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Nucleotide sequence of the attenuator region of the histidine operon of *Escherichia coli* K-12

(transcription regulation/restriction fragments/gene cloning)

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ABSTRACT **The attenuator region of the histidine operon of *Escherichia coli* K-12 has a potential coding capacity for two peptides, one of 16 amino acids and another of 30 amino acids. This region is followed by a perfect palindrome of 14 base pairs separated by five nucleotides. A G+C-rich region precedes and follows a possible transcription termination sequence. These features are compatible with a model in which active translation of a leader mRNA interferes with transcription termination, thus causing derepression of the histidine operon. The sequence of the region coding for the hypothetical 16-amino acid peptide is of particular relevance because it indicates the site and a possible mechanism of action of histidyl-tRNA^{his} in regulating histidine gene expression. Seven contiguous histidine codons are present within this sequence:**

5′ -ATG-ACA-CGC-GTT-CAA-TTT-AAA-
NH_2-Met -Thr -Arg -Val -Gln -Phe -Lys-

CAC-CAC-CAT-CAT-CAC-CAT-CAT-CCT-GAC-TAG-3′
His -His -His -His -His -His -His -Pro -Asp -COOH

The histidine (*his*) operon of *Escherichia coli* K-12, like that of *Salmonella typhimurium*, is composed of nine structural genes (1–3). Regulation of this cluster takes place at an operator-promoter region (4–8). A fine-structure map of this region has been established in *S. typhimurium* (6) and has been correlated with the different phenotypes of many distinct mutants (7, 8). The *his* operon of *E. coli* K-12 is under transcriptional control because the levels of *his* mRNA increase in several *his* regulatory mutants (5 and unpublished data). Unlike other biosynthetic operons, the *his* operon does not appear to be under control of a typical repressor protein. All available data from *S. typhimurium* indicate that the structure and the level of charged histidyl-tRNA^{his} directly affect the expression of the operon (9, 10). The isolation and characterization of *hisT* (4, 5) and other tRNA^{his} mutants (4 and unpublished data) in *E. coli* K-12 again point out the similarity of *his* operon regulation in the two species.

Termination of transcription seems to play an important role in the regulation of several operons (11). The concept of an attenuator site was originally proposed by Kasai (12) for the *his* operon of *S. typhimurium* and has since been intensively investigated in bacteriophage λ (13) and in the *trp* operon (14). Termination of transcription in the attenuator region may or may not depend on protein factors, in addition to RNA polymerase. *Rho*-dependent termination has been demonstrated, both *in vivo* and *in vitro*, for bacteriophage λ (15–17). In the *trp* operon of *E. coli*, termination appears to be *rho* dependent *in vivo* but not *in vitro* (18). In the case of the *his* operon, no role for *rho* can be demonstrated *in vitro* (12) or *in vivo* (19).

In order to elucidate the mechanism of regulation of *his* operon expression by histidyl-tRNA^{his} and the mechanism of transcription termination, we isolated and sequenced a DNA segment of the *his* operon of *E. coli* K-12 located between the RNA polymerase binding site and the amino-terminal region of the first structural gene, *hisG*.

MATERIALS AND METHODS

*Hin*dIII and *Bam*HI restriction endonucleases were purchased from New England Biolabs. *Bgl* II (20) and *Alu* I (21) were purified as described. *Apy* I endonuclease was purified from *Arthrobacter pyridolinis*.

Restriction fragments of the *E. coli his* operon were derived from plasmids *phis* 1 and *phis* 5 (22), derivatives of pBR313 (23). Binding of RNA polymerase to DNA was studied by the filter retention method as described by Reznikoff (24), at various concentrations of RNA polymerase (enzyme/DNA ratio 10:70); at the end of the incubation (15 min at 37°), heparin (200 μg/ml) was added and the mixture was further incubated for various times at 37° (1–30 min). The filter retention assay and electrophoretic analysis of bound DNA fragments have been described (24). Protection of restriction enzyme recognition sites was assayed by incubating nick-translated (25) [^{32}P]DNA fragments with RNA polymerase for 15 min at 37° under the conditions of the binding protocol (24). After addition of the restriction enzyme, the incubation was continued for 3 more hr at 37°, and the DNA then was analyzed by electrophoresis on a 5% acrylamide slab gel. Deoxyribonucleotide sequences were determined according to the method of Maxam and Gilbert (26).

All procedures using recombinant DNA were performed in accordance with the National Institutes of Health guidelines, pending approval of Italian official guidelines.

