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Cloning, Structure, and Expression of the *Escherichia coli* K-12 *hisC* Gene

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We used an expression vector plasmid containing the *Escherichia coli* K-12 histidine operon regulatory region to subclone the *E. coli hisC* gene. Analysis of plasmid-coded proteins showed that *hisC* was expressed in minicells. A protein with an apparent molecular weight of 38,500 was identified as the primary product of the *hisC* gene. Expression was under control of the *hisGp* promoter and resulted in very efficient synthesis (over 100-fold above the wild-type levels) of imidazolylacetolphosphate:L-glutamate aminotransferase, the *hisC* gene product. The complete nucleotide sequence of the *hisC* gene has been determined. The gene is 1,071 nucleotides long and codes for a protein of 356 amino acids with only one histidine residue.

The histidine operon of *Salmonella typhimurium* and *Escherichia coli* is a cluster of nine genes that maps at 44 min on the bacterial chromosome (4, 52). In the past this operon has been the subject of extensive studies concerning its genetics, biochemistry, and regulation (10, 13). More recent studies, which made use of recombinant DNA technology, have been directed to elucidate the mechanism of operon regulation (5, 19, 22, 27–29, 57) and the molecular structure and expression of the operon (2, 6, 7, 14, 16, 24, 25). Despite the extensive knowledge gained on this system, very little is known about the composition and structure of the primary gene products. In fact, the corresponding protein has been purified and characterized for only four of the nine genes: *hisG* (59), *hisD* (9, 33, 61), *hisC* (39, 41, 43), and *hisA* (37, 38). Moreover, only for the *hisG* (47) and *hisD* (31) gene products has the amino acid sequence been established.

Among these genes, the third structural gene, *hisC*, is of particular interest. Mutations in this gene exhibit a strong polarity gradient (21, 42). Mutations have been characterized between *hisD* and *hisC* which lead to the synthesis of a fused protein possessing both enzyme activities (48, 49, 62). A transcription initiation site (*hisBp*) which expresses the downstream genes is located within the *hisC* structural gene (25). The *hisC* gene codes for imidazolylacetolphosphate:L-glutamate aminotransferase (EC 2.6.1.9), which catalyzes the conversion of imidazolylacetolphosphate to L-histidinol phosphate, the eighth step in histidine biosynthesis (13).

In the present study we have determined the complete nucleotide sequence of the *hisC* gene, 1,071 base pairs (bp) long, coding for a protein of 356 amino acids with an apparent molecular weight of 39,315. The gene product was identified in a cell-free translation system by subcloning the gene in an expression vector which directs synthesis of the aminotransferase at high efficiency.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used are listed in Table 1. Construction of plasmids pCB3 (14), pHC9800 (25), and pHS10000 (16) has already been described. Details of the construction of histidine recombinant plasmids pHC730, pHC1600a, and pHC1600b are given below.

Media and growth conditions. The liquid media used were L broth (45) and minimal medium (58) supplemented with 0.5% glucose. Solid media contained 1.2% agar (Difco Laboratories, Detroit, Mich.) and were nutrient broth (45) and minimal medium (58) supplemented with 0.5 glucose. Amino acids were added at 0.5 mM, and L-histidine was added at 0.1 mM. Tetracycline and ampicillin were added to both liquid and solid media at 25 and 50 μg/ml, respectively.

Chemicals and enzymes. All amino acids and histidinol phosphate were obtained from Sigma Chemical Co., St. Louis, Mo. Acrylamide, methylene bisacrylamide, ammonium persulfate, *N,N,N',N'*-tetramethylenediamine, and agarose were from Bio-Rad Laboratories, Richmond, Calif. [γ-^{32}P]ATP (3,000 Ci/mmol), L-[^{35}S]methionine (800 Ci/mmol), and restriction endonucleases were purchased from Amersham Corp., Arlington Heights, Ill. *Pst*I was purified as previously described (8). Calf intestine alkaline phosphatase and T4 polynucleotide kinase were from Boehringer Mannheim Corp., New York, N.Y. T4 ligase was from Miles Laboratories Inc., Elkhart, Ind. Terminal deoxynucleotidyl transferase was from P-L Biochemicals. Unlabeled or ^{32}P-labeled pBR322 DNA digested with *Hin*fI was used as a molecular weight standard on acrylamide gels. A *Hin*dIII digest of λ DNA was used as a molecular weight standard on agarose gels.

