of the sequenced regions in the 24 min on the *E. coli* chromosome was done with EcoSeq, EcoMap (39), and the *E. coli* database collection (ECDC release 23 [51]). Translation of the gene encoding TMK, search of *E. coli* promoters, and codon usage analysis were done with PC/GENE version 6.5 (Intelligenetics). Comparison with DNA or protein databases and multiple sequence alignments were realized by use of the BLAST (2), ALIGN, CLUSTALW 1.5, and BLOCKMAKER (23) network services of the European Bioinformatics Institute (http://www.ebi.ac.uk) and the Baylor College of Medicine (http://kiwi.imgen.bcm.tcm.edu:8088).

Nucleotide sequence accession number. The nucleotide sequence of the *tmk* region (see Fig. 3) has been deposited in GenBank and assigned accession number U41456.

RESULTS

Subcloning and sequencing of the *tmk* gene. Since it has been shown previously that the *tmk* gene maps to approximately 24 min on the *E. coli* chromosome between the *pyrC*

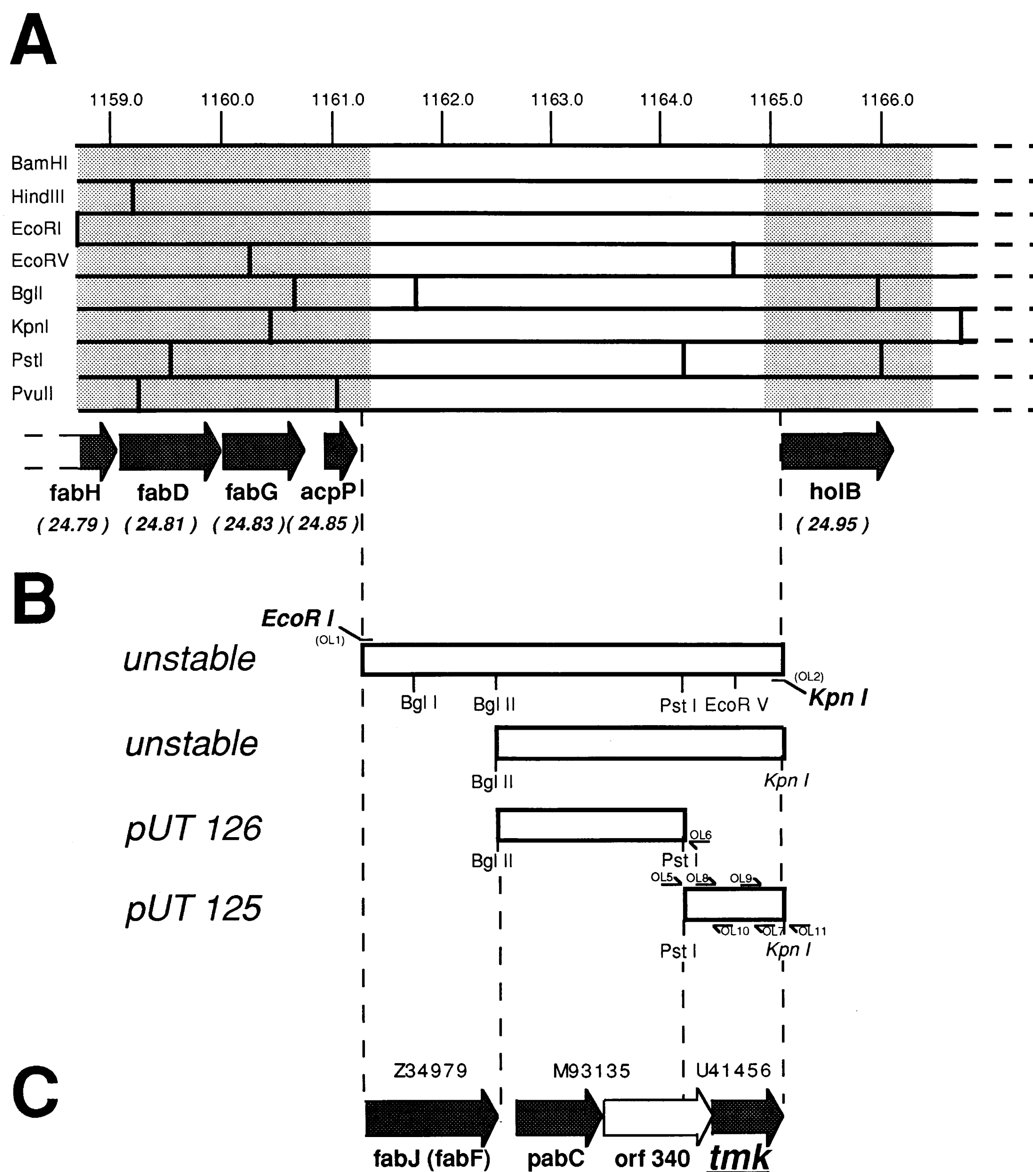

FIG. 2. Location of the *tmk* gene on the physical map of *E. coli*. (A) Section of the EcoMap (39) in the vicinity of 24.8 min contained in λ phage 236 (E9G1) of the *E. coli* genomic library (27). Scales are expressed in kilobase coordinates, and restriction enzymes are indicated to the left. Genetic position (51) and direction of transcription of the genes are indicated below the map. The genes *fabH*, *fabD*, *fabG*, *acpP*, and *holB* encode 3-ketoacyl-acyl carrier protein (ACP) synthase III, malonyl coenzyme A-ACP transacylase, 3-ketoacyl-ACP reductase, ACP, and δ′ subunit of DNA polymerase III holoenzyme, respectively. Shaded areas within the map correspond to sequenced regions reported before the beginning of this work. (B) Restriction map of the *E. coli* chromosomal fragment (open box) amplified by PCR and derivatives used to subclone the *tmk* gene. Only the relevant restriction sites are included. Oligonucleotides used to amplify by PCR or to sequence the pUT 125 and pUT 126 inserts are indicated (see Fig. 1 for oligonucleotide designations). (C) Gene organization in the *E. coli* chromosomal fragment amplified by PCR. Above the map are accession numbers of sequence data (see references 21 and 45 and present work) obtained from fragments covering the entire region.