RESULTS

A transducing derivative of phage λ carrying the first structural gene of the *his* operon of *E. coli* K-12 (λ *hisG*) was originally constructed *in vitro* by Borck *et al.* (27) from a *Hin*dIII digest of total *E. coli* DNA. This phage carries a 5300-base pair (bp) fragment of bacterial DNA that has been identified and isolated (22). Fig. 1*A* shows the restriction map of this fragment with endonucleases *Bgl* II, *Hpa* I, and *Pst* I. Within this fragment, there are three *Bgl* II sites, two *Hpa* I sites, and only one *Pst* I

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Abbreviations: bp, base pair(s); Amp^r, ampicillin resistant; Tet^r, tetracycline resistant.

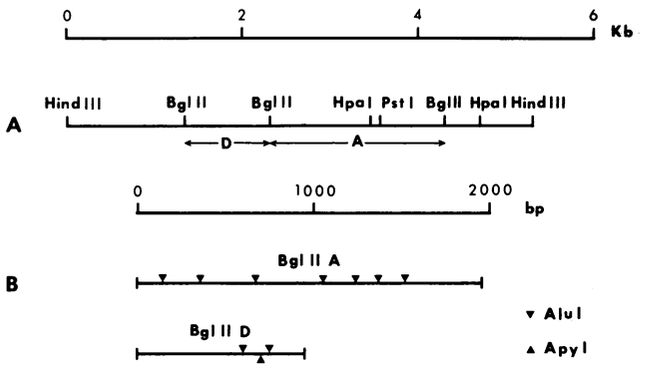

FIG. 1. (*A*) Restriction map of the 5300-bp *Hin*dIII DNA fragment carrying the *hisG* gene. (*B*) Restriction map of fragments *Bgl* II A and *Bgl* II D isolated from the 5300-bp *Hin*dIII *hisG* DNA. The orientation of these two fragments is shown in *A*. The single *Apy* I site of *Bgl* II D lies proximal to fragment *Bgl* II A. Kb, kilobases.

site. The identification of the *his* attenuator—i.e., the DNA region between the RNA polymerase binding site and the signal for the amino-terminal region of the first structural gene, *hisG*—was accomplished as follows. First, we located the *hisG* gene by cloning several DNA fragments into the pBR313 (23) vector, selecting for His^+ clones in an appropriate *hisG* host (FB190). Next, the RNA polymerase binding site was identified through protection against digestion by restriction endonucleases.

Location of the *hisG* Gene on the 2000-bp *Bgl* II A Fragment. The plasmid vector pBR313 (23) is a *col* E1 derivative carrying clusters of genes responsible for the resistance to the antibiotics ampicillin (Amp^r) and tetracycline (Tet^r). No restriction site for *Bgl* II is present in pBR313, whereas single restriction sites for *Hin*dIII and *Bam*HI are present in the promoter region and in the structural gene, respectively, of the *tet* cluster (23). We used these sites for introducing *hisG* DNA into the vector. We cloned into pBR313 the entire 5300-bp *Hin*dIII fragment or mixtures of DNA fragments thereof obtained with *Bgl* II. Transformants were selected for the expression of the $HisG^+$ phenotype. $HisG^+$ transformants were obtained both with the whole 5300-bp *Hin*dIII fragment and with the mixture of *Bgl* II fragments. Table 1 summarizes these data.

Representatives of both groups of transformants were isolated and analyzed. Those of group I contain a plasmid of about 14,000 bp; those of group II contain a plasmid of about 10,700 bp; pBR313 DNA itself is made up of about 8800 bp (23). On further analysis, the DNA of group I plasmids was shown to be composed of two *Hin*dIII fragments, the pBR313 linear DNA and the 5300-bp *hisG* fragment inserted at the *Hin*dIII site in both possible orientations (unpublished data). The DNA of group II plasmids was shown to have neither *Bam*HI nor *Bgl* II sites, as expected if a single *Bgl* II fragment had been inserted in the single *Bam*HI site of pBR313. The size of the plasmid (10,700 bp) suggests that the fragment inserted is *Bgl* II A. This has been confirmed by digestion with restriction endonucleases *Hpa* I and *Pst* I, both of which have a single site in the *Bgl* II A fragment (Fig. 1*A*). Assays of the phosphoribosyl-ATP synthetase activity (EC 2.4.2.17) (32), product of the *hisG* gene, demonstrate high levels of this enzyme in the transformants of group II (unpublished data). Therefore, the $HisG^+$ phenotype is due to the presence and expression of the entire *hisG* gene on fragment *Bgl* II A.