Enzymatic assays. Assay procedures for the histidine pathway enzyme imidazolylacetolphosphate:L-glutamate aminotransferase have been described elsewhere (40). Proteins were measured by the method of Lowry et al. (34).

DNA fractionation and labeling. Plasmid DNA was prepared as described elsewhere (17). For screening large numbers of transformants, plasmid DNA was purified by a rapid method described elsewhere (18). DNA fragments were isolated on and purified from preparative 5% acrylamide slab gels by electroelution as described elsewhere (35). 5'-terminal labeling of DNA fragments was performed with T4 polynucleotide kinase and [γ-^{32}P]ATP as described elsewhere (44), except that calf intestine alkaline phosphatase was used instead of bacterial alkaline phosphatase and the enzyme was inactivated by heating for 10 min at 75°C.

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TABLE 1. *E. coli* K-12 strains[a]

Strain	Relevant genotype or phenotype	Reference
FB1	Δ(*hisGDCBHAFIE*)*750 gnd rhaA*	25
FB8	Wild-type *E. coli* K-12 UTH1038	30
FB194	*hisG2743 recA56* (pCB3)	14
FB257	Δ(*hisGDCBHAFIE*)*750 gnd rhaA* (pHC9800)	25
FB273	Δ(*hisGDCBHAFIE*)*750 gnd rhaA* (pHC1600b)	This paper
FB283	Δ(*hisGDCBHAFIE*)*750 gnd rhaA* (pHC1600a)	This paper
DS998	*thr leu thi* λ^s $\phi 80^r$ Str^r	14

[a] Genetic symbols are those given by Bachmann (4).

Restriction maps. The restriction map of the plasmid DNA was determined by using restriction sites uniquely present in the vector or cloned DNA as described below. Fine mapping of the *Bgl*II 1,650-bp fragment was performed by double digestions with several restriction enzymes.

Cloning and transformation. Plasmid pHC730 was obtained by cloning a *Hin*fI 730-bp DNA fragment which contained the regulatory region of the *his* operon, including the primary promoter *hisGp* (57), into pBR322 (55) at the single *Pst*I site by G-C tailing (36). The DNA mixtures were used to transform bacterial strains as previously described (14). The presence and estimated copy number of plasmids in transformed cells used for enzyme assays were checked by dot-blot hybridization by the method of Thomas (56), with nick-translated pBR322 DNA used as the probe.

DNA sequencing. DNA sequencing was performed by the technique of Maxam and Gilbert (44). The 1,650-bp *Bgl*II fragment, whole or digested with several restriction enzymes, was terminally labeled at the 5′ end. Fragments labeled at only one end were obtained either by strand separation or by secondary restriction enzyme cleavage as described elsewhere (44).

Protein synthesis in minicells. The minicell-producing strain DS998 was transformed with purified plasmid DNA. Transformants containing plasmid pHC1600a, pHC1600b, or pHC9800 were selected on L broth plates containing tetracycline (10 μg/ml) or ampicillin (50 μg/ml) and grown overnight in L broth containing the same concentrations of antibiotics. Minicells were purified and labeled with L-[^{35}S]methionine as previously described (14). L-[^{35}S]methionine (10 to 20 μCi) was added to the minicells obtained from 10 ml of culture. Labeled minicells were diluted 1:1 with twofold-concentrated denaturing solution (0.13 M Tris hydrochloride, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol, 10% β-mercaptoethanol, and 0.002% bromophenol blue) (32) and immersed for 5 min in boiling water. Slab gel electrophoresis (10% polyacrylamide in sodium dodecyl sulfate) was done as described elsewhere (32). The gel was prepared for fluorography (12), dried under vacuum, and exposed to Kodak X-Omat film.

RESULTS

Subcloning of the *hisC* gene. We have previously reported the cloning of the entire *his* operon of *E. coli* K-12 in a pBR313 vector (25). The resulting plasmid, pHC9800 (18,500 bp), when digested with restriction endonuclease *Bgl*II, gave rise to a 1,650-bp fragment spanning an internal *Hin*dIII site. The distal region of the *hisC* gene (427 bp) was mapped between this *hin*dIII site and the distal *Bgl*II site (25). From previous estimates of the molecular size of the *hisC* gene product (41, 48), it was very likely that the entire *hisC* gene was contained in the *Bgl*II 1,650-bp fragment. We therefore isolated this fragment and established a detailed restriction map for it (Fig. 1).