and *ptsG* genes (3), we analyzed the sequenced regions in this area and found that a single unsequenced gap between the *acpP* (37) and *holB* (6, 11) genes could possibly enclose the *tmk* gene. The physical structure of the corresponding chromosomal region is presented in Fig. 2A. In light of this finding, we decided to excise the *acpP-holB* intergenic region from Kohara phage λ E9G1 (phage 236) (27) by PCR amplification. The PCR-regenerated DNA fragment obtained (~4 kb) was digested by *Eco*RI and *Kpn*I, sites which were included in extra bases appended to the 5′ end of each PCR primer and ligated into pZEO SV1 digested by *Eco*RI and *Kpn*I. After transformation of *E. coli* by the ligation mixture, we were unable in repeated attempts to obtain transformants harboring the expected size of the recombinant plasmid, suggesting that the amplified region was somehow toxic or lethal to *E. coli*. Further subcloning of the amplified region was accomplished with the restriction sites shown in Fig. 2B. The same instability was obtained by cloning the PCR-amplified product reduced to a 2.5-kb *Bgl*II-*Kpn*I fragment into pUT 58 (digested by *Bgl*II and *Kpn*I). Subcloning of a 1.6-kb *Bgl*II-*Pst*I fragment into pUT 106 (digested by *Bgl*II and *Pst*I) or a 0.9-kb *Pst*I-*Kpn*I fragment into pAPT 110 (9) (digested by *Pst*I and *Kpn*I) gave rise to stable recombinant plasmids named pUT 126 and pUT 125, respectively.

A temperature-sensitive mutant of *tmk*, named TD205, was isolated some time ago from *E. coli* LD0181 on the basis of the

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         <-... M93135 ->]
                 Pst I
1        GGTATGCGCCTGCAGACCGACCCGACCGTGATTTACGGGATGGGAGAGCGTTATAATGGCAAACTTTCTCGTGCAGAC
         G  M  R  L  Q  T  D  P  T  V  I  Y  G  M  G  E  R  Y  N  G  K  L  S  R  A  D
79       CTGGAAACGCCGACAGCGTATAACACCTATACCATTACCGGTCTGCCGCCAGGTGCGATAGCGACGCCGGGGGCGGAT
         L  E  T  P  T  A  Y  N  T  Y  T  I  T  G  L  P  P  G  A  I  A  T  P  G  A  D
157      TCGCTGAAGGCTGCTGCGCATCCGGCAAAAACGCCGTATCTCTATTTTGTGGCCGATGGTAAAGGTGGTCACACGTTT
         S  L  K  A  A  A  H  P  A  K  T  P  Y  L  Y  F  V  A  D  G  K  G  G  H  T  F
                                                                   rbs
235      AATACCAATCTTGCCAGTCATAACAAGTCTGTGCAGGATTATCTGAAAGTGCTTAAGGAAAAAA ATG CGC AGT AAG
         N  T  N  L  A  S  H  N  K  S  V  Q  D  Y  L  K  V  L  K  E  K  N  A   Q   *
                                                                          M   R   S   K
311       TAT ATC GTC ATT GAG GGG CTG GAA GGC GCA GGC AAA ACT ACC GCG CGT AAT GTG GTG GTT
          Y   I   V   I   E   G   L   E   G   A   G   K   T   T   A   R   N   V   V   V

371       GAG ACG CTC GAG CAA CTG GGT ATC CGC GAC ATG GTT TTC ACT CGG GAA CCT GGC GGT ACG
          E   T   L   E   Q   L   G   I   R   D   M   V   F   T   R   E   P   G   G   T

431       CAA CTT GCC GAA AAG TTA AGA AGC CTG GTG CTG GAT ATC AAA TCG GTA GGC GAT GAA GTC
          Q   L   A   E   K   L   R   S   L   V   L   D   I   K   S   V   G   D   E   V

