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Cloning and expression of the 1.3S biotin-containing subunit of transcarboxylase

(gene cloning/bacterial expression vectors/biotin holoenzyme synthetase)

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ABSTRACT **We have cloned the gene coding for the 1.3S biotin-containing subunit of transcarboxylase (EC 2.1.3.1) from *Propionibacterium shermanii*. Transcarboxylase is a well-characterized enzyme composed of 30 polypeptides of three different types: twelve 1.3S biotinyl subunits, six 5S dimeric outer subunits, and one 12S hexameric central subunit. In propionic acid fermentation, the enzyme catalyzes the transfer of a carboxyl group from methylmalonyl-CoA to pyruvate in two partial reactions. The 1.3S subunit binds the outer and central subunits of the enzyme together, and its biotin serves as carboxyl carrier between subsites on the central and outer subunits where each partial reaction occurs. The cloned gene has been expressed in *Escherichia coli*, and the 1.3S subunit accumulates to 7% of total cellular protein. The foreign protein is recognized and biotinated by biotin holoenzyme synthetase of *E. coli*. The identifications of the gene and its product were confirmed by four independent approaches: DNA sequence analysis, immunoprecipitation, incorporation of labeled biotin, and measurement of enzymatic activity in the first partial reaction.**

Transcarboxylase (EC 2.1.3.1) is a biotin-containing multisubunit enzyme (1.2×10^6 M_r) involved in propionic acid fermentation (for review, see ref. 1). In the propionic acid bacteria, the enzyme (E) catalyzes the transfer of a carboxyl group, through biotin, from methylmalonyl-CoA to pyruvate to form oxalacetate. The complete reaction (C) is the summation of two partial reactions (A and B):

$$\text{Methylmalonyl-CoA} + \text{E-biotin} \overset{12S}{\rightleftharpoons} \text{propionyl-CoA} + \text{E-biotin-COO}^- \quad \text{(A)}$$

$$\text{E-biotin-COO}^- + \text{pyruvate} \overset{5S}{\rightleftharpoons} \text{E-biotin} + \text{oxalacetate} \quad \text{(B)}$$

$$\text{Methylmalonyl-CoA} + \text{pyruvate} \rightleftharpoons \text{propionyl-CoA} + \text{oxaloacetate.} \quad \text{(C)}$$

Transcarboxylase is composed of 30 polypeptides of three different types. Partial reaction A takes place on the central, CoA-ester-binding, 12S subunit, a cylindrical hexamer of identical polypeptides (60,000 M_r). Each polypeptide has two CoA ester binding sites. The second partial reaction (B) occurs on the outer, keto-acid-binding, 5S subunits, of which there are three at each end of the central cylinder. Since each 5S subunit is a dimer of identical polypeptides (60,000 M_r), there are a total of 12 polypeptides of this type within each enzyme. The third type of subunit, the 1.3S polypeptide (12,000 M_r), binds the outer subunits to the central subunit, and its covalently bound biotin (as ε-biotinyllysine, biocytin) oscillates carboxyl groups between binding sites on the 12S and 5S subunits where partial reactions A and B occur. There are 12 1.3S subunits within each holoenzyme. Each subunit and polypeptide type has been isolated and assayed separately after pH-dependent dissociation of the enzyme, and the polypeptides can then be reassembled to form fully active enzyme (2, 3). These features make transcarboxylase an attractive model system for studies of structure/function relationships in complex enzymes.

The flexible 1.3S biotinyl subunit of transcarboxylase is well characterized, and several of its functions have been associated with specific regions within the 123 amino acid polypeptide. Studies using tryptic peptide fragments and synthetic peptides have indicated that the NH_2 terminus of the 1.3S polypeptide is involved in the association of central (12S) to outer (5S) subunits (4–6). A synthetic peptide containing amino acid residues 1–14 interacts with the 12S subunit, and a tryptic fragment consisting of amino acid residues 2–26 is sufficient to promote binding of both 12S and 5S subunits (6). Biotin is attached to amino acid residue lysine-89 of the 1.3S polypeptide by biotin holoenzyme synthetase, and this post-translational modification is essential to production of enzymatically active 1.3S subunit (7–9). Biotin holoenzyme synthetases are responsible for addition of biotin to all biotin enzymes, and in all known cases, the biocytin (Bct) is located within the amino acid sequence Ala-Met-Bct-Met (5, 10, 11) and at a distance of 35 amino acids from the COOH-terminus of the polypeptide (12). It is not known if these features of primary sequence are involved in recognition by the synthetase or if they reflect a general mechanism of action of biotin enzymes.