The *Bgl*II 1,650-bp fragment was subcloned in plasmid pHC730, a pBR322 derivative containing the *E. coli* K-12 *his* regulatory region (Fig. 2). The fragment was cloned in the unique *Bgl*II site of the vector located between the *hisGp* promoter and the attenuator (57). The ligated DNA mixture was used to transform strain FB1 harboring a deletion of the entire *his* operon. Recombinants were identified by a rapid plasmid procedure (see Materials and Methods) and by sizing the DNA on agarose gels. Several recombinant plasmids were digested with *Hin*dIII to establish the relative orientation of the insert with respect to the vector pHC730.

Two plasmids harboring the insert in opposite orientation were retained for further study and were named pHC1600a and pHC1600b, respectively (Fig. 2).

Expression of the *hisC* gene product. To demonstrate that the *Bgl*II 1,650-bp fragment contained the entire *hisC* gene, we measured and compared the enzyme levels of several strains harboring different plasmids. The *hisC* gene can only be expressed if there is a functional promoter in the correct orientation. We therefore anticipated that aminotransferase activity would be detected in strain FB283 (pHC1600a) but not in strain FB273 (pHC1600b) (Fig. 2), and this was indeed

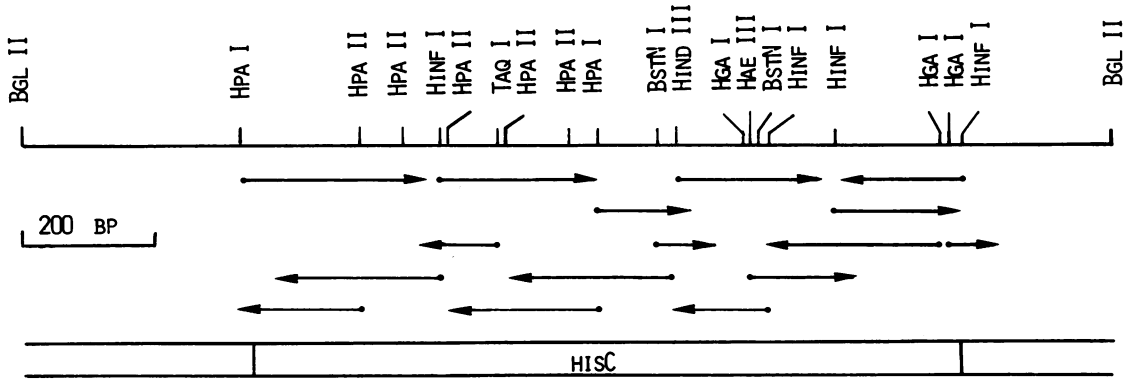

FIG. 1. Restriction endonuclease cleavage sites and sequencing strategy for the *Bgl*II 1,650-bp fragment containing the *hisC* structural gene. Arrows indicate the direction and distance that each fragment was sequenced; solid circles at the beginning of the arrows indicate the position of the ^{32}P label at the 5′ end. Only restriction sites used for the sequencing experiments are shown. The relative position of the *hisC* gene is indicated on the bottom line.