491       ATT ACC GAT AAA GCC GAA GTT CTG ATG TTT TAT GCC GCG CGC GTT CAA CTG GTA GAA ACG
          I   T   D   K   A   E   V   L   M   F   Y   A   A   R   V   Q   L   V   E   T
         [L01483->                      -
551       GTC ATC AAA CCA GCT CTG GCT AAC GGC ACC TGG GTG ATT GGC GAT CGC CAC GAT CTC TCC
          V   I   K   P   A   L   A   N   G   T   W   V   I   G   D   R   H   D   L   S
                                      C                                   AC
611       ACT CAG GCG TAT CAG GGC GGC GGA CGT GGT ATT GAC CAA CAT ATG CTG GCA ACA CTG CGT
          T   Q   A   Y   Q   G   G   G   R   G   I   D   Q   H   M   L   A   T   L   R
                                                      -
671       GAT GCT GTT CTC GGG GAT TTT CGC CCC GAC TTA ACG CTC TAT CTC GAT GTT ACC CCG GAA
          D   A   V   L   G   D   F   R   P   D   L   T   L   Y   L   D   V   T   P   E
                          G                                           [L04577->
731       GTT GGC TTA AAA CGC GCG CGT GCG CGC GGC GAG CTG GAT CGT ATT GAG CAA GAA TCT TTC
          V   G   L   K   R   A   R   A   R   G   E   L   D   R   I   E   Q   E   S   F

791       GAT TTC TTT AAT CGC ACC CGC GCC CGC TAT CTG GAA CTG GCA GCA CAA GAT AAA AGC ATT
          D   F   F   N   R   T   R   A   R   Y   L   E   L   A   A   Q   D   K   S   I

851       CAT ACC ATT GAT GCC ACC CAG CCG CTG GAG GCC GTG ATG GAT GCA ATC CGC ACT ACC GTG
          H   T   I   D   A   T   Q   P   L   E   A   V   M   D   A   I   R   T   T   V
                                                        Kpn I
911       ACC CAC TGG GTG AAG GAG TTG GAC GCA TGA GATGGTAcc
          T   H   W   V   K   E   L   D   A   *
                                          M   R   W   Y ..
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FIG. 3. Nucleotide sequence and predicted amino acid sequence of the 949-bp DNA fragment containing the *tmk* gene. Initial and terminal codons as well as the putative ribosome binding site of the *tmk* gene are underlined or overlined, respectively. Accession numbers of sequence data related to this DNA fragment and observed differences are indicated above the nucleotide sequence.

level of resistance to 2′,3′-dideoxythymidine (10). Using this criterion for the presence of the *tmk* gene in the cloned inserts, we observed that the restoration of the dideoxythymidine sensitivity of the TD205 strain occurred only among the pUT 125 transformants (data not shown). Therefore, the complete sequence in both directions of the pUT 125 insert was determined. By using DNA from Kohara's λ clone 236 as the template, the corresponding region was then resequenced to confirm that no errors were generated by the PCR process during the initial amplification.

DNA sequence analysis. The entire nucleotide sequence of the 0.94-kb *Pst*I-*Kpn*I DNA insert carried by pUT 125 and the deduced amino acid sequences of predicted open reading frames (ORFs), transcribed in the same direction, are shown in Fig. 3. The *tmk* coding region from nucleotides 299 to 940 predicts a protein of 213 amino acids and molecular weight (M_r) of 23,783, which is in close agreement with that of the previously identified dTMP kinase enzymes. This ORF is preceded by a strong consensus ribosome binding site (44). Neither promoter consensus −35 and −10 regions upstream of the *tmk* gene nor a putative transcription terminator was found in this sequence. Additional data obtained from sequencing pUT 126 (data not shown) have revealed that the sequence upstream of *tmk* (Fig. 3) constitutes the C-terminal portion of a possible ORF previously described by Green et al. (21) with an 11-base overlap between the termination codon and the beginning of *tmk*. Similarly, the stop codon of *tmk* overlaps the ATG initiation codon of the *holB* gene (6, 11). The nucleotide sequence presented in Fig. 3 differs by a few bases from the uncharacterized sequence reported by Carter et al. (6) upstream of the *holB* gene that we show here corresponds to the C-terminal part of the *tmk* ORF. The arrangement of these three ORFs is suggestive of an operon, and overlapping sequences could be indicative of translational coupling.

The codon usage of the *tmk* gene was examined to determine the frequency of 23 rare codons identified from a survey of 25 nonregulatory *E. coli* genes (28). The rare codons occur at a frequency of 19.6%, 1.6-fold higher than the average, and may contribute to a low gene expression by translational modulation as suggested for *dnaG* (28).