Restriction analysis of the DNA of several group II *hisG* plasmids with *Hpa* I and *Pst* I showed that in all cases the *Bgl* II A fragment is oriented clockwise, as are the *tet* genes. This result suggested to us that *hisG* expression might not be under the control of the *his* promoter but rather under the control of the *tet* promoter. In order to verify this point we cloned the purified *Bgl* II A fragment into the *Bam*HI site of pBR313, selecting for ampicillin resistance and scoring for tetracycline sensitivity. The scheme of the experiment is depicted in Fig. 2. Redigestion of ligated DNA with *Bam*HI (step 3) was carried out in order to lower the background of wild-type recombinant pBR313 plasmids, taking advantage of the fact that recombinant hybrid plasmids are resistant to *Bam*HI cleavage (see legend of Table 1). The results are summarized in Table 2. Of the tetracycline-sensitive colonies, only 50% were able to express the $HisG^+$ phenotype. From the data of Table 2 it can be calculated that the background of wild-type recombinant plasmids, exhibiting a tetracycline-sensitive phenotype, cannot be higher than 2%.

These results show that expression of *hisG* in plasmids of group II is possible only in 50% of the clones. Because the $HisG^+$ plasmids we analyzed are all oriented in the same way, we conclude that expression of *hisG* is possible only when the orientation is in the direction of transcription of the *tet* genes. This, in turn, suggests that the promoter of the *his* operon may not reside in the 2000-bp *Bgl* II A fragment.

Location of the *his* Promoter on the 950-bp *Bgl* II D Fragment. In order to verify that the *his* promoter is not located on the *Bgl* II A fragment we carried out experiments with purified *E. coli* RNA polymerase *in vitro*. A mixture of DNA fragments obtained by cleaving the 5300-bp *Hin*dIII fragment with *Bgl* II (see Fig. 1) was incubated with RNA polymerase. Incubation conditions and detection of bound fragments on

Table 1. Cloning of *hisG* DNA into pBR313 vector

Exp.	Vector DNA	Passenger DNA	Transformation efficiency, no. Amp^r clones/ μg DNA	Amp^r $HisG^+$ clones, no./ μg DNA
1	pBR313 *Hin*dIII	*Hin*dIII	200,000	1000
2	pBR313 *Bam*HI	*Hin*dIII + *Bgl* II	200,000	160

In Exp. 1, 1 μg of the purified 5300-bp *Hin*dIII fragment was ligated (28) to 1 μg of pBR313 DNA digested with *Hin*dIII. Ligation was carried out in 50 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$/10 mM dithiothreitol/0.2 mM ATP/bovine serum albumin (50 μg/ml) with 0.04 unit of T4 polynucleotide ligase, in a total volume of 0.1 ml, for 16 hr at 15°. Incubation was stopped by heating for 10 min at 70°. In Exp. 2, 1 μg of the purified 5300-bp *Hin*dIII fragment was first digested with *Bgl* II and the mixture was ligated to 1 μg of pBR313 DNA digested with *Bam*HI. *Bam*HI (G↓GATCC) and *Bgl* II (A↓GATCT) recognition sequences (29, 30) share the four internal bases and their sticky ends can therefore be joined by T4 polynucleotide ligase as suggested by Pirrotta (30) with the loss of each of the two sites. The ligation mixtures were incubated with *E. coli* K-12 strain FB 190 (*hisG*2743, *recA*56) made competent for transformation (31). Transformed clones were selected on glucose minimal ampicillin (50 μg/ml) plates; overall transformation efficiency was measured on LB ampicillin (50 μg/ml) plates.