The amino acid sequence of the 1.3S subunit has been determined (5), providing a means by which the gene may be isolated and then modified. We used the amino acid sequence to design homologous synthetic oligonucleotides, which were used as radiolabeled hybridization probes to isolate, from *Propionibacterium shermanii*, the gene coding for the 1.3S polypeptide. The gene has been expressed in *Escherichia coli* to produce biologically active 1.3S subunits. This activity requires recognition of the foreign protein by *E. coli's* biotin holoenzyme synthetase and attachment of biotin.

MATERIALS AND METHODS

Synthetic Oligonucleotides. The oligonucleotide mixture TC_1 was synthesized by K. J. Collier (Hoffmann–La Roche, Nutley, NJ) using the phosphotriester method (13). It contains four 14-mers, each complementary to one of the possible nucleotide sequences coding for amino acid residues 88–92 of the 1.3S subunit. The mixture includes the complement of the sequence shown from base pair (bp) 262 to bp 275 in Fig. 1. The oligonucleotide mixture TC_2 is complementary to all possible nucleotide sequences coding for amino acid residues 23–27 and was synthesized by a modification of the phosphoramidite method (14). It is a mixture of sixteen 14-mers, one of which is the complement of the sequence

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Abbreviations: Bct, biocytin (ε-biotinyllysine); bp, base pair(s); kb, kilobase pair(s).

from bp 67 to bp 80 in Fig. 1. For use as hybridization probes, oligonucleotides were end labeled with [γ-^{32}P]ATP (ICN Biomedicals; 7000 Ci/mmol; 1 Ci = 37 GBq) and polynucleotide kinase (New England Biolabs) (ref. 15, pp. 122–123) to a specific activity of approximately 2×10^9 cpm/μg.

Construction and Screening of *P. shermanii* Library. Genomic DNA was purified from anaerobically grown *P. shermanii* (strain W52). Cells (5 g, wet weight) were resuspended in 1 M sorbitol/0.1 M EDTA, pH 9, and digested with zymolyase (0.5 mg/ml; Miles) and lysozyme (1 mg/ml; Worthington) for 1 hr at 37°C. Pelleted cells were crushed with a mortar and pestle and rinsed in 0.1 M Tris·HCl, pH 7.5/10 mM EDTA/1% $NaDodSO_4$. Cellular debris was removed by centrifugation, and the supernatant was digested with proteinase K (0.1 mg/ml; Merck) for 1 hr at 37°C. The digestion was followed by extractions with phenol and a precipitation with ethanol. The preparation was resuspended in 10 mM Tris·HCl, pH 7.5/100 mM NaCl and digested with RNase A (0.1 mg/ml; Millipore) for 30 min at room temperature. $NaDodSO_4$ was added to 0.2% prior to redigestion with proteinase K (0.1 mg/ml). The DNA was again extracted with phenol and precipitated in ethanol. The genomic DNA was used to prepare a minilibrary of 1.6- to 1.9-kilobase-pair (kb) *Pst* I fragments. DNA was digested to completion with *Pst* I (International Biotechnologies, New Haven, CT) and subjected to preparative gel electrophoresis. A gel slice containing fragments of 1.6–1.9 kb was excised, and the DNA was eluted (16). The purified *Pst* I fragments were inserted into the *Pst* I site of pUC9 (ref. 15, p. 246) and used to transform *E. coli* HB101 (17). Positive colonies were identified by duplicate filter hybridizations (18) to the ^{32}P-end-labeled probe, TC_1, using previously published conditions (12). Hybridizations and washes were performed at 35°C. Approximately 6% of the transformants contained DNA that hybridized to the oligonucleotide probe.

Plasmid Subclones. The initial plasmid isolate, pTC1.3, contains a 1.7-kb *Pst* I fragment in the *Pst* I site of pUC9 (19), and all subclones are derived from this plasmid insert. Ligations (ref. 15, p. 246), transformations (17), and small- and large-scale plasmid purifications (20) were performed by following published procedures, using DNA modification enzymes obtained from International Biotechnologies, Boehringer Mannheim, or P-L Biochemicals. The plasmid pTC1.3B was constructed, for future ease of manipulations, by *Pst* I digestion and religation of pTC1.3, and it contains the identical insert in the opposite orientation. The *Pst* I insert of pTC1.3 was inserted into the *Pst* I site of pKK223-3 (Pharmacia) to form the plasmid ptac1.3, in which the insert is in the proper orientation for expression from the *tac* promoter of the vector. Digestion of pTC1.3B with *Bam*HI and *Hin*dIII released the plasmid insert and flanking polylinker for insertion into the *Bam*HI and *Hin*dIII sites of pPLc28 (21), forming the plasmid pPL1.3, in which the insert is in the proper orientation for expression from the λ P_L promoter of the vector. The plasmid pTC1.3t contains a shortened insert consisting almost entirely of 1.3S subunit gene sequences in the vector pUC9. It was constructed by insertion of a segment of the pTC1.3 insert, containing one *Pst* I end and one *Sfa*NI end that was made blunt with T4 DNA polymerase, into the *Pst* I and *Sma* I sites of pUC9.