Protein sequence analysis. The deduced amino acid sequence of *tmk* was compared with previously determined sequences of several cellular and viral TMKs as well as two uncharacterized gene products from *Haemophilus influenzae* Rd and *Bacillus subtilis*, recently identified by systematic sequencing (14, 35). The alignment presented here (Fig. 4) is more complete than previously published dTMP kinase alignments (24, 30, 50, 55). The inclusion of the *E. coli* TMK (and

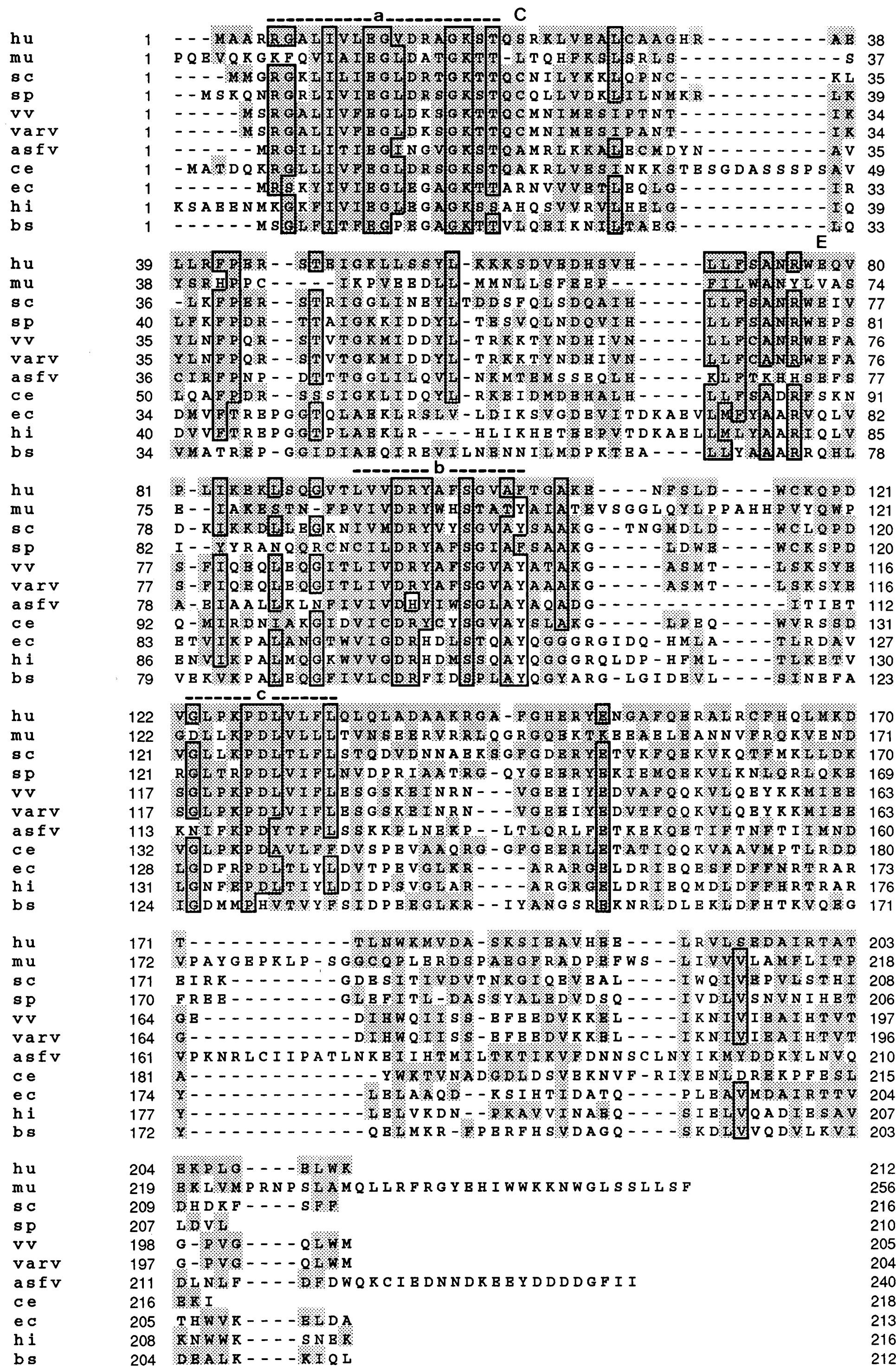

FIG. 4. Protein sequence comparison of dTMP kinases. TMK proteins from human (hu) (24), murine cell line (mu) (30) (from amino acid 176 to the terminus), *S. cerevisiae* (sc) (26), *S. pombe* (sp) (1), vaccinia virus (vv) (46), variola virus (varv; EMBL accession number X67118), African swine fever virus (asfv) (55), *Caenorhabditis elegans* (ce) (54), *E. coli* (ec; this work), and *H. influenzae* (hi) (14) (from amino acid 22 to the terminus) and *B. subtilis* (bs) (35)-related ORFs were aligned by use of the CLUSTAL program. Overlined regions represent conserved motifs. Boxed residues indicate identical residues, and shaded regions indicate homologous residues. The C and E residues, discussed in the text, are indicated above the alignment.

