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J. Bacteriol. **1995, 177(6):1461.**

JOURNAL OF BACTERIOLOGY, Mar. 1995, p. 1461–1469
0021-9193/95/$04.00+0

Vol. 177, No. 6

The End of the *cob* Operon: Evidence that the Last Gene (*cobT*) Catalyzes Synthesis of the Lower Ligand of Vitamin B_{12}, Dimethylbenzimidazole

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Received 1 September 1994/Accepted 5 January 1995

The *cob* operon of *Salmonella typhimurium* includes 20 genes devoted to the synthesis of adenosyl-cobalamin (coenzyme B_{12}). Mutants with lesions in the promoter-distal end of the operon synthesize vitamin B_{12} only if provided with 5,6-dimethylbenzimidazole (DMB), the lower ligand of vitamin B_{12}. In the hope of identifying a gene(s) involved in synthesis of DMB, the DNA base sequence of the end of the operon has been determined; this completes the sequence of the *cob* operon. The *cobT* gene is the last gene in the operon. Four CobII (DMB^-) mutations mapping to different deletion intervals of the CobII region were sequenced; all affect the *cobT* open reading frame. Both the CobT protein of *S. typhimurium* and its *Pseudomonas* homolog have been shown in vitro to catalyze the transfer of ribose phosphate from nicotinate mononucleotide to DMB. This reaction does not contribute to DMB synthesis but rather is the first step in joining DMB to the corrin ring compound cobinamide. Thus, the phenotype of *Salmonella cobT* mutants conflicts with the reported activity of the affected enzyme, while *Pseudomonas* mutants have the expected phenotype. J. R. Trzebiatowski, G. A. O'Toole, and J. C. Escalante Semerena have suggested (J. Bacteriol. 176:3568–3575, 1994) that *S. typhimurium* possesses a second phosphoribosyltransferase activity (CobB) that requires a high concentration of DMB for its activity. We support that suggestion and, in addition, provide evidence that the CobT protein catalyzes both the synthesis of DMB and transfer of ribose phosphate. Some *cobT* mutants appear defective only in DMB synthesis, since they grow on low levels of DMB and retain their CobII phenotype in the presence of a *cobB* mutation. Other mutants including those with deletions, appear defective in transferase, since they require a high level of DMB (to activate CobB) and, in combination with a *cobB* mutation, they eliminate the ability to join DMB and cobinamide. Immediately downstream of the *cob* operon is a gene (called ORF in this study) of unknown function whose mutants have no detected phenotype. Just counterclockwise of ORF is an asparagine tRNA gene (probably *asnU*). Farther counterclockwise, a serine tRNA gene (*serU* or *supD*) is weakly cotransducible with the *cobT* gene.

Salmonella typhimurium makes cobalamin (vitamin B_{12}) during anaerobic growth (17). Most of the cobalamin synthetic genes are organized into a single operon (*cob* or *cbi*) mapping at min 41 (3, 18). The genetic map of the *cob* operon can be divided into three distinct regions, each of which includes mutations with characteristic phenotypes. Part I has 17 genes (*cbi*) needed for cobinamide (Cbi) synthesis; strains with mutations in this region (CobI) can synthesize vitamin B_{12} when Cbi is provided. Part II of the operon includes mutations that prevent vitamin B_{12} synthesis unless 5,6-dimethylbenzimidazole (DMB) is provided (CobII), suggesting that this region contains the genes for DMB synthesis. Part III includes genes for joining Cbi and DMB to produce functional adenosyl-cobalamin (Ado-B_{12}); these mutants (CobIII) cannot make vitamin B_{12} even when both DMB and Cbi are provided. The standard view of the pathway is depicted in Fig. 1.

A fine-structure genetic map of the *cob* or *cbi* operon shows that the three types of mutations are ordered as parts I, III, and II in a single operon (3, 18). The nucleotide sequences of parts I and III have been determined (27). On the basis of sequence homology to genes of known function in *Pseudomonas denitrificans* (for a recent review, see reference 2), it has been possible to assign functions to many of the genes in parts I and III of the *cob* operon. Mutations in the CobIII region have been analyzed genetically and biochemically, demonstrating that the *cobU* and *cobS* open reading frames correspond to part III of the genetic map (11, 24, 35). These two genes have been assigned the functions diagrammed in Fig. 1. The *cobC* gene indicated in Fig. 1 was discovered by Charlotte Grabau (13, 14, 26) and was assigned the depicted phosphatase activity by O'Toole et al. (25). Mutations in part II of the operon were thought to be defective in DMB synthesis and genetic analysis of CobII mutations suggested that this region encodes a single polypeptide with three or four functionally distinct regions (11). We provide evidence that the CobT protein catalyzes the synthesis of DMB in addition to its demonstrated ability to perform the nicotinate mononucleotide (NaMN): DMB phosphoribosyltransferase reaction.

Riboflavin is the precursor of DMB in propionibacteria (16) and since the *Salmonella* vitamin B_{12} pathway seems similar to that of propionibacteria (32), riboflavin is likely to be the precursor in *S. typhimurium*. The conclusions drawn here support predictions made by Renz and coworkers that enzymes involved in synthesizing DMB are likely to be tightly associated with the enzyme that transfers phosphoribose to DMB (16).

MATERIALS AND METHODS

Bacterial strains and plasmids. All strains used in this study (listed in Table 1) are derivatives of *S. typhimurium* LT2, except for strains LE392 and MC1061 and their derivatives which carry plasmids and are derived from *Escherichia coli* K-12. Strain MC1061 was used to harbor plasmids, and strain LE392 was used for propagation of λ phage. Plasmid pPC2 is a derivative of the pACYC184 vector obtained from Bob Weiss and includes a 7.2-kb *Eco*RI DNA fragment with part II of the *Salmonella cob* operon. The pKZ vector was kindly provided by Bob

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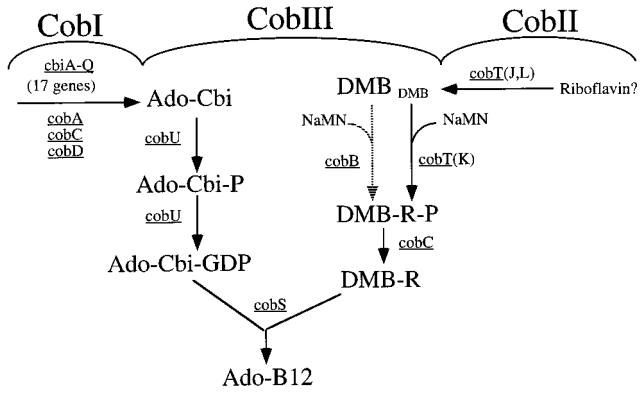

FIG. 1. Vitamin B_{12} synthetic pathway. CobI enzymes perform the synthesis of Cbi (Ado-Cbi). The CobII reactions synthesize DMB (presumably from riboflavin). CobIII activities join Ado-Cbi and DMB to form Ado-B_{12}. Only *cobT* mutations cause a DMB requirement for vitamin B_{12} synthesis; thus, we suggest that all CobII reactions may be catalyzed by the CobT protein (see text). The *cobB* and *cobT* genes are proposed to provide alternative NaMN:DMB ribosyltransferases. We argue that the CobB enzyme requires a high concentration of DMB (indicated by large print) and the CobT enzyme has a strong affinity for DMB and thus can act with a low concentration of DMB (indicated by small print).