Bacterial Strains. *E. coli* strains used for plasmid propagation and expression were HB101 [F^-, *hsdS20*(r_B^-, m_B^-), *supE44, ara-14, galK2, lacY1, proA2, rpsL20* (streptomycin resistant), *xyl-5, mtl-1*, λ^-, *recA13*] (22); JM105 (Δ*lac–pro, thi, strA, endA, sbcB15, hspR4,* F′*traD36, proAB*$^+$, *lacI*^{q}ZΔM15) (23), K-12ΔH1ΔTrp (M72 streptomycin resistant *lacZ*am, Δ*bio–uvrB*, Δ*trpEA2* [λ*N*am7–*N*am53 *c*I857 ΔH1]) (21), and M5219 (M72 *lacZ*am *trp*am streptomycin resistant [λ*bio*252 *c*I857 ΔH1]) (21).

Immunoprecipitation. Cells (*E. coli* HB101) were grown in M9 minimal medium (24) to midlogarithmic phase and labeled with [^{35}S]methionine (Amersham) for 3 hr. Cells were pelleted and lysed by boiling in 4% $NaDodSO_4$/10 mM dithiothreitol. The lysate was diluted with phosphate-buffered saline (140 mM NaCl/3 mM KCl/8 mM Na_2HPO_4/2 mM KH_2PO_4, pH 7.2) containing 1% Triton X-100, 0.5% sodium deoxycholate and 0.5% $NaDodSO_4$, and incubated overnight with a rabbit antiserum to 1.3S subunit (25) prior to precipitation with staphylococcal protein A (IgGsorb, Enzyme Center, Cambridge, MA) (26). Precipitates were solubilized in protein loading buffer [10% (vol/vol) glycerol/0.1 M dithiothreitol/2% $NaDodSO_4$/80 mM Tris·HCl, pH 6.8/0.002% bromophenol blue) and electrophoresed in $NaDodSO_4$/12% polyacrylamide gels (27). Gels were treated with 1 M sodium salicylate/1% glycerol and dried prior to fluorography (28).

Incorporation of Labeled Biotin. Cells were grown in M9 minimal medium containing [^{14}C]biotin (Amersham; 56 mCi/mmol) at approximately 100 ng/ml. In induced cultures, both [^{14}C]biotin and inducer were added at the time of inoculation. Approximately 1 ml (0.7 OD_{600} unit) of late logarithmic phase cells were lysed in protein loading buffer (same as above), and total cell extracts were electrophoresed in $NaDodSO_4$/15% polyacrylamide gels (27). After being stained with Coomassie blue, gels were prepared for fluorography as described above. Levels of 1.3S subunit accumulation were determined by densitometry.

Purification of 1.3S Subunit. The subunit was partially purified from a sonic extract of pTC1.3-containing *E. coli* HB101 by ammonium sulfate fractionation and chromatography on DEAE-cellulose. Procedures used were those previously described for subunit purification from *P. shermanii* (5).

RESULTS AND DISCUSSION

Molecular Cloning of the Gene Coding for the 1.3S Biotin-Containing Subunit of Transcarboxylase. From the known amino acid sequence of the 1.3S subunit (5), two mixtures of oligonucleotides complementary to independent regions of the gene were synthesized (see *Materials and Methods* and Fig. 1). Genomic DNA was first analyzed in Southern (29) blot experiments. *P. shermanii* DNA was digested with various restriction enzymes, subjected to electrophoresis in agarose gels, and transferred in duplicate to nitrocellulose filters (ref. 15, pp. 383–385). The ^{32}P-labeled oligonucleotides were used as probes in filter hybridizations, and both mixtures hybridized to 1.7-kb fragments in lanes containing *Pst* I-digested DNA (data not shown). One of the synthetic oligonucleotide mixtures was used as a probe in hybridizations to a *P. shermanii* library of 1.6- to 1.9-kb *Pst* I fragments in the plasmid pUC9. Several positive transformants were identified, and one, containing a plasmid designated pTC1.3, was selected for further analysis. A portion of the plasmid insert containing the 1.3S gene was sequenced (30), and the nucleotide sequence was found to be entirely consistent with the previously published amino acid sequence (Fig. 1; ref. 5).