two other putative TMKs from bacteria) confirms the highlight of three conserved motifs. The first one (*a* motif) containing the consensus motif found in nucleoside triphosphate (NTP)-binding proteins (GXXXXGKS/T) is the most conserved, demonstrating its importance for enzymatic activity and ATP binding. The second conserved region (*b* motif), essential for catalytic activity, lies in the nucleoside or nucleotide binding site including the consensus DRY tripeptide found in the previously published TMKs (except African swine fever virus TMK). The *E. coli* and two other putative TMK-encoding

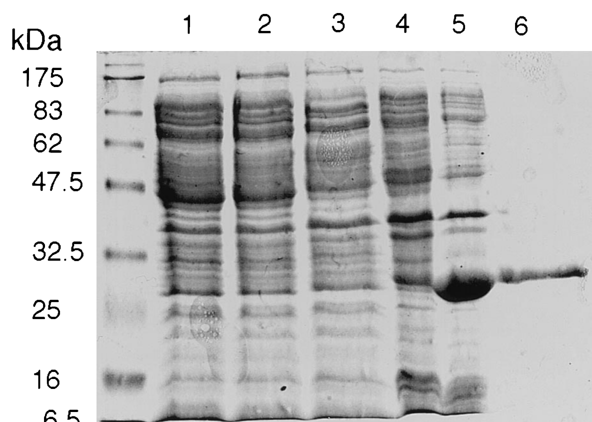

FIG. 5. SDS–12% PAGE analysis of *E. coli* overproducing dTMP kinase cell extracts. Lanes: 1, untransformed *E. coli* TD205; 2, *E. coli* TD205 transformed by pUT 125; 3, *E. coli* TD205 transformed by pUT 136; 4, uninduced *E. coli* BL21(DE3) transformed by pET-HisTmk; 5, induced *E. coli* BL21(DE3) transformed by pET-HisTmk; 6, purified TMK. Values on the left side indicate the molecular mass markers.

prokaryotic sequences differ from eukaryotic TMKs by the presence within this motif of H (or F) in place of Y. The significance of the third domain (*c* motif), including the highly conserved P residue, remains to be determined. Additional conserved residues found in previously published TMK sequence alignments might also be considered for enzyme function. Among these, the important glutamate residue (E-75) for the activity of the yeast enzyme is replaced by a Q-80 residue in *E. coli* (and other putative prokaryotic sequences), which is a conservative substitution. Because *E. coli* TMK does not contain any cysteine residue, we definitively conclude, as suggested by Huang et al. (24), that TMKs do not require such a residue close to the glycine-rich loop (*a* motif) for active conformation of the enzyme.

Identification of the product of the *tmk* gene in *E. coli*. To identify the product of the *tmk* gene, two different plasmid constructions were necessary to achieve a significant yield of the active TMK protein in *E. coli*. In a first attempt to produce the TMK protein in *E. coli*, the *tmk* gene was PCR amplified from pUT 125 with oligonucleotide 3 and oligonucleotide 4 PCR primers (Fig. 1) to create a *Nco*I-*Rsr*II fragment upon restriction enzyme digestion. The modified structural gene was then inserted into pUT 106 under the control of the constitutive synthetic EM7 promoter (unpublished results) in place of the *Nco*I-*Rsr*II fragment containing the *Sh ble* gene (Zeo^r determinant [12]) to give pUT 136. Analysis of total soluble proteins from cell lysates of TD205 transformed by pUT 136 compared with pUT 125-transformed or parent TD205 cells did not reveal an increase in a protein of the expected size (M_r 24,000) by SDS-PAGE (Fig. 5), although the thymidylate activity was high in crude extracts (see below). We finally succeeded in the isolation of the *tmk* gene product by using a His-tagged expression system (7) derived from the T7 promoter-based vectors of Studier et al. (49). The modified *tmk* gene from pUT 136 (*Nco*I-*Bam*HI fragment) was inserted into the *Nco*I-*Bam*HI cloning sites of the pET-His vector, creating a histidine tag at the N terminus of the *tmk* gene product to give pET-HisTmk. This plasmid was introduced into the BL21 (DE3) host strain containing an IPTG-inducible T7 RNA polymerase gene. After induction, the transformed cells overproduced an IPTG-dependent protein that appeared by SDS-PAGE to have the expected molecular mass of about 25 kDa

TABLE 2. TMK activity in *E. coli* overexpressing the *tmk* gene[a]

Extract or TMK	Quantity (μg of protein)	^{3}H-nucleotide formed (cpm)		
		dTDP	dTTP	dTDP + dTTP
E. coli cell extracts				
TD205	1	7	27	34
TD205(pUT 125)	1	24	439	463
TD205(pUT 136)	1	1,090	5,529	6,619
Purified TMK	0.1	1,759	0	1,759

[a] The levels of TMK activity were assayed as described in Materials and Methods. The amounts of [^{3}H]dTDP and [^{3}H]dTTP formed derived from a single experiment and are representative of the values obtained with two other separate experiments using fresh cell extracts assayed in parallel.