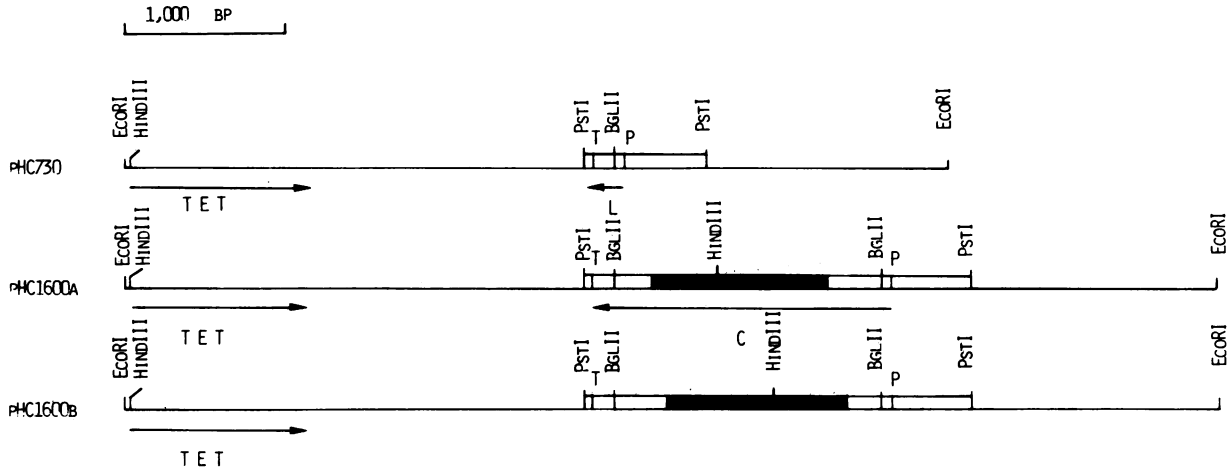

FIG. 2. Physical map of the recombinant histidine plasmids. The circular plasmid DNAs were schematically linearized at the unique *Eco*RI site of vector pBR322. Solid bars represent cloned fragments. Arrows indicate the orientation of the tetracycline resistance genes (TET) and the transcripts initiated at the *hisGp* (P) promoter and terminated at the *his* attenuator (T), *his* leader RNA (L), and *hisC* mRNA (C). Plasmid pHC730 contains the *his* operon regulatory region (57). Plasmids pHC1600a and pHC1600b contain the *Bgl*II 1,650-bp fragment, including the *hisC* structural gene (solid area), cloned in the unique *Bgl*II site of pHC730 in opposite orientations. The *Hin*dIII sites used to verify the relative orientation of the insert are also shown.

the case (Table 2). Strain FB257 harboring the complete histidine plasmid had about 20-fold-higher levels of enzyme than the wild-type strain FB8 did, presumably due to the presence of multiple copies of the plasmid (11, 25, 26). Strain FB283 had sixfold-higher aminotransferase levels than FB257. This was not due to differences in the plasmid copy number, since the copy number was found to be comparable in different strains (see Materials and Methods). The increase reflects expression of *hisC* from the *hisGp* promoter in the absence of the attenuator.

To identify the *hisC* gene product, the different plasmids were used to transform the minicell-producing strain DS998, and the in vivo-synthesized proteins were analyzed on sodium dodecyl sulfate-polyacrylamide gels (32) (Fig. 3). Plasmid pHC9800 synthesized several proteins, since it contained the entire *his* operon (lane 2). Plasmid pCB3 (lane 5) is a *his*-proximal plasmid which only expresses the *hisG* and *hisD* gene products of molecular weight 33,500 and 47,000, respectively (14). pHC1600a (lane 4) but not pHC1600b (lane 3) directed the synthesis of a single protein, coincident with one present in pHC9800, with an apparent molecular weight of 38,500. The protein apeared as a rather faint band compared with the other *his*- and plasmid-specific products, probably due to the low methionine content of the aminotransferase (see below). A band of the same size was also present when pHS10000, a plasmid containing the entire *his* operon of *S. typhimurium* (16), was used to direct protein synthesis in minicells (data not shown). This protein should correspond to the *hisC* primary gene product. The molecular weight of the aminotransferase subunit has been inferred to be 29,500 by ultracentrifugation (41). Anomalous slower migration on denaturing gels has been observed (48). Un-

TABLE 2. Levels of aminotransferase in *E. coli* K-12 strains

Strain	Relevant genotype (plasmid)	Aminotransferase activity[a]
FB1	Δ*his*	<0.1
FB8	Wild type	0.4
FB257	Δ*his* (pHC9800)	7.5
FB273	Δ*his* (pHC1600b)	<0.1
FB283	Δ*his* (pHC1600a)	45.0

[a] Enzyme-specific activity is expressed as described by Martin et al. (40). Cells were grown in L broth (45).