The 1.3S subunit gene is positioned within the insert such that the beginning of protein coding sequence is 40 bp from one of the *Pst* I sites. In pTC1.3, the 1.3S subunit sequence is in the same transcriptional orientation as the *lacZ′* gene of pUC9; therefore, transcription of the plasmid insert from the *lac* promoter will utilize the coding strand of the 1.3S subunit gene. Within the insert, there is a termination codon at −8 (see Fig. 1), meaning that the *lacZ* translation product terminates upstream of 1.3S subunit-encoding sequences and that production of a fusion protein containing the NH_2 terminus of β-galactosidase is not possible. In addition, the *lacZ* and 1.3S protein coding sequences are not in the same

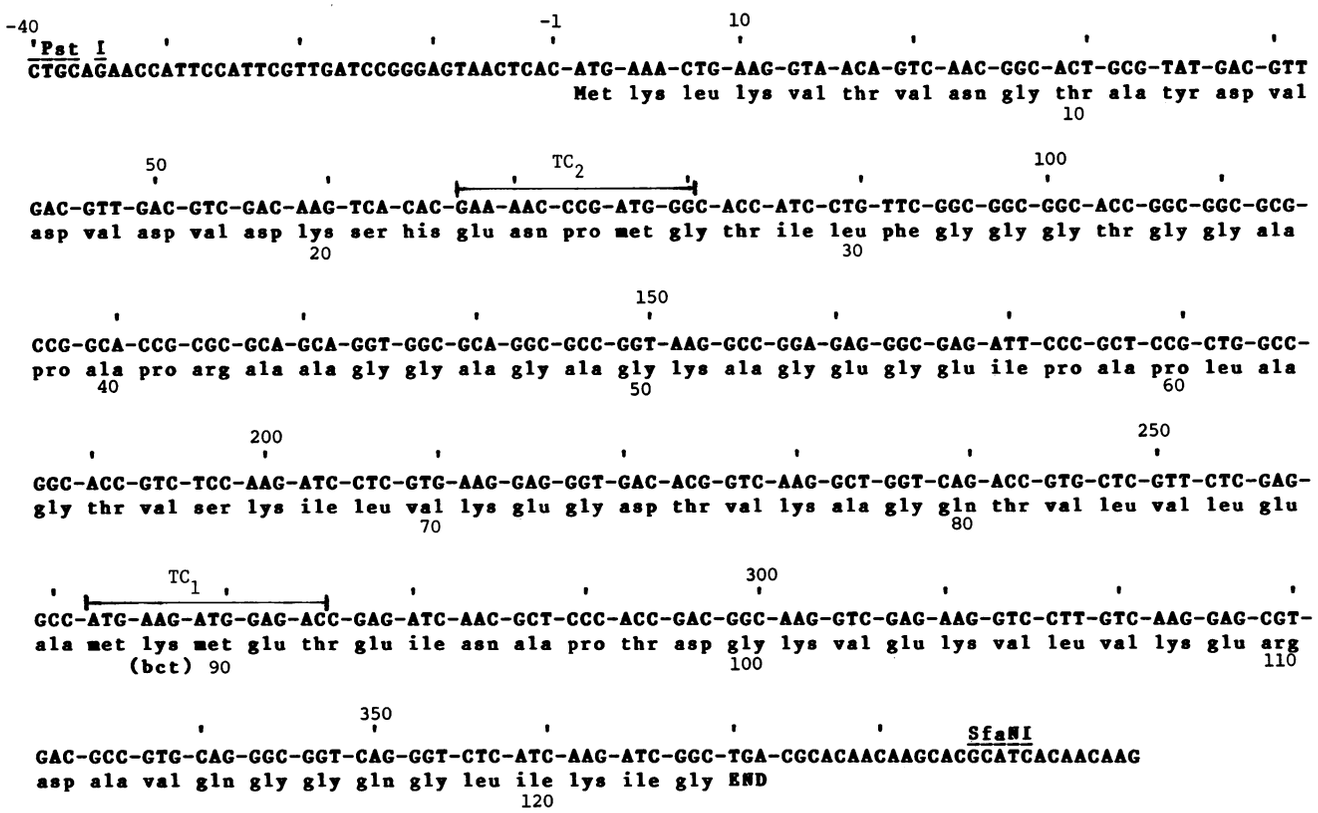

FIG. 1. DNA and amino acid sequences of the 1.3S subunit. The upper line is the noncoding strand DNA sequence of the 1.3S subunit gene and short segments of flanking sequence, as determined from the plasmid pTC1.3 (30). The beginning of the displayed sequence is one end of the 1.7-kb *Pst* I fragment isolated from *P. shermanii*. The other *Pst* I end is *ca.* 1.3 kb downstream of the 3′ flanking sequence shown. The previously determined amino acid sequence (5) is aligned below the DNA sequence. The positions within the gene of sequences complementary to the oligonucleotide probes, TC_1 and TC_2, are shown. The positions of restriction sites discussed in the text are also indicated. In the plasmid pTC1.3, the 1.3S subunit gene is in the proper orientation for expression from the *lac* promoter of pUC9. The reading frame of a *lacZ* translation product is such that nucleotides −40 and −39 are the second and third bases of a codon (19). In this reading frame, there is a termination codon (TAA) beginning at −8. The reading frames required by *lacZ* and 1.3S are not the same.