Weiss and has a pUC origin. The pKZ plasmids listed in Table 1 contain subclones of the 7.2-kb *Eco*RI DNA fragment. Plasmid pMJ2 contains the 4.2-kb *Eco*RI-*Sca*I fragment of the 7.2-kb *Eco*RI fragment subcloned and supplied by Michael Johnson.

Media, growth conditions, and β-galactosidase assays. For bacterial culture, the rich medium was nutrient broth (0.8%; Difco) with 85 mM NaCl. The minimal medium was the E medium of Vogel and Bonner (36). For λ phage growth, the NZCM medium (21) supplemented with 0.2% maltose was used. The SM buffer (21) was used to store or dilute bacteriophage λ. For β-galactosidase assays, NCE (no citrate E medium) was used with carbon sources at the concentrations described previously (3). The concentrations of antibiotics and the growth conditions for aerobic and anaerobic culture were described previously (3). The β-galactosidase assays were performed as described by Miller (22).

Cloning of part II of the operon. When efforts to clone part II of the operon by complementation from standard plasmid libraries were unsuccessful, the following approach was used. An oligonucleotide (pr2-1 [5′-CCGCCTTCCCGC ATCGATAATAAAA-3′]) was chosen on the basis of known sequence in the distal end of part III of the operon just downstream of an *Eco*RI site (27). This oligonucleotide was used to probe plaques of a λgt7 library obtained from Brian Nichols. This library contains *Eco*RI fragments of the *Salmonella* chromosome. A plaque which hybridized to the probe was identified, and phage from this plaque included a 7.2-kb *Eco*RI fragment. A Southern blot confirmed that this plaque carried an insert derived from part II of the *cob* operon (see Results). The plasmid pMJ2, constructed by Michael Johnson, contains the 4.2-kb *Eco*RI-*Sca*I subfragment which complements CobII mutations. The pKZ subclones were picked from among a pool of shotgun subclones of the sonication subfragments of the 7.2-kb *Eco*RI fragment.

All molecular manipulations, including Southern blotting, in vitro plaque hybridization, λ phage isolation and purification, enzyme digestion, and DNA ligation, were performed as described by Maniatis et al. (21).

TABLE 1. List of strains used in this study

Strain	Genotype	Plasmid
TR6583	*metE205 ara-9*	
TR6612	*ara-9 polA2*	
TT2069	*zea-607*::Tn*10 supD*(su-1) *his-527 leu-414*	
TT10857	*metE205 ara-9 cobT62*::MudJ	
TT11257	*metE205 ara-9 cobT104*::MudJ	
TT11262	*metE205 ara-9 cobT109*::MudJ	
TT11263	*metE205 ara-9 cobT110*::MudJ	
TT11780	*metE205 ara-9 zea-1872*::Tn*10d*Tc	
TT12744	*metE205 ara-9 cob-290*	
TT12745	*metE205 ara-9 cob-291*	
TT12748	*metE205 ara-9 cob-236*::Tn*10d*Tc *cobT294*	
TT12749	*metE205 ara-9 cob-236*::Tn*10d*Tc *cobT295*	
TT12750	*metE205 ara-9 cob-236*::Tn*10d*Tc *cobT296*	
TT12757	*metE205 ara-9 cob-236*::Tn*10d*Tc *cobT303*	
TT13712	*metE205 ara-9 zea-3666*::MudF	
TT14205	*metE205 ara-9 cob-310 cob-236*::Tn*10d*Tc *recA1*	
TT14561	*serB1465*::MudJ *leuA414 fels2*$^-$ *hsd* r$^-$ m$^+$ *recA1*	
TT17643	DE*1077* (*metE*) *ara-9 zea-3777*::Tn*10d*Tc	
TT17703	DE*1077* (*metE*) *ara-9 cobT701*::Tn*10d*Tc	
TT17874	DE*1077* (*metE*) *ara-9* ORF-1::Kan^r	
TT17876	DE*1077* (*metE*) *ara-9* ORF-3::MudJ	
TT17877	DE*1077* (*metE*) *ara-9* ORF-4::MudJ	
TT17878	DE*1077* (*metE*) *ara-9* ORF-4::MudJ *zea-3777*::Tn*10d*Tc	
TT17879	DE*1077* (*metE*) *ara-9* ORF-4::MudJ *cobT111*::Tn*10*	
TT17881	DE*1077* (*metE*) *ara-9 zea-3790*::Tn*10d*Tc	
TT17886	*metE205 ara-9 cobB1206*::MudJ	
TT17890	*metE205 ara-9 cobB1206*::MudJ *cob-291*	
TT17891	*metE205 ara-9 cobB1206*::MudJ *cob-236*::Tn*10d*Tc *cobT294*	
TT17892	*metE205 ara-9 cobB1206*::MudJ *cob-236*::Tn*10d*Tc *cobT295*	
TT17893	*metE205 ara-9 cobB1206*::MudJ *cob-236*::Tn*10d*Tc *cobT296*	
TT17894	*metE205 ara-9 cobB1206*::MudJ *cob-236*::Tn*10d*Tc *cobT303*	
MC1061	*araD139* Δ(*ara leu*)*7697* Δ*lacX74 galU galK rpsL hsr hbm*$^+$	
LE392	*supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1*	
TT17868	*metE205 ara-9 cob-291 srl-202*::Tn*10*	pLacPO-cobT, pMS421(LacI^q)
TT17869	Same as MC1061	pPC2
TT17870	Same as MC1061	pKZ1610
TT17871	Same as MC1061	pKZ1623
TT17872	Same as MC1061	pKZ1614
TT17873	*metE205 ara-9 cob-291 recA1*	pMJ2

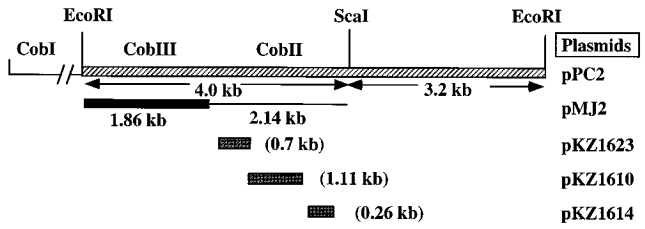

FIG. 2. Subcloning and sequencing of the CobII region. Plasmid pPC2 contains a 7.2-kb *Eco*RI fragment (hatched bar); pMJ2 carries the 4.0-kb *Eco*RI-*Sca*I subfragment (subcloned by Michael Johnson). The thick black bar represents the previously sequenced region, and the thin line is the sequence presented in this work. The pKZ plasmids contain subcloned fragments of the 7.2-kb *Eco*RI insert. Sequence data was first obtained from pKZ subclones. Plasmids pPC2 and pMJ2 were used to complete the sequence for both strands.