(Fig. 5). The high level of overproduction of the His tag TMK fusion protein enabled us to purify, as described in Materials and Methods, 5.5 mg of a highly purified enzyme as judged in Fig. 5 from 200 ml of culture. An in vitro enzyme assay was used to measure the levels of TMK activity from both cell extracts of TD205 expressing the cloned *tmk* gene and the highly purified His tag protein (Table 2). Extracts of TD205 containing pUT 125 exhibited a moderate increase of TMK activity in comparison with that of the basic cellular mutated TMK enzyme from the untransformed strain. In contrast, the activity of the TMK increased over 350-fold in cells containing the high-copy-number plasmid pUT 136. In addition, the highly purified protein was found active in this assay, suggesting that the His tag was not interfering with the catalytic function of the protein.

Functional complementation of *S. cerevisiae cdc8* mutation. The TMK of the yeast *S. cerevisiae* is encoded by the *cdc8* gene (26, 42). The *TK* gene of human herpes simplex virus type 1, which encodes a protein with both TK (thymidine kinase) and TMK activities (8), has been shown to complement the dTMP kinase deficiency when introduced into *cdc8* mutants in an expression vector (42). Functional complementation of *cdc8* mutants was also used to demonstrate the cloning of other eukaryotic dTMP kinase cDNA (50). To provide evidence that the *E. coli tmk* gene encodes an active enzyme, we examined whether its expression in a temperature-sensitive *cdc8* mutant of *S. cerevisiae* could complement the *cdc8* defect at the nonpermissive temperature.

The PCR-regenerated DNA fragment containing the modified *tmk* gene was digested by *Msc*I and *Rsr*II and ligated into the *E. coli*-yeast shuttle vector pUT 377 digested by *Msc*I and *Rsr*II (Fig. 6) to give pUT 391. This plasmid together with a negative control, pUT 377, was transformed into the temperature-sensitive *cdc8* strain CMY 616, and transformants were selected at the permissive temperature of 23°C. All Ura$^+$ transformants from pUT 391 and none from pUT 377 replicated onto YEPD plates and screened for growth at 32°C displayed the Tmk$^+$ phenotype. Some of them were streaked onto nonselective plates to obtain Ura$^-$ colonies that became temperature sensitive (Tmk$^-$), indicating that temperature complementation was mediated by the presence of the plasmid pUT 391. Thus, the *E. coli tmk* gene clearly does complement the *cdc8* mutant, and the *E. coli* TMK protein is able to substitute for the defective yeast enzyme.

DISCUSSION

In this study, we have isolated the *E. coli tmk* gene by subcloning and sequencing part of the *E. coli* W3110 chromosomal DNA insert from the λ phage 236 (E9G1) of the Kohara collection, confirming the previous map location of the gene.