reading frame (see Fig. 1 legend). Therefore, as it is positioned in pTC1.3, a translation product of the 1.3S subunit gene will contain the NH_2 terminus of the authentic subunit.

Immunoprecipitation of 1.3S Protein. Expression of 1.3S polypeptide in pTC1.3 containing cells was demonstrated by immunoprecipitation. *E. coli* cells (HB101) containing pTC1.3 were labeled with [^{35}S]methionine, and cell extracts were incubated with an antiserum to 1.3S subunit (25). IgG was precipitated by addition of protein A, and samples were fractionated in $NaDodSO_4$/polyacrylamide gels and subjected to fluorography. The major immunoprecipitable peptide comigrated with authentic 1.3S subunit and was present only in cultures containing the plasmid pTC1.3 (Fig. 2), indicating that the plasmid-encoded gene for the 1.3S subunit is expressed.

Incorporation of Labeled Biotin. Extracts of cells grown in the presence of [^{14}C]biotin were subjected to electrophoresis in $NaDodSO_4$/polyacrylamide gels (Fig. 3*A*). Fluorography revealed the presence of two labeled proteins in pTC1.3-containing cells (Fig. 3*B*, lane b). The more slowly migrating band is also present in HB101 control extracts (compare Fig. 3*B*, lane f), and its approximate M_r of 20,000 corresponds to the size of the biotin-containing subunit of acetyl-CoA carboxylase (31), an endogenous biotin enzyme in *E. coli*. The intensely labeled band at 12,000 M_r is present only in extracts of plasmid-containing cells and comigrates with authentic 1.3S subunit. Incorporation of labeled biotin was also observed in cells containing a plasmid with a 400-bp insert, in which the *ca.* 1.3 kb of unidentified *P. shermanii* sequences downstream of the 1.3S subunit gene were eliminated (pTC1.3t; Fig. 3*B*, lane c), thus indicating that biotin holoenzyme synthetase of *E. coli* is capable of biotin attachment. Reduced accumulation of 1.3S polypeptide is observed with pTC1.3t (compare lanes b and c in Fig. 3). The only difference between pTC1.3 and pTC1.3t is in the transcribed sequences downstream of the 1.3S subunit gene. A possible explanation of the reduced accumulation is that the pTC1.3t transcript has reduced stability.

These results demonstrate that *E. coli* biotin holoenzyme synthetase is able to recognize and biotinate the foreign protein, presumably at lysine-89. Biotin holoenzyme synthetases of various organisms, including mammals and bacteria, are known to have a broad specificity of action (32–34). As mentioned in the introduction, several biotin enzymes have been shown to share certain features of amino acid sequence. The introduction of modifications within the conserved region can be used to analyze requirements for proper recognition by synthetases.

Assay of Enzymatic Activity. The 1.3S polypeptide was partially purified from pTC1.3-containing *E. coli* extracts to test enzymatic activity. An enriched 1.3S protein preparation was used in partial reaction A in an *in vitro* assay that measures transfer of [^{14}C]carboxyl groups from [^{14}C]methylmalonyl-CoA to 1.3S subunits (35). For the assay, the partially purified protein was combined with [^{14}C]methylmalonyl-CoA in the presence of 12S subunit purified from *P. shermanii*. [^{14}C]Methylmalonyl-CoA is stable, and ^{14}C transferred to the 1.3S subunit as a carboxyl group is labile in the presence of acid and heat, thus allowing the reaction to be assayed by the reduction in cpm (Fig. 4). The pTC1.3-encoded protein fully substituted for authentic 1.3S subunit of *P. shermanii* in partial reaction A. Therefore, the plasmid-