DNA sequencing. The *Eco*RI-*Sca*I fragment (Fig. 2) overlaps the previously known sequence (27) by about 1.86 kb. New sequence was determined by sequencing with universal primers adjacent to the cloning site of plasmid pKZ. The oligonucleotide pr2-1 (described above) was used as a sequencing primer to determine the intersection of the new and old sequence. Custom-made primers were used to obtain the remaining sequences of the *Eco*RI-*Sca*I fragment by using pPC2 or pMJ2 as the template. The nucleotide sequences of both strands were determined completely and compiled by the Genetics Computer Group sequence analysis software package (9).

The dideoxy-chain termination procedure (29) was used for DNA sequencing. All enzymes and chemicals for sequencing were purchased from United States Biochemical Corporation. Routine sequencing was done with Sequenase version 2.0 (United States Biochemical Corporation). Compressions and other ambiguities were resolved with 7-deaza-GTP reactions. Custom-made primers were obtained from Robert Schackmann at the University of Utah Sequence Facility.

PCR and cycle sequencing. Insertion mutations in the CobII region were localized by PCR and cycle sequencing as described previously (7). Briefly, primers were designed corresponding to the ends of the insertion element and from the CobII region upstream and downstream of the insertion site. Four PCRs were set up with pairwise combinations of primers (one from the insertion element and one from the nearby chromosomal region). The lengths of the PCR products showed the approximate sites of the insertions and the orientations of the insertions. Cycle sequencing was done with the purified PCR products described above; this provided the exact border sequences of the inserts.

Oligonucleotides used for PCRs are listed in Table 2. MuL and MuR are primers corresponding to the left and right ends of the MudJ element, respectively (15). Primer T-I corresponds to the 66-bp inverted repeat at both ends of Tn*10* (37). Primers T-L and T-R are unique sequences just inside the element from the 66-bp inverted repeat at the left and right ends of Tn*10*, respectively. Primers pr2-2, pr2-3, pr2-4, and pr2-5 were designed to match certain sites of the CobII region.

Cloning of the single *cobT* gene. A single *cobT* gene was amplified by PCR and cloned into the *Bgl*II and *Eco*RI sites of the plasmid vector pLacPO (constructed by Elliot Altman (1). This places the gene under the control of the wild-type *lac* regulatory elements. The DNA polymerase used for PCR cloning of *cobT* is *Pfu*, which has a 3′-to-5′ proofreading exonuclease activity and possesses a 12-fold-higher fidelity than that of *Taq* polymerase (8). Plasmid pMJ2 (described above) was used as the template; PCR was initiated with two oligonucleotide primers: cT-F (5′-TACTATAGATCT**ATG**AGCCGATTGTTGCGGACG-3′), which has a *Bgl*II site (underlined) before the start codon ATG (bold), and primer cT-R (5′-ATATATGAATTC**TTA**TGTTGCGTTTGCGTTCCC-3′), which has a *Eco*RI site following the terminator codon TAA (bold). The PCR fragment generated was cut with *Bgl*II and *Eco*RI and ligated between the *Bgl*II and *Eco*RI sites of the pLacPO vector. The PCR-cloned *cobT* gene was used for complementation tests.

TABLE 2. Oligonucleotides used in PCRs

Oligonucleotides	Sequence
pr2-2	5′-GTCTGGCGCGCCATTTTCGTG-3′
pr2-3	5′-TGGTTTTGATCTGGTCGGGAT-3′
pr2-4	5′-TTTGGCATACCAGGGCGT-3′
pr2-5	5′-ATCGCCATATGCAAATAG-3′
T-I	5′-GACAAGATGTGTATCCACCTTAAC-3′
T-R	5′-ACCTTTGGTCACCAACGCTTTTCC-3′
T-L	5′-TCCATTGCTGTTGACAAAGGGAAT-3′
MuL	5′-ATCCCGAATAATCCAATGTCC-3′
MuR	5′-GAAACGCTTTCGCGTTTTTCGTGC-3′

Inserting a kanamycin resistance gene into the chromosome ORF gene. The plasmid pMJ2 which includes the entire gene which we designate ORF was opened at the unique site *Bst*XI located about 250 bp downstream of the start codon. A 1.5-kb DNA fragment which contains a kanamycin resistance gene (10) was inserted at this site, and the plasmid was closed by T4 DNA ligase. This construct was electroporated into strain TT14561 (lacking the host restriction enzyme but able to perform host methylation) and then transduced into *S. typhimurium* TR6612, a *polA* mutant that does not support plasmid replication (38). The kanamycin resistance gene of the plasmid can be inherited only by recombination into the chromosome, using the sequences (3 and 1 kb) which flank the kanamycin resistance gene. Phage lysates from Kn^r transformants were used to transduce strain TR6583, selecting Kn^r transductants. Linkage analysis showed that the Kn^r phenotype is over 96% linked to mutations in the *cobT* gene. PCR and cycle sequencing confirmed that the Kn^r gene is at the *Bst*XI site of the ORF gene.

Identification of Mud-*lac* fusions in ORF. Many Mud-*lac* insertions that are linked to *cob* mutations but show no *cob* phenotype have been isolated (3). These insertion mutations were screened in search of a Mud-*lac* fusion to the ORF gene. Of 54 Mud-*lac* fusions screened, 6 showed over 95% cotransduction with a *cobT* mutation. Strains with these tightly linked Mud-*lac* fusions were plated on succinate (1%) 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates or glucose (0.2%) X-Gal plates; four showed blue color, suggesting that the inserted *lacZ* gene was expressed by a chromosomal promoter. Finally, PCR and cycle sequencing showed that three of the identified Mud-*lac* fusions carried an insertion within the ORF gene and are in the correct orientation for expression by the ORF promoter. The Mud-*lac* fusions in ORF were used to study regulation of the ORF gene.

Linkage analysis and genetic methods. Linkage of the *supD* gene to the *cobT* gene was determined by transduction crosses. The high-frequency generalized transducing phage mutant of phage P22, HT105/1 *int-201* (30) was used to produce phage lysates. For all transductional crosses, cells (2×10^8) and phage (10^7 to 10^8) were mixed and incubated nonselectively in NB medium for about 30 to 60 min (to allow expression of donor drug resistance genes) before plating on selective medium. Nonselective green indicator plates were used to purify phage-free transductant clones (6). The clear-plaque P22 mutant H5 was used to test the phage sensitivity of the purified transductants. Replica printing was performed to score coinheritance of Cob phenotypes with the selected drug resistance.