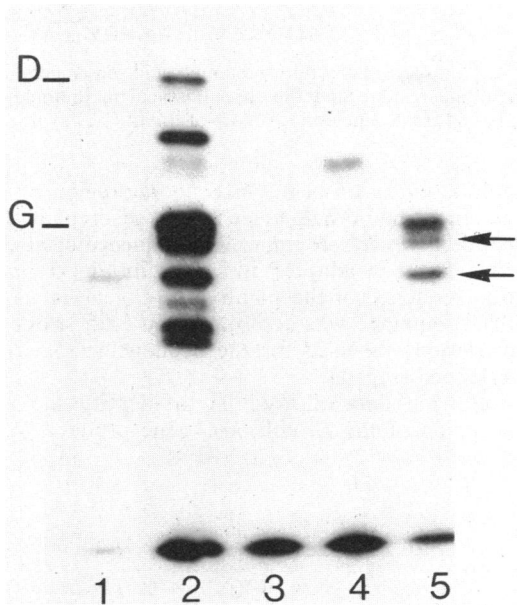

FIG. 3. Protein synthesis in minicell-producing strains transformed with histidine plasmids. ^{35}S-labeled proteins were analyzed on 10% sodium dodecyl sulfate-polyacrylamide gels by electrophoresis and fluorography (see the text). Lanes show proteins synthesized in cells carrying pBR322 (lane 1), the complete histidine plasmid pHC9800 (25) (lane 2), *hisC* plasmids pHC1600b (lane 3), pHC1600a (lane 4), and *his*-proximal plasmid pCB3 (lane 5). The bands corresponding to histidinol dehydrogenase (D), the product of the *hisD* gene, and to phosphoribosyl synthetase (G), the product of the *hisG* gene, are indicated. Molecular weight markers were run in parallel. The vector plasmid-specific ampicillin resistance proteins (molecular weights 31,000 and 28,000, respectively [51]) are indicated by arrows.

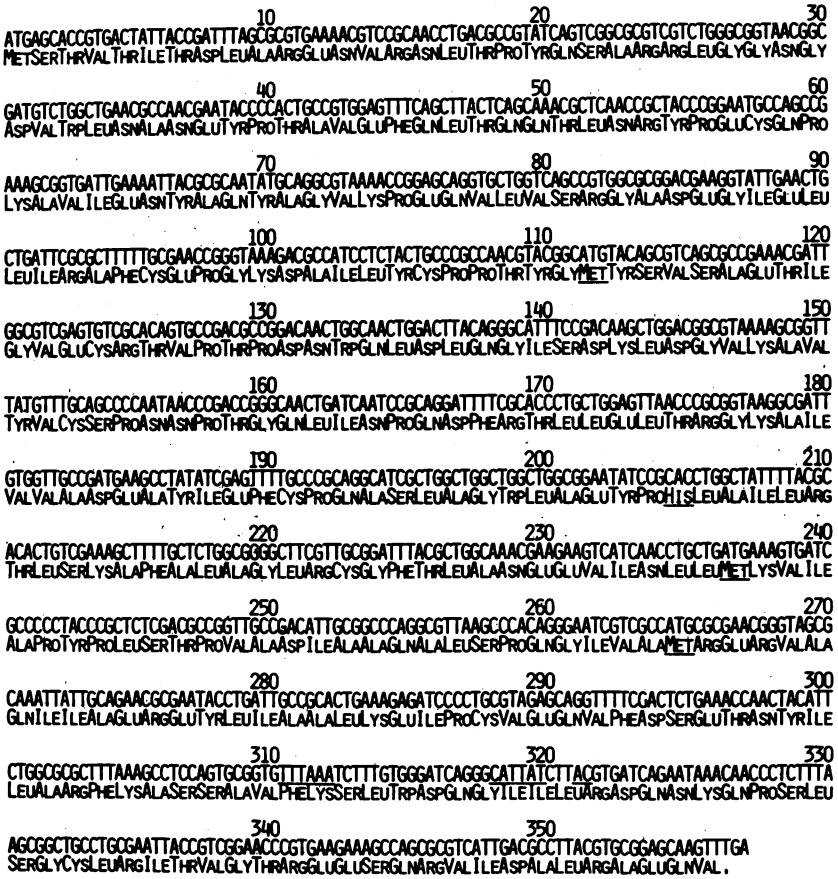

FIG. 4. Complete nucleotide and amino acid sequence of *E. coli hisC*. Amino acids are numbered from the initial methionine residue. The single histidine residue and the three internal methionine residues are underlined. The −35 (TTTAAA) and Pribnow box (CATTAT) sequences and the 5′ nucleotides (AC) of the mRNA synthesized from the internal *hisBp* promoter (25) are also underlined.

equivocal identification of the *hisC* coding region by nucleotide sequence should help clear up this discrepancy.

Nucleotide and derived amino acid sequence of *hisC*. The sequencing strategy adopted is shown in Fig. 1, and the nucleotide sequence of the putative *hisC* gene is shown in Fig. 4. The sequence was determined on both strands, and each restriction site used for the sequencing experiments was overlapped (Fig. 1).