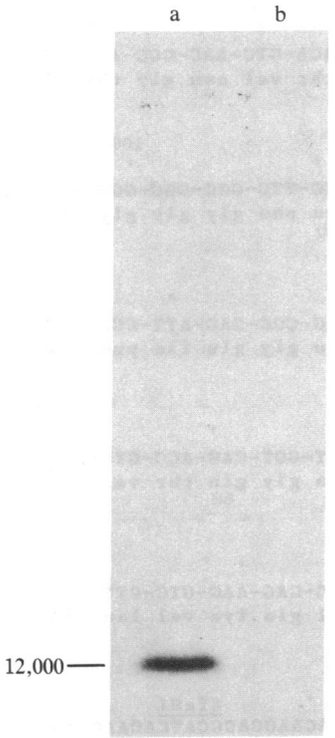

FIG. 2. Immunoprecipitation of 1.3S subunit. Cells (*E. coli* HB101) were labeled with [^{35}S]methionine, and immunoprecipitation was performed with an antiserum to 1.3S subunit and protein A. A fluorogram of precipitated material that was fractionated by NaDodSO$_4$/polyacrylamide gel electrophoresis is shown. Lane a, cells containing the recombinant plasmid, pTC1.3; lane b, cells containing the plasmid vector, pUC9.

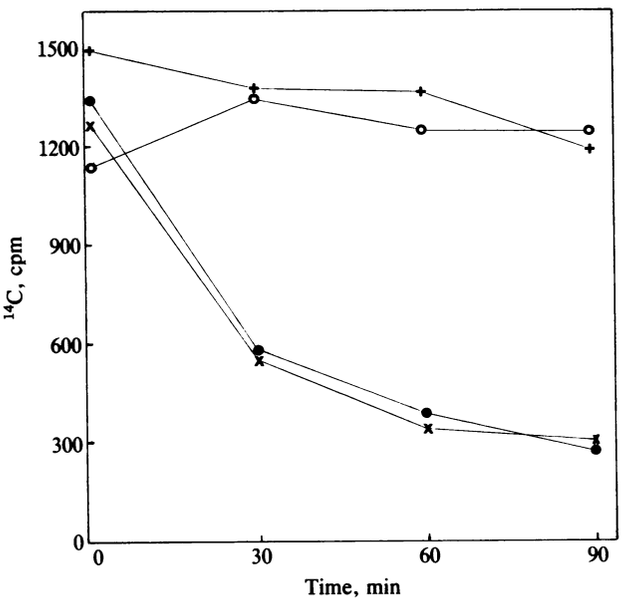

FIG. 4. Enzymatic activity of 1.3S subunit purified from *E. coli*. Partially purified 1.3S polypeptide from pTC1.3-containing HB101 was used in an *in vitro* assay of partial reaction A. The 12S subunit of transcarboxylase purified from *P. shermanii* and [^{14}C]methylmalonyl-CoA were incubated with 1.3S subunit purified from *P. shermanii* or from *E. coli* cells containing the plasmid pTC1.3. Carboxyl transfer was assayed as a decrease in heat-and-acid-stable cpm. ●, 12S subunit and 1.3S subunit from *P. shermanii*; ×, 12S subunit of *P. shermanii* and 1.3S subunit expressed in *E. coli*; +, 1.3S subunit (*E. coli*) alone; ○, 12S subunit (*P. shermanii*) alone.

encoded protein is biotinated by *E. coli* synthetase, and functional subunit is produced.

Regulated Expression of 1.3S Polypeptide. The isolated fragment containing the 1.3S subunit gene contains 40 bp of DNA upstream of the beginning of protein coding sequence and approximately 1.3 kb of sequence downstream of the gene. The 1.7-kb fragment, originally cloned in pUC9 (pTC1.3), was inserted in two plasmids containing strong inducible promoters. The plasmid pPLc28 contains the λ P_L promoter; regulation of expression of cloned sequences is