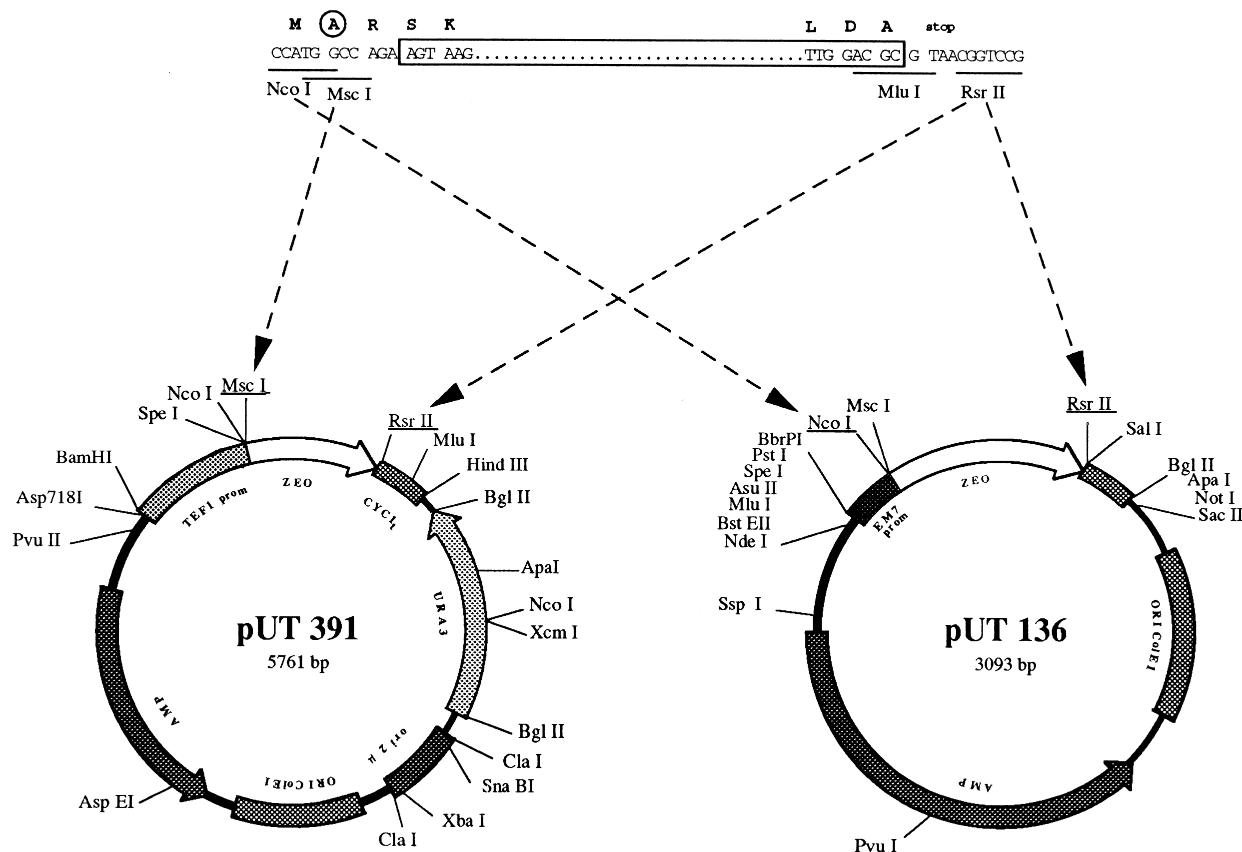

FIG. 6. DNA restriction endonuclease maps of pUT 391 and pUT 136 plasmids. Vectors pUT 377 and pUT 106 are drawn as circles, and the PCR *E. coli tmk* gene insert is indicated by a line. The insert is a 0.6-kb *Msc*I-*Rsr*II or *Nco*I-*Rsr*II fragment. Above the plasmid maps, an open box represents the conserved part of the *tmk* gene. Nucleotide changes introduced by PCR at the extremities as well as the additional amino acid (circled A residue) are indicated.

The *tmk* gene located immediately upstream of the δ′ subunit of DNA polymerase III holoenzyme encoding *holB* gene was specifically identified as the structural gene for the enzyme TMK by functional complementation of a *S. cerevisiae cdc8* (*tmk*) mutant and by specific enzymatic assays of the corresponding protein produced in *E. coli*. The molecular mass of the *tmk* gene product (23.78 kDa) is in agreement with that of previously identified TMK subunits. However, Nelson and Carter (33) have shown that the purified TMK from *E. coli* B has a molecular mass estimated to be 65 kDa (±5%) by gel filtration and sucrose gradient centrifugation. Since a common characteristic of *E. coli* pyrimidine nucleotide-synthesizing enzymes is their oligomeric structure, we postulate that *E. coli* TMK is an homotrimer, but further experiments have to be carried out with the purified TMK in the presence or absence of ATP to confirm the structure of the active enzyme. In addition, analysis of the deduced amino acid sequence of this gene product has revealed features in common with all previously identified TMKs from eukaryotes and viruses, including three conserved structural motifs. The first motif (*a* motif) at the N terminus is the hallmark of known TMKs. It contains the motif GXXXXGKS/T, which was termed the P loop (41), involved in the binding of ATP. This NTP-binding motif, evolutionarily conserved in all NTPases involved in the main biochemical processes (20, 52), is preceded by a glutamate residue in the case of the TMK family. The second conserved region (*b* motif) lies in the nucleotide or nucleoside binding site containing the consensus DRY triplet found in the enzymes that catalyze only TMK kinase activity. A distinctive feature of the *E. coli* TMK in this domain is the presence of a DRH triplet (in place of DRY) as found in the *Herpesviridae* thymidine kinases (4), which possess associated TMK and deoxycytidine kinase activities (38). Herpes simplex virus type I TK and TMK activities have a common active site including the DRH triplet (31). We have not yet determined whether the *E. coli* TMK possesses an additional TK activity but, if it is the case, it is not sufficient to genetically complement a TK-deficient *E. coli* transformed by the *tmk*-overexpressing plasmid pUT 136 (our unpublished results). The significance of the last conserved region (*c* motif) remains open to speculation. A possible involvement of this domain in protein oligomerization or allosteric regulation by nucleotides has yet to be demonstrated.

The genes encoding other *E. coli* nucleoside monophosphate kinases specific for AMP, CMP, GMP, and UMP have been isolated and sequenced (5, 15, 17, 43). Unlike UMP kinase which is specific for UMP, the other enzymes are active with the corresponding 2′ deoxyribonucleotides. An amino acid sequence comparison of *E. coli* TMK with AMP, CMP, and GMP kinases (data not shown) has revealed significant regions of homology spanning *a* and *c* motifs as well as the invariant glycine residue (G-93 of the TMK enzyme) close to the *b* motif. No significant similarity of *E. coli* UMP kinase with *E. coli* TMK was found as previously described for other *E. coli* nucleoside monophosphate kinase (15). In fact, this enzyme appears more related to the aspartokinase family (43).