Nucleotide sequence accession number. The nucleotide sequence reported here has been submitted to GenBank and has been assigned the locus STYCOBII and the accession number L35477.

RESULTS

Cloning of the CobII region of the *cob* operon. Phage λ clones carrying the end of the *cob* operon were identified by plaque hybridization with a probe whose sequence is at of the distal end of the previously known *cob* sequence. The insert of this λ clone extends beyond known *cob* sequence (see Materials and Methods). Southern hybridization was done to confirm that the fragment includes the promoter-distal end of the *cob* operon. The cloned 7.2-kb *Eco*RI fragment was labeled with [γ-^{32}P]ATP and used for hybridization to *Eco*RI-digested DNAs from both wild-type and CobII mutant strains (Fig. 3B). The probe hybridized with a 7.2-kb chromosome fragment from both wild-type cells and from a CobII point mutant (Fig. 3A, lanes 2 and 4). It hybridized with a smaller fragment of DNA from CobII deletion mutants (Fig. 3A, lanes 1 and 3). Two hybridizing fragments are seen for each of the CobII:: MudJ insertion mutants (lanes 5 and 6). The MudJ element is about 11.4 kb long and has one *Eco*RI site. The sizes of these two hybridizing bands add up to about 18.6 kb, the combined size of the insertion element (11.4 kb) and the chromosomal *Eco*RI fragment (7.2 kb). These results demonstrate that the cloned 7.2-kb fragment includes the CobII region.

Open reading frames in the distal end of the *cob* operon. The reported sequence data completes the *cobT* gene. The nucleotide sequence of part of the *cobT* gene was reported previously (27), and the first base of the *cobT* gene is arbitrarily assigned bp 1 in Fig. 4. The complete *cobT* gene encodes a 37.8-kDa protein of 367 amino acids. The amino acid sequence can be aligned with the amino acid sequence of the CobU

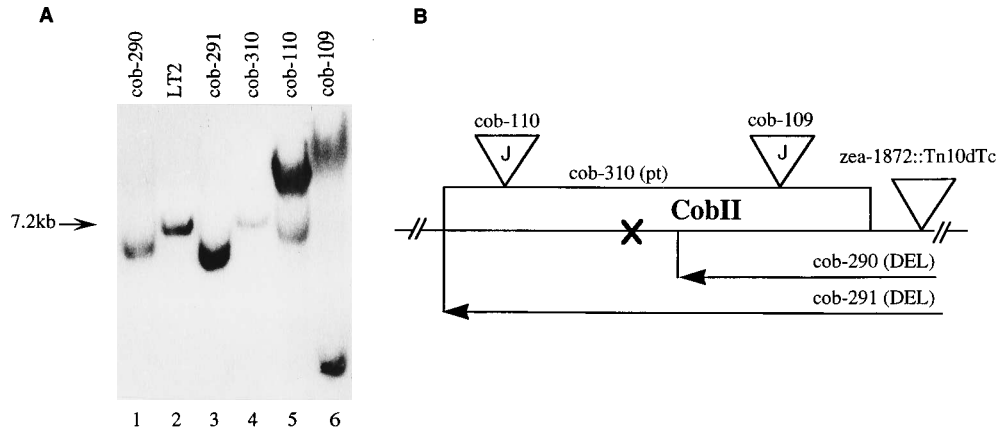

FIG. 3. Southern blot of CobII mutants. (A) The 7.2-kb *Eco*RI fragment identified from the phage was labeled with [γ-^{32}P]ATP and used to probe *Eco*RI-digested DNA from wild-type and mutant strains. Lanes 1 and 3 contain deletion mutants; lane 2 contains wild-type LT2; lane 4 contains a point mutant; lanes 5 and 6 contain MudJ insertion mutations. The arrow indicates the 7.2-kb *Eco*RI fragment. (B) Diagram of the CobII mutations used. pt, point mutation.

protein of *P. denitrificans* (5) over the complete length of both proteins (Fig. 5); the two sequences show 33% identity. Additional homologs of this sequence are found in *Rhizobium meliloti* (34% identity over the aligned sequence) (23) and *E. coli* (70% identity) (20).

Just beyond the *cobT* gene is an open reading frame which we designate ORF. Like the *cob* operon, ORF is transcribed counterclockwise; it encodes a 34.2-kDa protein of 310 amino acids. The inferred amino acid sequence of ORF is very similar to one encoded by a gene in *E. coli* mapping at the same chromosomal position (20). A partial sequence (35 amino acids) from *Klebsiella aerogenes* shows 70% identity to ORF (31). Another partial sequence, reported for *Caenorhabditis elegans* (GenBank accession number T00301), shows 70% identity over 80 amino acids of reported sequence). The closeness of this match suggests that the sequence reported for *C. elegans* may actually be derived from a bacterial contaminant.

Downstream of ORF is a palidromic sequence which can be arranged in two alternative conformations (4). Conformation I has a free energy of −17.2 kcal/mol and is not followed by a run of U residues; conformation II has a free energy of −19.9 kcal/mol and is followed by a run of 6 U residues. It seems likely that conformation II may serve to terminate ORF transcripts.

An asparagine tRNA gene is found beyond the terminator of ORF and is transcribed clockwise. The sequence of this tRNA gene is identical to that of the *E. coli* genes *asnU*, *asnV*, and *asnT*, but the flanking sequences suggest that the gene near *cob* may correspond to the *asnU* gene of *E. coli* (12).

All insertion mutations with a CobII phenotype affect the *cobT* gene and are complemented by a minimal *cobT* plasmid. Detailed genetic mapping and complementation tests of CobII mutations were reported previously (11). The point mutations in this region of the map are interspersed between insertion mutations that are shown (below) to affect the *cobT* open reading frame. The complementation data suggested that this region encodes a single protein, since many point mutations failed to complement all other CobII mutations. However, three groups of contiguous point mutations (*cobJ*, *cobK*, and *cobL*) showed intragenic complementation, suggesting that the protein has several independently functional domains (see below).

Four Mud insertion mutations in the CobII region were localized by PCR amplification and precisely mapped by cycle sequencing (Fig. 4). These four insertions are mixed among point mutations of the four intragenic complementation groups on the genetic map (11). All known DMB auxotrophic mutations (CobII) map at the distal end of the *cob* operon, and all those tested affect the *cobT* open reading frame.