The following data allowed us to identify the amino-terminal region of the *E. coli hisC* gene product. We had previously determined the amino-terminal sequence of the first 19 amino acid residues of the *S. typhimurium* wild-type aminotransferase by successive Edman degradations (49). Very recently, Riggs and Artz determined the nucleotide sequence of the *hisD-hisC* gene border of *S. typhimurium* (50). The nucleotide and amino acid sequences of *S. typhimurium* were aligned with the corresponding regions of *E. coli* (Fig. 5). An identical Shine-Dalgarno box (AAGGAG) (54) preceded the ATG initiation codon in both species. Both the amino acid and nucleotide sequences were very similar,

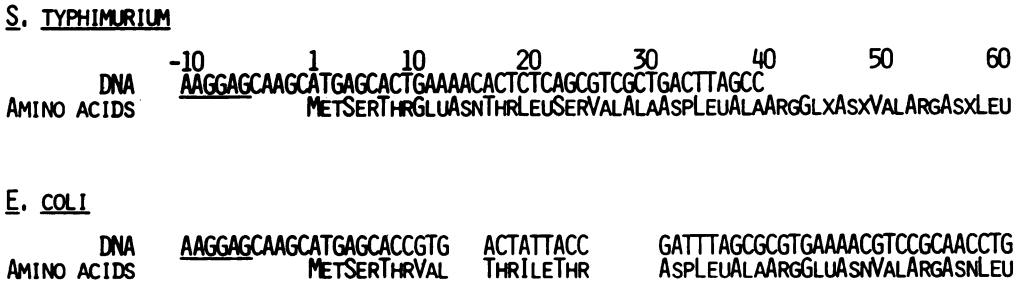

FIG. 5. Amino-terminal region of *S. typhimurium* and *E. coli hisC* genes. The *S. typhimurium* DNA sequence is from Riggs and Artz (50), and the amino acid is sequence from Rechler et al. (49). The two identical ribosomal binding sites are underlined. The two sequences were aligned for maximum homology. Nucleotides are numbered from the first nucleotide in the ATG initiation codon of *S. typhimurium*. The *E. coli* gene is 9 nucleotides shorter in the region between nucleotides 11 and 30.

except that the region of DNA 21 bp long corresponding to residues 4 to 10 in *S. typhimurium* was only 12 nucleotides long in *E. coli* and all but one of the corresponding four amino acids were different from those present in *S. typhimurium*. In view of this difference it is likely that the amino-terminal region of the aminotransferase is not important for proper function.

Starting from the ATG initiation codon, there was one open reading frame 1,071 nucleotides long which could be decoded in a polypeptide of 356 amino acids (Fig. 4). The carboxyl-terminal region of the gene and the terminal amino acid valine have been identified previously (25, 43). The most striking features of the amino acid composition of the *hisC* gene product are the near absence of histidine residues (only one) and the paucity of methionine condons (only three) (Table 3). The apparent molecular weight of the aminotransferase was calculated to be 39,339. The codon preferences observed in the *hisC* gene of *E. coli* (Fig. 6) are in agreement with general trends that have been observed in other *E. coli* biosynthetic genes (23). The most striking ones are the nonuse of codon ATA for isoleucine, the absence or rare use of codons CGA, CGG, AGA, and AGG for arginine, and the preference for codon CTG for leucine.

DISCUSSION

The DNA sequence reported here establishes the primary structure of the enzyme coded for by the *hisC* gene of *E. coli*. The product of the *hisC* gene, imidazolylacetolphosphate:L-glutamate aminotransferase, in *S. typhimurium* has been extensively characterized by physical and chemical studies (39, 41, 43, 48, 49). These studies established that the enzyme is a dimer composed of two identical subunits. The accuracy of the sequence can be verified by several criteria. First, the molecular weight of the native protein was found to be 59,000 by sedimentation equilibrium (41). However, molecular weight determinations under denaturing conditions by either sedimentation equilibrium (43) or sodium dodecyl sulfate-acrylamide gel electrophoresis (48) gave values in the 40,000 range. The molecular weight deduced from the amino acid composition of the sequenced gene, 39,315, is consistent with those found under denaturing conditions. Second, the amino acid composition as predicted by the DNA sequence is in excellent agreement with that observed for the protein isolated from *S. typhimurium* (43) (Table 3) except for histidine (three residues in *S. typhimurium* versus one in *E. coli*) and isoleucine (16 residues versus 24). Third, our amino-terminal sequence can be aligned with the first 19 residues of the *S. typhimurium* enzyme determined by sequential degradation (49) and with the nucleotide sequence of the *S. typhimurium hisD-hisC* gene border (50) (Fig. 5). Fourth, the carboxyl-terminal residue of *S. typhimurium* aminotransferase (43) is identical to that predicted by the DNA sequence. Finally, the relative frequency of codons in *hisC* of *E. coli* is in very good agreement with the rules governing codon usage in *E. coli* for biosynthetic gene products (23).