Examination of the nucleotide sequence of *tmk* identified a

strong potential ribosome binding site but no consensus promoter elements. Nevertheless, since the *tmk* gene is cloned opposite to the transcriptional direction from the *lac* promoter in the pUT 125 vector, the expression observed by enzymatic assay should come from a weak promoter inside the upstream ORF. The sequence of this ORF was determined for 255 bp upstream from the *Pst*I site in Fig. 3 (data not shown) and analyzed for similarity to known *E. coli* DNA sequences by use of the BLAST network service of the European Bioinformatics Institute. It was found to be identical to that of a portion of an unidentified ORF downstream of the *pabC* gene (21). The complete identity of the DNA sequence as well as the final comment reported by Siggaard-Andersen et al. (45) provides independent confirmation of the reposition of the *pabC* gene (previously mapped to 25 min on the *E. coli* chromosome) clockwise of and just downstream of the *fabJ* (*fabF*) gene. The uncharacterized gene (ORF 340) between *pabC* and *tmk* was analyzed for similarity in the GenBank database. Except for the previously mentioned homology (21) of the N-terminal portion with the membrane-bound *E. coli* CDP-diacylglycerol pyrophosphatase and a weak homology of the central portion with a cell division gene (*ftsX* [19]) involved in a septum-membrane-protein complex (Septalsome), no significant homology with characterized proteins was found. This ORF is not preceded by a Shine-Dalgarno sequence but contains another complementary region to the 16S rRNA (spanning the ATG) involved in translation initiation efficiency (48). Cloning of the entire ORF 340 gene into a pUC derivative seems to lead to an instability in *E. coli*. If it is a consequence of the overexpression of the gene product or the presence of a chi recombinational hot spot (47) found at the N-terminal part of the gene remains an open question. Maximum homology (73%) of the ORF 340 occurred with a putative ORF of *H. influenzae* Rd (HI 0457) falsely related to *E. coli* ADC lyase encoded by *pabC* (14). The predicted termination codon of the ORF 340 upstream of *tmk* overlaps by 11 bp the ORF of *tmk*, and similarly, the stop codon of *tmk* overlaps the initiation codon of the downstream *holB* gene. This genetic organization is characteristic of genes that are translationally coupled and that exist in an operon. The same kind of regulation was recently described (16) for the *E. coli* thymidylate synthase-encoding gene (*thyA*), another important gene of the de novo dTTP metabolic pathway. Such a phenomenon seems to be a common feature in *E. coli* (13), correlating the intergenic distance and the expression level of the downstream gene. The high percentage of rare codons in both *tmk* and *holB* genes supports this hypothesis of coregulated expression.

Figure 2A and C display genes and their organization in the 24-min region of the *E. coli* chromosome around the *tmk* gene. Data of this report together with the recently published *fabJ* sequence permit the filling in of the unsequenced gap of this region (between *acpP* and *holB* genes), creating a new contiguous region joining the previously determined ECDO24.51 and ECDO24.85 contiguous regions (51). It is interesting to note that the three genes downstream of *pabC*, from ORF 340 to *holB*, were found to be similar in organization and sequence to those (HI0457, HI0456, and HI0455) in the corresponding region (accession number L42023, from 475.771 to 478.440 [complement]) of the *H. influenzae* Rd chromosome (14). In addition, the putative *tmk* gene (accession number D26185, from 71050 to 71685) of *Bacillus subtilis* was found in the vicinity of an ORF (from 72556 to 73542) similar to *E. coli holB* (35).

Studies in prokaryotic and eukaryotic systems have demonstrated the functional and/or physical associations between the enzymes of deoxy NTP (dNTP) synthesis complex and the components of the DNA replication apparatus (replisome for prokaryotes or replitase for eukaryotes) (36). With such a compartmentation, dNTPs are synthesized by sequential reactions in the immediate vicinity of their use and are more efficiently incorporated. Another advantage is the possible coordinated activation or inhibition of the enzymes of the complex by local concentrations of substrates.

The closed genetic location of *E. coli* TMK and δ′ subunit of DNA polymerase III holoenzyme-encoding genes is to our knowledge the first example of physically and potentially coregulated linked genes involved in the dNTP-synthesizing complex and replication apparatus, which may reflect the coordination between dNTP synthesis and DNA replication. The molecular mechanisms of the regulation of *tmk* gene expression and the importance of the linkage with *holB* remain subjects for further studies. The fact that now we possess both the isolated gene and the purified protein should allow us to further analyze the possible involvement of TMK in other cellular functions, as suggested for AMP and UMP kinases, as well as its activity on various pyrimidine (monophosphate) compounds, including pyrimidine derivative drugs like AZT (3′-azido-3′-deoxythymidine).

ACKNOWLEDGMENTS

We are grateful to J. M. Louarn for the gift of Kohara's bacteriophage λ E9G1, J. M. François, J. A. Fuchs, B. Chen, and P. Polard for providing us with *S. cerevisiae* CMY616, *E. coli* TD205, and pET-His and pAPT 110 plasmids, respectively, and F. Drocourt for excellent secretarial help.

Support was provided by Ensemble Contre le Sida (Fondation pour la Recherche Médicale).

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