For complementation tests, we have cloned a minimal *cobT* gene following PCR amplification. Only the coding sequence of the *cobT* gene was amplified (see Materials and Methods). The cloned *cobT* gene is expressed by the wild-type *lac* promoter and is inducible by isopropyl-β-D-thiogalactopyranoside. When induced, the single *cobT* gene complements all CobII mutations tested, including a deletion that covers almost the entire genetically defined gene and extends outside the distal end of the operon (strain TT17868). O'Toole and coworkers

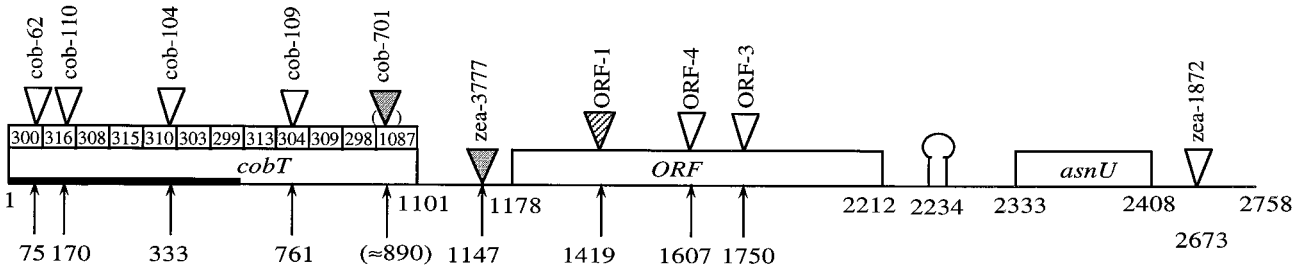

FIG. 4. Open reading frames in the CobII region. The start of the *cobT* gene is arbitrarily assigned number 1. The thick line is the previously determined sequence, and the thin line is the sequence reported here. Boxed numbers just above the *cobT* gene are allele numbers of CobII point mutations. Triangles represent insertions. The stem-loop downstream of ORF is a putative rho-independent terminator. The arrows indicate the exact base positions of insertion mutations. The precise position of insertion *cobT701* with respect to nearby point mutations is not known.

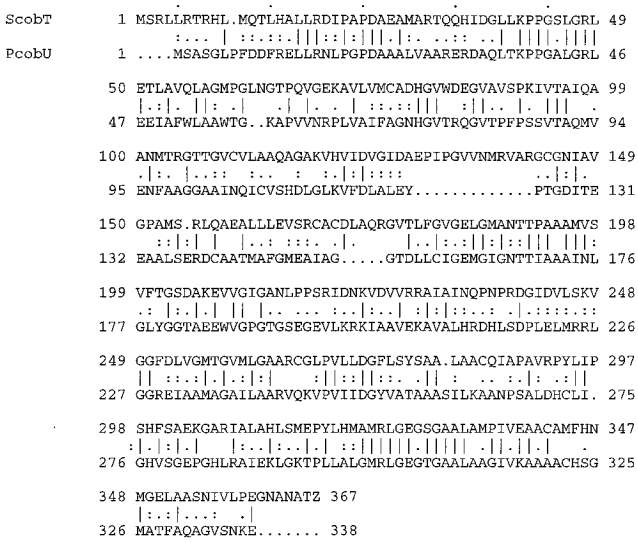

FIG. 5. Alignment of *Salmonella* CobT (ScobT) and *Pseudomonas* CobU (PcobU) amino acid sequences. Identical residues are indicated by vertical lines, and similar residues are indicated by dots between the sequences. Gaps (····) are shown. The identity is 32.8%, and similarity is 57%. The GenBank accession number for the Pseudomonas *cobU* gene is M62868.

have demonstrated that clones of the *cobU* and *cobS* genes (located promoter proximal to the *cobT* gene) do not correct the defect of *cobT* mutations (24). These results indicate that *cobT* is the only gene affected by mutations with a CobII phenotype.

Insertion mutations in ORF have no Cob phenotype. To test whether the ORF gene distal to the *cobT* gene plays a role in vitamin B_{12} synthesis, we inserted a kanamycin resistance gene into the ORF and recombined this constructed mutation into the *Salmonella* chromosome. Under both aerobic and anaerobic conditions, this constructed null mutation does not show any defect in vitamin B_{12} synthesis (data not shown).

Furthermore, we have isolated three MudJ insertions in ORF and determined their exact positions by PCR and cycle sequencing. None of these insertions shows a Cob phenotype. The Tn*10d*Tc insertion between the *cobT* gene and ORF also has no phenotypic defect. These findings indicate that ORF is not essential for vitamin B_{12} synthesis.

ORF is not expressed as part of the *cob* operon. To determine whether ORF is transcribed as part of the *cob* operon, we assayed the β-galactosidase activity of an ORF::*lac* fusion in cells grown with or without oxygen (Table 3). Under both aerobic and anaerobic conditions, the ORF gene is constitutively expressed at a basal level (about 15 Miller units). When propanediol was added, no obvious induction was observed under aerobic conditions in glucose medium. There was a ca. twofold induction by propanediol when cells were grown aerobically in succinate-containing medium and anaerobically in both glucose- and pyruvate-fumarate-containing media. This induction is very low in comparison to the 10- to 20-fold induction seen for a *cobT*::*lac* fusion. The weak induction of ORF by propanediol was eliminated by the *cobT111*::Tn*10* insertion, suggesting that there is some read-through of a termination site between the *cobT* gene and ORF. This is probably a rho-dependent terminator, since no stem-loop structure was detected in the 78-bp interval between the *cobT* gene and the ORF. The basal level of ORF expression may be due to a promoter located between *cobT* and a Tn*10d*Tc insertion in the space between the *cobT* and ORF genes. We suggest this because the *zea-3777*::Tn*10d*Tc insertion (30 bp upstream of ORF) abolishes all expression of an ORF::*lac* fusion, but the distal *cobT111*::Tn*10* does not abolish basal ORF expression. No promoters have been found by inspection of the sequence between the *cobT* and ORF genes.

The *supD* locus is cotransducible with the *cobT* gene. The *supD* (*serU*) locus encodes a $tRNA^{Ser}$ which can give rise to suppressors of the amber codon UAG (34). In a *serU132* (*supD32*) mutant of *E. coli*, the CGA anticodon of wild-type $tRNA_2^{Ser}$ is replaced by CUA in the suppressor tRNA. In *E. coli*, the *serU* gene maps at 43 min (2054 kb), and the *asnT* gene is located at a similar position (2054 kb). Two extra copies of asparagine tRNA genes (*asnU* and *asnV*) are located at 2070 and 2072 kb (19). The finding that an asparagine tRNA gene (*asnU*) lies just beyond ORF suggested that the *supD* locus in *S. typhimurium* might be closer than suspected from the genetic map (28). Transductional linkage analysis based on 200 to 300 transductants for each cross is presented in Fig. 6. The insertion *cobT61*::MudA is about 5% cotransducible with a *supD* amber suppressor mutation. A *zea-3666*::MudF insertion downstream of *cobT* (80% linked to the *cobT* gene) showed 14% cotransduction with a Tn*10* closely linked to *supD*.