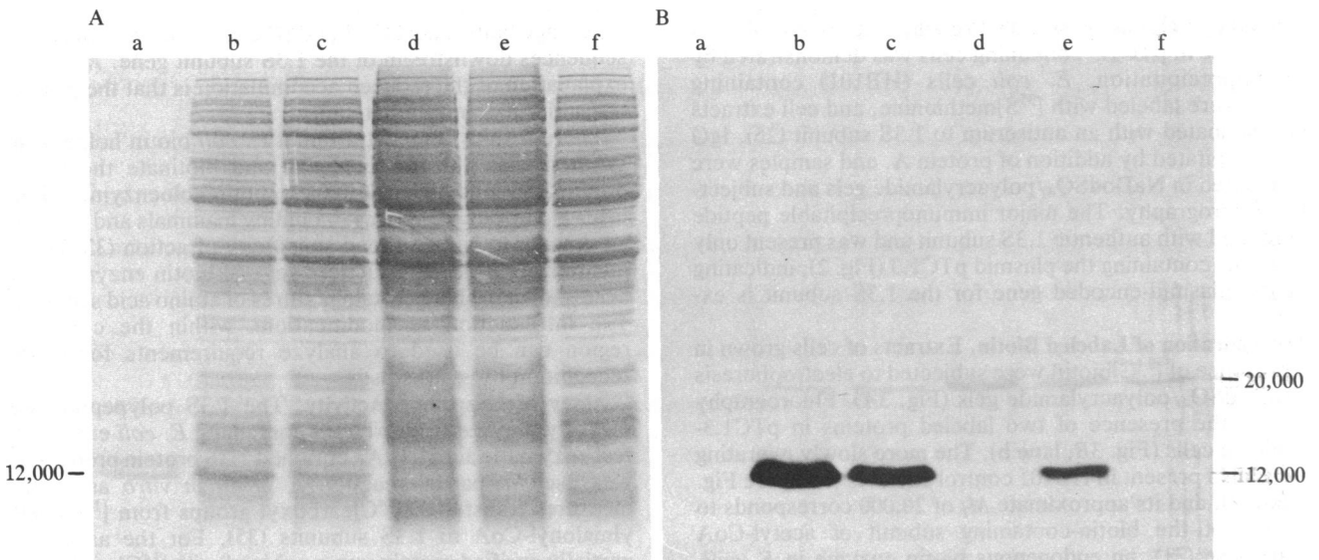

FIG. 3. Accumulation of 1.3S subunit and incorporation of [^{14}C]biotin. Cells were grown at 42°C in the presence of [^{14}C]biotin, and total cell extracts were fractionated by NaDodSO$_4$/polyacrylamide gel electrophoresis. (*A*) Coomassie blue staining pattern of the gel; (*B*) resulting fluorogram. Lane a, 1.3S subunit (approximately 0.5 nmol) purified from *P. shermanii*. The protein is apparent only in panel A. The upper band is the intact polypeptide. The COOH terminus (approximately five amino acids) is highly susceptible to proteolysis (5), and in this preparation, most of the protein is in the short form (lower band). The plasmid-encoded proteins present in total cell extracts of lanes b, c, and e (more readily observed in *B*) comigrate with the short form of the 1.3S subunit. Lane b, pTC1.3 in HB101; lane c, pTC1.3t in HB101; lane d, ptac1.3 in JM105 (uninduced); lane e, ptac1.3 in JM105 in the presence of 2 mM isopropyl β-D-thiogalactoside; lane f, HB101.

controlled by the presence of a temperature-sensitive repressor (c*I*857), such as in the λ lysogen of *E. coli*, ΔH1ΔTrp. The 1.7-kb fragment was introduced into pPLc28, in the proper orientation for expression of the 1.3S subunit gene, to obtain the plasmid pPL1.3. The vector pKK223-3 contains the *tac* promoter, and expression is repressed in a *lacI*^{q} strain such as JM105. Expression from the *tac* promoter is induced by addition of isopropyl β-D-thiogalactoside. The *tac*-containing recombinant is called ptac1.3. Regulation of expression was observed in strains containing the recombinant inducible plasmids. Regulated expression of 1.3S subunit from ptac1.3 is shown (Fig. 3*B*, lanes d and e); similar levels of expression were observed with pPL1.3 (data not shown). With the inducible plasmids, levels of accumulation of 1.3S polypeptide were lower than the level observed with pTC1.3 in HB101, where 1.3S polypeptide accumulates to 7% of total cellular protein when cells are grown at 42°C. (Accumulation is reduced by about 50% in cells grown at 37°C, for unknown reasons.) There are several parameters that may affect levels of accumulation, including plasmid copy number, rates of transcription and translation, and transcript and protein stability. Although these variables have not been investigated, it was observed that the depressed accumulation observed with pPL1.3 was strain specific, and the accumulation level rose to that observed with pTC1.3 when pPL1.3 was introduced into HB101. The increased expression was at the expense of regulation.

The absence of detectable expression of 1.3S subunit from inducible plasmids maintained in the repressed state indicates that the isolated *P. shermanii* fragment does not contain a transcriptional promoter that functions in *E. coli*. A promoter must be supplied upstream of the 1.3S subunit gene to achieve appreciable levels of expression of 1.3S polypeptide. Once transcribed, the upstream flanking sequence of the cloned *P. shermanii* fragment is able to support translation, presumably through the use of a potential Shine–Delgarno sequence of GGAG beginning at −12.

Concluding Remarks. The gene coding for the 1.3S subunit of transcarboxylase can be expressed at high levels in *E. coli* to form enzymatically functional subunit. Modifications within the gene can be constructed to assess requirements for subunit interactions, for synthetase recognition, and for carboxyl transfer through biotin. The question of recognition by biotin holoenzyme synthetase can be addressed by altering the distance of the biocytin from the COOH terminus of the polypeptide and by introducing single amino acid changes within the highly conserved sequence flanking the biocytin.