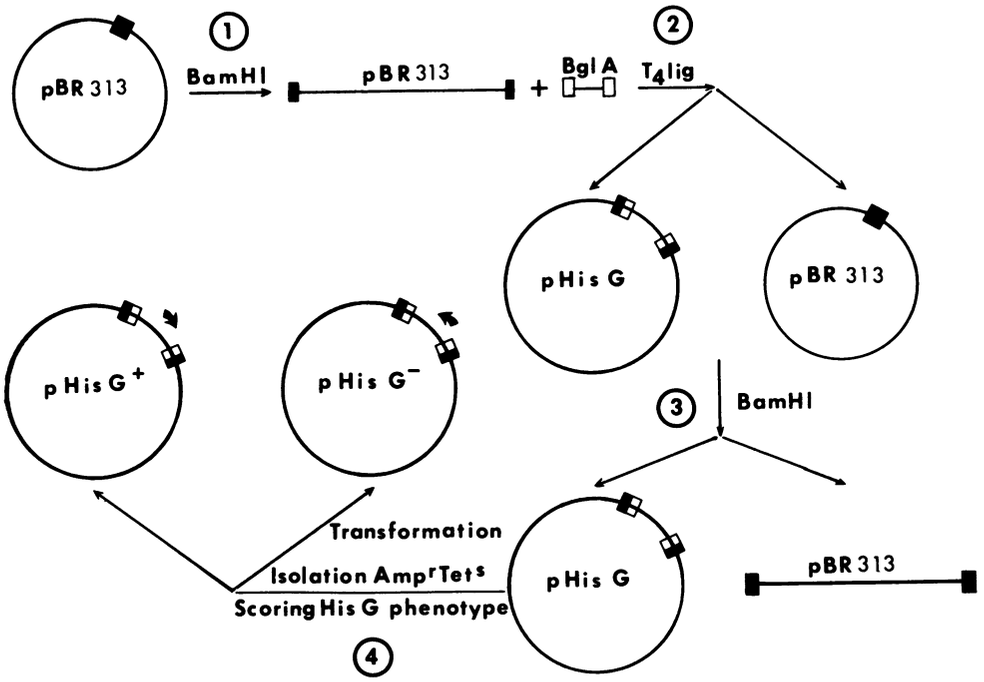

FIG. 2. Schematic representation of the protocol for cloning the purified *Bgl* II A fragment into the *Bam*HI site of pBR313. See text and legend to Table 2 for experimental details. Step 3, redigestion with *Bam*HI, was carried out to decrease the background due to re-formation of wild-type nonrecombinant pBR313. Transformed Amp^r tetracycline-sensitive (Tet^s) clones (step 4) are scored for the HisG$^+$ phenotype and for the orientation of *hisG* gene in the *Bam*HI site. ■, *Bam*HI recognition sequence (located in the *tet* gene cluster); □, *Bgl* II recognition sequence; ◧, *Bgl* II–*Bam*HI hybrid recognition sequence, which is resistant to both endonucleases.

nitrocellulose filters was carried out as described by Reznikoff (24). From these experiments it could be shown that fragment *Bgl* II D forms an initiation complex with RNA polymerase (unpublished data). These data agree with the results of the cloning experiment and indicate that the *his* promoter region is in fragment *Bgl* II D. In order to locate more precisely the binding site(s) for RNA polymerase in the *Bgl* II D fragment, we digested this fragment with several restriction enzymes and investigated whether RNA polymerase could protect any of the restriction sites from digestion. Digestion of *Bgl* II D with restriction endonuclease *Apy* I generated two fragments (700 and 250 bp, respectively) (Fig. 3, lane A). The location of this restriction site in *Bgl* II D is shown in Fig. 1*B*. When the *Bgl* II D fragment was incubated with this enzyme in the presence of purified RNA polymerase the cleavage site was protected (Fig. 3, lane B). These results indicate that the binding site for RNA polymerase lies 250 bp away from the left terminus of the *Bgl* II A fragment.

Nucleotide Sequence of the Attenuator Region of the *his* Operon. The results reported in the previous sections suggest that the *his* attenuator site lies in the region between the right-hand terminus of the *Bgl* II D fragment and the left-hand terminus of the *Bgl* II A fragment (Fig. 1*A*). We therefore sequenced this region. Fragments *Bgl* II A and D were terminally labeled in the 5′ position (33) and cleaved with *Alu* I endonuclease (see Fig. 1*B*). The terminally labeled fragments (a 200-bp fragment from *Bgl* II D and a 150-bp fragment from *Bgl* II A) were separated on a 5% acrylamide slab gel, eluted, and sequenced by the technique of Maxam and Gilbert (26). The sequence of the antisense strand is shown in Fig. 4. Numbering of the nucleotides starts at the *Bgl* II site between fragments A and D and increases in both directions, the positive value toward the *hisG* gene and the negative toward the RNA polymerase binding site.

Several features of this sequence are unique and deserve mention. (*i*) Starting at nucleotide −83, there is a 51-bp stretch