The conflict between the phenotypes of *cobT* mutants and the reported activity of the CobT enzyme. The CobT protein has been reported to catalyze the transfer of ribose phosphate from NaMN to DMB to form α-ribazole-5′-phosphate (35). The proposed pathway is depicted in Fig. 1, and the structures of the key molecules are shown in Fig. 7. A mutant lacking the phosphoribosyltransferase activity ascribed to CobT protein would be expected to show a CobIII phenotype, the inability to make vitamin B_{12} even when both DMB and Cbi are provided. Mutants of *P. denitrificans* lacking the enzyme are unable to make vitamin B_{12} even when DMB is provided, suggesting that they have the predicted CobIII phenotype (5). However, *Salmonella cobT* mutants show a CobII phenotype; they are still able to join Ado-Cbi and DMB to form vitamin B_{12} but cannot

TABLE 3. Regulation of ORF

Strain	Relevant genotype	β-Galactosidase activity (Miller units) under the following conditions:							
		Aerobic				Anaerobic			
		Glucose		Succinate		Glucose		Pyruvate-fumarate	
		None	Pd[a]	None	Pd	None	Pd	None	Pd
TT17877	ORF-4::*lac*	15	18	11	21	14	26	17	38
TT17878	ORF-4::*lac zea-3777*::Tn*10*dTc	1	<1	1	1	<1	<1	1	<1
TT17879	ORF-4::*lac cobT111*::Tn*10*	12	10	9	11	8	9	11	10
TT10857	*cobT*::*lac*	16	31	54	610	1	24	67	550

[a] Pd, propanediol.

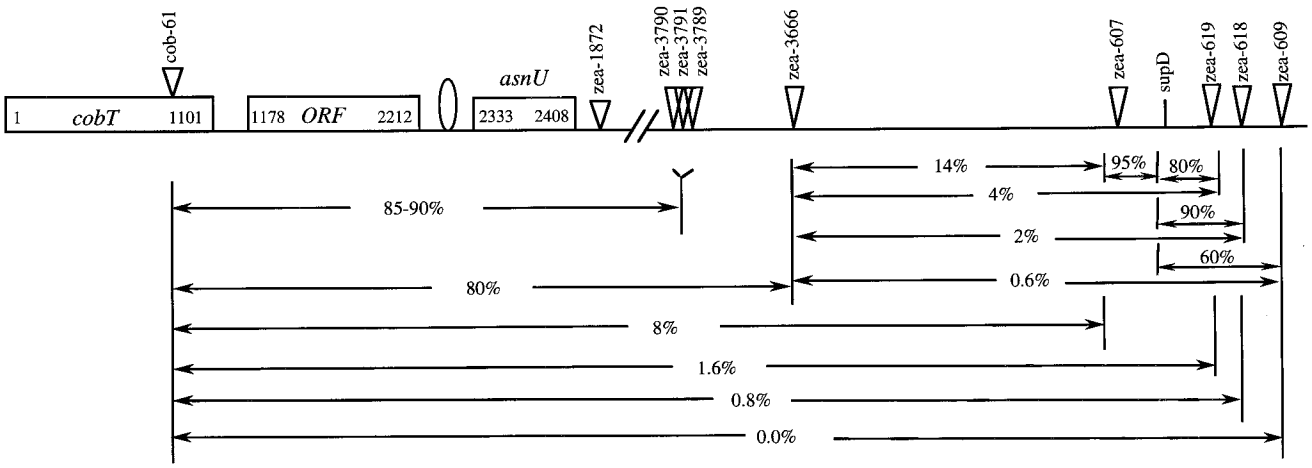

FIG. 6. The *supD* locus is cotransducible with *cobT*. Triangles represents insertions used in the transductional linkage analysis. Linkages between different sites are indicated by the numbers between the arrows.

make vitamin B_{12} unless DMB is provided exogenously. Understanding this paradox should give some insights into vitamin B_{12} synthesis.

One explanation of the situation has been offered by Trzebiatowski et al. (35) who propose the existence of a second phosphoribosyltransferase with a very high K_m for DMB. The existence of this enzyme would explain why *cobT* mutants can still join DMB and Ado-Cbi. The DMB requirement of these mutants is explained if the alternative enzyme has a low affinity for DMB and is active only when a very high level of exogenous DMB is provided. Two pieces of evidence support this explanation. (i) A very high concentration of DMB (3×10^{-4} M) is required to allow *cobT* deletion mutants to make vitamin B_{12}. (ii) Trzebiatowski et al. have isolated a mutant (*cobB1206*::MudJ) that appears to lack the predicted alternative transferase. That is, the mutation alone has no phenotype, but when added to a strain with a *cobT* deletion, it converts the CobII phenotype to the CobIII phenotype expected for strains lacking all transferase activity (Fig. 1). While their explanation is likely to be correct, it raises several questions. Why did not the normal DMB synthetic enzymes create sufficient DMB to activate the alternative enzyme? If *cobT* is not involved in DMB synthesis, why have no true DMB synthesis mutants been isolated in the exhaustive mutant hunts that have been conducted?

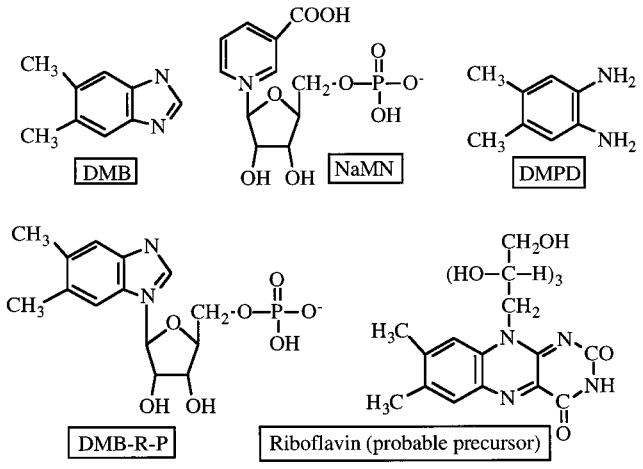

FIG. 7. Structures of DMB and related compounds. NaMN donates ribose phosphate to DMB to form α-ribazole-5′-phosphate. Riboflavin is a possible precursor of DMB. Dimethyl pheylene diamine (DMPD) can satisfy the DMB requirement of mutations in the J and L regions of *cobT* (11).

Evidence that the CobT protein has multiple functions. Complementation studies of point mutations with a CobII phenotype indicated that they all affect a single protein; that is, some recessive point mutations failed to complement all other CobII mutations (11). However, the pattern of intragenic complementation was interesting. Four regions of the gene were distinguished (designated J, K, L, and M). Point mutations in the M region failed to complement all others. Point mutations in the J, K, and L regions failed to complement other mutations in their own region but complemented mutations in the other two groups. These results suggested a protein with four domains of different functions. The J, K, and L regions appear to perform distinct independent functions while the M region is required for all activities. This interpretation of the complementation results is diagrammed in Fig. 8.