The presence of very few histidine residues in the aminotransferase from both *S. typhimurium* and *E. coli* is striking. While on average there are 3 histidine residues per 100 amino acids in proteins, the aminotransferase possesses less than 1 histidine residue per 100 amino acids. Analysis of the *trp* operon has shown that proximal genes contain very few tryptophan residues (46). This has been interpreted as being due to the existence of a mechanism by which, in conjunction with expression of the distal genes from an internal promoter (60), the cells would recover quickly from a decreased capacity to synthesize the amino acid. This mechanism seems to operate also in the *his* operon. In fact,

TABLE 3. Amino acid composition of *hisC* gene product

Amino acid	No. of residues in:	
	S. typhimurium[a]	*E. coli*
Ala	35	38
Arg	22	21
Asx	36	30
Cys	9	9
Glx	49	47
Gly	21	20
His	3	1
Ile	16	24
Leu	40	37
Lys	10	12
Met	3	3
Phe	10	9
Pro	21	21
Ser	17	18
Thr	22	20
Trp	4	4
Tyr	11	14
Val	29	27

[a] Values from Martin et al. (43), corrected for the predicted molecular weight of 39,461.

TTT	8	TCT	3	TAT	5	TGT	1
TTC	1	TCC	2	TAC	9	TGC	8
TTA	8	TCA	0	TAA	0	TGA	1
TTG	1	TCG	4	TAG	0	TGG	4
CTT	2	CCT	0	CAT	0	CGT	8
CTC	3	CCC	5	CAC	1	CGC	11
CTA	0	CCA	2	CAA	7	CGA	1
CTG	23	CCG	14	CAG	14	CGG	1
ATT	16	ACT	3	AAT	4	AGT	1
ATC	8	ACC	8	AAC	11	AGC	8
ATA	0	ACA	2	AAA	10	AGA	0
ATG	4	ACG	7	AAG	2	AGG	0
GTT	6	GCT	5	GAT	6	GGT	4
GTC	9	GCC	13	GAC	9	GGC	11
GTA	4	GCA	5	GAA	18	GGA	3
GTG	8	GCG	15	GAG	8	GGG	2

FIG. 6. Codon usage in *E. coli hisC*.

the genes beyond *hisC* are expressed from the internal promoter *hisBp* (3, 20, 25, 53), and the histidine content of the three proximal genes is rather low in both *S. typhimurium* and *E. coli* (*hisG*, four residues; *hisD*, five residues; and *hisC*, three and one residue, respectively) (31, 47; C. B. Bruni, unpublished data).

We were able to positively identify the product of the *hisC* gene of *E. coli* by examining the in vivo protein synthesis products of plasmids pHC1600a and pHC1600b. In these plasmids the structural *hisC* gene was cloned between the primary *hisGp* promoter and the *his* attenuator (Fig. 2). The *hisC* gene was expressed only when transcription was initiated at the *hisGp* promoter and the structural gene was in the correct orientation (Fig. 3, Table 2). Moreover, the protein was synthesized at higher efficiency in pHC1600a than in plasmid pHC9800, in which the *hisC* gene retains its physiological position (third structural gene in the operon, downstream of the attenuator). The sixfold increase in the expression of the aminotransferase in strain FB283 over that in strain FB257 can be easily explained if it is remembered that termination of transcription at the *his* attenuator is very efficient both in vitro (22) and in vivo (30). The synthesis of aminotransferase increased over 100-fold with respect to wild-type levels when the *hisC* gene was cloned in vector pHC730. The high level of expression of the cloned *hisC* gene in this vector was probably dependent, aside from the inherent strength of the *hisGp* promoter, on the presence of the very efficient *his* attenuator downstream of the cloned and transcribed sequences. It has, in fact, been shown that efficient transcription from cloned promoters interferes with plasmid replication, and hence gene dosage and expression, and that these effects can be abolished by placing an efficient transcription terminator downstream (1, 15). This system seems very useful for subcloning, expressing, and identifying the still poorly characterized distal *his* gene products.

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