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1. Wood, H. G. (1979) *CRC Crit. Rev. Biochem.* **7**, 143–160.
2. Wood, H. G., Ahmad, F., Jacobson, B., Chuang, M. & Brattin, W. (1975) *J. Biol. Chem.* **250**, 918–926.
3. Bahler, C., Goss, N. H., Poto, E. M. & Wood, H. G. (1981) *Biochem. Int.* **3**, 349–358.
4. Ahmad, F., Jacobson, B., Chuang, M., Brattin, W. & Wood, H. G. (1975) *Biochemistry* **14**, 1606–1611.
5. Maloy, W. L., Bowien, B. U., Zwolinski, G. K., Kumar, K. G., Wood, H. G., Ericsson, L. H. & Walsh, K. A. (1979) *J. Biol. Chem.* **254**, 11615–11622.
6. Kumar, G. K., Bahler, C. R., Wood, H. G. & Merrifield, R. B. (1982) *J. Biol. Chem.* **257**, 13828–13834.
7. Lane, M. D., Young, D. L. & Lynen, F. (1964) *J. Biol. Chem.* **239**, 2858–2864.
8. Wood, H. G., Harmon, F. R., Wuhr, B., Hubner, K. & Lynen, F. (1980) *J. Biol. Chem.* **255**, 7397–7409.
9. Craft, D. V., Goss, N. H., Chandramouli, N. & Wood, H. G. (1985) *Biochemistry* **24**, 2471–2476.
10. Rylatt, D. B., Keech, D. B. & Wallace, J. C. (1977) *Arch. Biochem. Biophys.* **183**, 113–122.
11. Sutton, M. R., Fall, R. R., Nervi, A. M., Alberts, A. W., Vagelos, P. R. & Bradshaw, R. A. (1977) *J. Biol. Chem.* **252**, 3934–3940.
12. Freytag, S. O. & Collier, K. J. (1984) *J. Biol. Chem.* **259**, 12831–12837.
13. Miyoshi, K., Arentzen, R., Hoang, L. & Itakura, K. (1980) *Nucleic Acids Res.* **8**, 5507–5517.
14. Carathers, M. H., Beaucage, S. I., Efcavitch, J. W., Fisher, E. F., Goldman, R. A., deHaseth, P. L., Mandecki, W., Matteucci, M. D., Rosendahl, M. S. & Stabinsky, Y. (1982) *Cold Spring Harbor Symp. Quant. Biol.* **47**, 411–418.
15. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
16. Vogelstein, B. & Gillespie, D. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 615–619.
17. Hanahan, D. (1983) *J. Mol. Biol.* **166**, 557–580.
18. Hanahan, D. & Meselson, M. (1980) *Gene* **10**, 63–67.
19. Vieira, J. & Messing, J. (1982) *Gene* **19**, 259–268.
20. Birnboim, H. C. (1983) *Methods Enzymol.* **100**, 243–255.
21. Remaut, E., Stanssens, P. & Fiers, W. (1981) *Gene* **15**, 81–93.
22. Boyer, H. W. & Roulland-Dussoix, D. (1969) *J. Mol. Biol.* **41**, 459–472.
23. Yanisch-Perrone, C., Vieira, J. & Messing, J. (1985) *Gene* **33**, 103–119.
24. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), p. 431.
25. Berger, M. & Wood, H. G. (1976) *J. Biol. Chem.* **251**, 7021–7034.
26. Tucci, A., Goldberger, G., Whitehead, A. S., Kay, R. M., Woods, D. E. & Colten, H. R. (1983) *J. Immunol.* **131**, 2416–2419.
27. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
28. Chamberlain, J. P. (1979) *Anal. Biochem.* **98**, 132–135.
29. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503–517.
30. Maxam, A. M. & Gilbert, W. (1980) *Methods Enzymol.* **65**, 499–560.
31. Fall, R. R., Alberts, A. W. & Vagelos, P. R. (1975) *Biochim. Biophys. Acta* **379**, 496–503.
32. Kosow, D. P., Huang, S. C. & Lane, M. D. (1962) *J. Biol. Chem.* **237**, 3633–3639.
33. Rominger, K. L. (1964) Dissertation (University of Munich, Munich, Federal Republic of Germany).
34. McAllister, H. C. & Coon, M. J. (1966) *J. Biol. Chem.* **241**, 2855–2861.
35. Chuang, M., Ahmad, F., Jacobson, B. & Wood, H. G. (1975) *Biochemistry* **14**, 1611–1619.