Strains carrying mutations within the *cobT* gene that are satisfied by a low level of DMB. To pursue this interpretation of the complementation results, we tested strains carrying mutations in each of these regions for their DMB requirement. A representative mutation from each class is shown in Table 4. Strains with point mutations in regions K and M, like those with deletion mutations, required a high level of DMB (3×10^{-4} M) to permit vitamin B_{12} synthesis. This was true of all strains with mutations in the M region. Some strains with mutations in the K region were satisfied by 10-fold-less DMB, and one strain with a mutation in the K region (*cobT293*) required only 3×10^{-9} M DMB. It is likely that this mutation is extremely leaky and that all strains with mutations in the K region retain residual phosphoribosyltransferase activity (see below). Surprisingly, all nonpolar point mutations in the J and L regions could make vitamin B_{12} when the strains were supplied with a 1,000-fold-lower concentration of exogenous DMB (3×10^{-7} M). In general, strains with mutations in the L region appeared to require slightly more DMB than those with mutations in the J region (data not shown). If the high level of DMB required by *cobT* deletion mutants is needed to activate the alternative enzyme (*cobB*) as suggested by Trzebiatowski et al. (35), then the *cobT* mutants that grow on a low concentra-

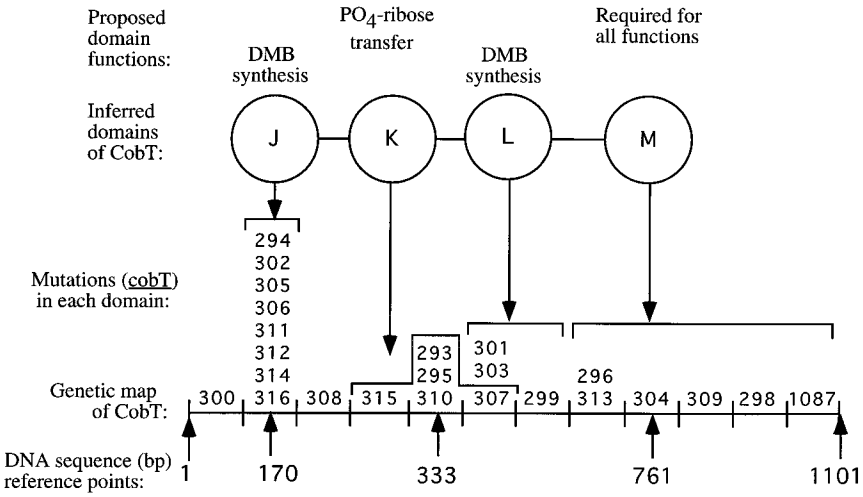

FIG. 8. Functions proposed for various regions of the CobT protein. The complementation groups J, K, L, and M were defined previously and the point mutations in each group were positioned by deletion mapping (11). The approximate sequence position of each group was determined by sequencing insertion mutations mapping in the indicated deletion interval.

tion of DMB appear to retain transferase activity but lack some other activity needed for vitamin B_{12} synthesis. Since the J and L mutants make vitamin B_{12} when a low level of DMB is provided, we suggest that they have lost only the ability to make DMB and that this synthesis is a normal activity of the CobT enzyme. We propose that K mutants have lost only transferase activity while deletions and M point mutants have lost both activities.

Some *cobT* mutants appear to retain transferase activity and are defective only in DMB synthesis. We tested this possibility further by combining various *cobT* point mutations with a *cobB* mutation (generously provided by Jorge Escalante). As reported by Trzebiatowski et al., a *cobT* deletion mutant ($CobII^-$) gained a CobIII phenotype when the *cobB* mutation (no phenotype alone) was introduced (35). We confirmed this and found that point mutations in the *cobT* regions K and M behaved similarly (Table 4). Double mutants of *cobB* and mutations in the K region showed a $CobIII^-$ phenotype (failure to grow on Cbi plus DMB) when scored early but were able to develop a $CobIII^+$ phenotype later. This is probably due to a partial loss of phosphoribosyltransferase activity. Mutations in the J and L regions retained their CobII phenotype in the presence of a *cobB* mutation. Their ability to join DMB and Cbi (despite the *cobB* mutation) suggests that J and L mutants have transferase activity that could not have been provided by the *cobB* gene. The continued CobII phenotype (DMB requirement) of J and L mutants cannot reflect use of DMB to activate the alternative transferase, since that alternative is eliminated by the *cobB* mutation. This result demonstrated (if indirectly) that the CobT enzyme can synthesize DMB in addition to its demonstrated transferase activity.

The compound dimethylphenylenediamine (DMPD) (Fig. 7) was reported to substitute for DMB in supporting growth of some *cobT* mutations (11). We confirmed that the mutants which are fed this compound are those with a lesion in the J or L region, which we concluded had lost only the ability to synthesize DMB. With more time, all *cobT* mutants were able to grow on DMPD, including those with a deletion of the entire gene. We propose that DMPD is not a synthetic intermediate but is converted to DMB by an alternative route. Apparently, conversion of DMPD produces insufficient DMB to activate the CobB enzyme, so only CobT mutants with normal transferase activity are able to use it well.

DISCUSSION

The distal end of the *cob* operon, which had not been previously sequenced, includes mutants that can synthesize vitamin B_{12} only if provided with an exogenous source of DMB, the lower ligand of the vitamin B_{12} molecule. The sequence of this region was determined in hopes of characterizing the biosynthetic gene(s) for DMB. The sequence revealed that the *cobT* gene, only part of which had been sequenced previously, is the last gene in the operon. Several insertion mutations with

TABLE 4. Separable functions of the CobT protein

Strain	Relevant genotype[a]	Minimum required DMB concn (M)	Phenotype in *cobB* background[b]	Growth[c] on DMPD
TT12745	*cob-291* deletion	3×10^{-4}	$CobIII^-$	−
TT12748	*cobT294* J	3×10^{-7}	$CobII^-$ $CobIII^+$	+
TT12749	*cobT295* K	3×10^{-4}	$CobIII^-$	−
TT12757	*cobT303* L	3×10^{-7}	$CobII^-$ $CobIII^+$	+
TT12750	*cobT296* M	3×10^{-4}	$CobIII^-$	−

[a] All strains with the mutations listed are phenotypically CobII when tested alone. Thus, all can make vitamin B_{12} if DMB is provided.
[b] The CobIII phenotype is failure to make vitamin B_{12} even when DMB and Cbi are provided. In these mutants, the CobII phenotype (ability to make DMB) cannot be scored by growth phenotype. The CobII phenotype is the failure to make vitamin B_{12} unless DMB is supplied.
[c] −, no growth occurs; +, growth occurs.

a CobII phenotype were sequenced to demonstrate unequivocally that they all affect the *cobT* gene. The only other open reading frame in the area was shown to be functionally unrelated to vitamin B_{12} synthesis.

The fact that all mutations with a CobII phenotype affect the *cobT* gene conflicts in two ways with the biochemically assigned activity of the protein, transfer of phosphoribose from NaMN to DMB to form α-ribazole-5′-phosphate (5, 35). First, *cobT* mutants are still able to join Cbi and DMB, so they must have another enzyme able to perform the phosphoribose transfer (Fig. 1), and second, they need exogenous DMB to perform this synthesis, suggesting a defect in DMB synthesis. The conflict seems to exist only in *S. typhimurium*, since mutants of *P. denitrificans* lacking the homolog of the CobT enzyme are unable to make vitamin B_{12} even when DMB is provided. This could be explained if *P. denitrificans* lacked the CobB alternative phosphoribosyltransferase.

Trzebiatowski and coworkers (35) explained these conflicts by proposing that *S. typhimurium* produces a second transferase with a high K_m for DMB. This would explain the ability of *cobT* mutants to join Cbi and DMB; the DMB requirement of *cobT* mutants would be caused by needing a high level of DMB to stimulate the second transferase. They supported their suggestion by isolating mutants (*cobB*) likely to be defective in the predicted second phosphoribosyltransferase. Double mutants (*cobT cobB*) are unable to join DMB and Ado-Cbi, consistent with a complete lack of the phophoribosyltransferase. While we think their explanation is correct, it appears to be incomplete. If the CobT enzyme is not involved in DMB synthesis, why have the real DMB synthetic enzymes not been revealed by the exhaustive mutant hunts that have been conducted?

We provide genetic evidence that the *cobT* enzyme is responsible for DMB synthesis in addition to its transferase activity. Particular alleles of the *cobT* gene appear to retain transferase activity but still require DMB. Two regions of the *cobT* gene include mutations that appear to be defective only in DMB synthesis. Since mutations in these two regions complement each other, it is possible that at least two DMB synthetic reactions are catalyzed; this is a minimal estimate, since it depends on the available number of alleles. The failure to isolate CobII mutations mapping to genes other than *cobT* suggests that the entire synthesis of DMB (probably from riboflavin) is accomplished by the CobT enzyme. Since the CobU protein of *P. denitrificans* shows sequence similarity to the CobT protein over its entire length, it seems likely that this protein also accomplishes the synthesis of DMB in *Pseudomonas* species.

The finding that the CobT protein catalyzes both DMB synthesis and transfer of ribose phosphate fulfills a prediction made 15 years ago by Renz and coworkers (16). On the basis of labeling studies done in propionibacteria, they demonstrated that the two nitrogen atoms in the central ring of riboflavin (Fig. 7) have different probabilities of attachment to the ribose of DMB nucleotide. In free DMB, these two nitrogen atoms are chemically indistinguishable because of the symmetry of DMB (Fig. 7). Therefore, it would be impossible to maintain preferential attachment of one of these atoms to ribose. This led them to conclude that about 20% of the synthesized DMB is not released as a free intermediate. Renz predicted that the transferase must be associated closely with the DMB synthetic enzyme. That prediction is fulfilled if a single polypeptide is able to catalyze both DMB synthesis and the transferase reaction. Also consistent with this finding is Renz's observation that the synthesis of DMB is stimulated by the addition of nicotinate to the reaction mixture. This suggests that nicotinate (or a derived compound such as NaMN) might allosterically regulate the synthetase. This possibility is easy to visualize if a single protein catalyzes both the synthesis of DMB and the NaMN-dependent transfer of ribose phosphate.

It is remarkable but not unprecedented that a protein of average size (40 kDa) is able to catalyze so many reactions. A hypothetical scheme for DMB synthesis suggested by Renz involves five reactions and the transferase would be a sixth. However, some of the synthetic reactions might occur in a concerted way. The proposed scheme does not include as an intermediate DMPD (which can be used in place of DMB by some *cobT* mutants). However, a folate-dependent addition of one carbon between the amino groups of DMPD might generate DMB by a means that is independent of the CobT enzyme. An unattractive feature of Renz's proposed pathway is its requirement for molecular oxygen; *S. typhimurium* can synthesize vitamin B_{12} (and therefore DMB) under completely anaerobic conditions.

There are several other multifunctional enzymes in the vitamin B_{12} synthetic pathway. Probably most remarkable is the CysG enzyme which methylates two sites on uroporphyrinogen III to form precorrin-2, a precursor of both vitamin B_{12} and siroheme. The same CysG polypeptide catalyzes ring oxidation and iron insertion in the synthesis of siroheme (33). Another bifunctional enzyme is encoded by *cobU* and catalyzes two sequential reactions (kinase and GDP transferase) in the CobIII part of the pathway (24) (Fig. 1). A third example may be encoded by the *cobC* gene, which was discovered by Charlotte Grabau (13, 14, 26, 27) and recently reported to encode a phosphatase for DMB-ribose phosphate, as shown in Fig. 1 (25). The reported activity predicts a CobIII phenotype for *cobC* mutations. In fact, these mutants can perform the CobIII reactions but appear blocked prior to cobyric acid in the CobI portion of the pathway. If the reported activity is correct, this protein may also prove to have multiple functions.

A search of the GenBank data base with TFASTA revealed homologs of the CobT and ORF proteins in some other organisms. In *E. coli*, which lacks the CobI genes, the CobIII and CobII regions are organized in the same way as those of *S. typhimurium*; *E. coli* also has an adjacent ORF homolog and an asparagine tRNA gene (20). In *K. aerogenes*, a homolog of ORF is found near the *nacC* (nitrogen assimilation control protein) and there is also an asparagine tRNA gene nearby (31). It is not known whether the *cob* genes of *K. aerogenes* map in this region. The homolog of the *cobT* gene in *R. meliloti* is next to the *dgkA* (diacylglycerol kinase) gene, as it is in *P. denitrificans* (23).

In *E. coli*, one asparagine tRNA gene is located near the *serU* gene and the other two are about 16 kb away (19). If *S. typhimurium* has a similar number of *asn* tRNA genes, we suppose that *asnU* and *asnV* are close together near the *cob* operon and we have sequenced only the *asnU* gene; presumably the *asnT* gene is nearer the *serU* (*supD*) gene which is weakly linked to the *cobT* gene.

ACKNOWLEDGMENTS

We thank Brian Nichols and Bob Weiss for their help in the initial cloning of this region and Michael Johnson for construction of one of the subclones. Jorge Escalante kindly supplied the *cobB* mutation. Elliott Altman constructed the expression vector and helped in the PCR cloning of *cobT*. Jeffery Lawrence helped with computer analysis. Tom Bobik gave helpful comments throughout this project and suggested testing various *cobT* mutants for the threshold concentration of DMB required for growth.

This work was supported by NIH grant GM34804.

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