Table 2. Cloning of purified *Bgl* II A fragment into the *Bam*HI site of pBR313

DNA in the ligation mixture	Redigestion with *Bam*HI after ligation	Amp^r clones, no./10 ng pBR313	Tet^s, %	HisG$^+$ among Tet^s, %
pBR313 *Bam*HI	+	20	30	0
pBR313 *Bam*HI + *Bgl* II A	+	1,000	95	49.2
pBR313 *Bam*HI	−	>10,000	ND	ND
pBR313 *Bam*HI + *Bgl* II A	−	>10,000	ND	ND

pBR313 DNA (0.1 μg) digested with *Bam*HI was ligated to itself or to 0.2 μg of the purified *Bgl* II A fragment (1:10 molar ratio). *Bgl* II A fragment was purified from group I plasmid DNA (22) digested with *Bgl* II, by sucrose density gradient centrifugation (unpublished data). See legend to Table 1 for ligation conditions. At the end of the ligation reaction the DNA mixture was heated for 10 min at 70° and then redigested with *Bam*HI. Transformation was carried out in FB 190 (*hisG*2743, *recA*56) on LB ampicillin (50 μg/ml) plates. Amp^r clones were tested for tetracycline sensitivity on LB tetracycline (25 μg/ml) plates. Two hundred tetracycline-sensitive (Tet^s) colonies were scored for the HisG$^+$ phenotype by replica plating on glucose minimal ampicillin plates with or without histidine (0.1 mM). ND, not determined.

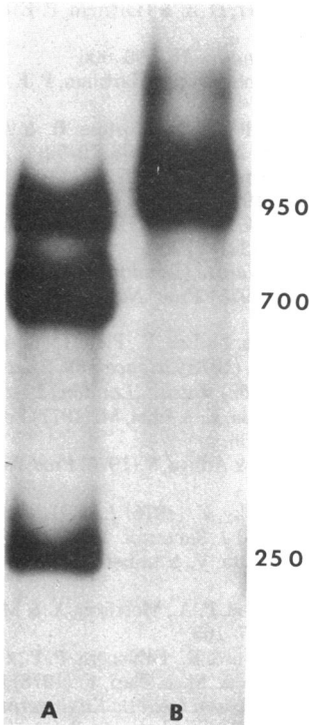

FIG. 3. Protection of the *Apy* I restriction site in purified *Bgl* II D fragment by RNA polymerase, shown by 5% acrylamide slab gel electrophoretic pattern obtained after digestion of *Bgl* II D DNA with endonuclease *Apy* I in the absence (lane A) or presence (lane B) of *E. coli* RNA polymerase. The 950-bp band in lane A represents non-digested DNA.

that could code for a 16-amino acid peptide. Seven contiguous histidine codons (four CAT and three CAC) are present within this region. An amber terminator, TAG, is present in phase at the end of this stretch. (*ii*) Another ATG triplet is present at position −21 in a frame different from that of the "histidine peptide" and could generate a 30-amino acid peptide; an amber codon terminates this sequence at position +70. (*iii*) Another ATG triplet, in the same frame as the "histidine peptide," is present at nucleotide +17 and is followed at +26 by a TGA terminator. (*iv*) Starting at position +30 and ending at position +62, there is a perfect G+C-rich palindrome of 14 bp separated by five nucleotides. Immediately following the palindrome there is an A+T-rich region followed by another smaller G+C-rich region. (*v*) Finally, at position +112 there is an ATG triplet that might signal the amino terminus of the *hisG* gene product.

DISCUSSION

We have identified and sequenced a portion of the control region of the histidine operon of *E. coli* K-12 located between the RNA polymerase binding site and the 5′ end of the first *his* structural gene. Although there is as yet no proof that this region is transcribed, its location and its similarity in structure to that of the leader region of the *trp* operon (34) strongly suggest a regulatory role for this region. As in the case of the *trp* operon leader region, where a sequence coding for a 14-amino acid peptide has been demonstrated (35), the *his* attenuator region carries the coding information for a 16-amino acid peptide. Whereas the peptide encoded by the *trp* leader contains two tryptophan codons, the *his* attenuator encodes for seven contiguous histidine codons. This large number of histidine codons must have great significance in the regulation of the *his* operon. It has been shown that the AUG of the *trp* leader peptide can serve as a translation initiation codon *in vivo* (36). Whether the ATG of the putative *his* attenuator peptide also represents a translation initiation site *in vivo* is not yet known.

Another similarity between the *his* and *trp* sequences, although again the structure is more pronounced in the case of *his*, is the presence of a 32-bp sequence with the potential of forming a stem and loop. Whereas in the case of the *trp* operon a sequence of seven uridine residues is present at the transcription termination site and follows the stem and loop structure (35), the *his* attenuator contains an A+T-rich region (TTTATATAATTA) at a homologous site (Fig. 4). The significance of this difference cannot be assessed now, although it is tempting to speculate that it involves the difference in efficiency and mechanism of transcription termination.

The sequence of the *his* attenuator of *E. coli* strongly suggests that translation of the histidine-rich peptide represents the mechanism that senses the concentration of charged histidyl-tRNA^{his} in the cell. Derepression of the *his* operon results when

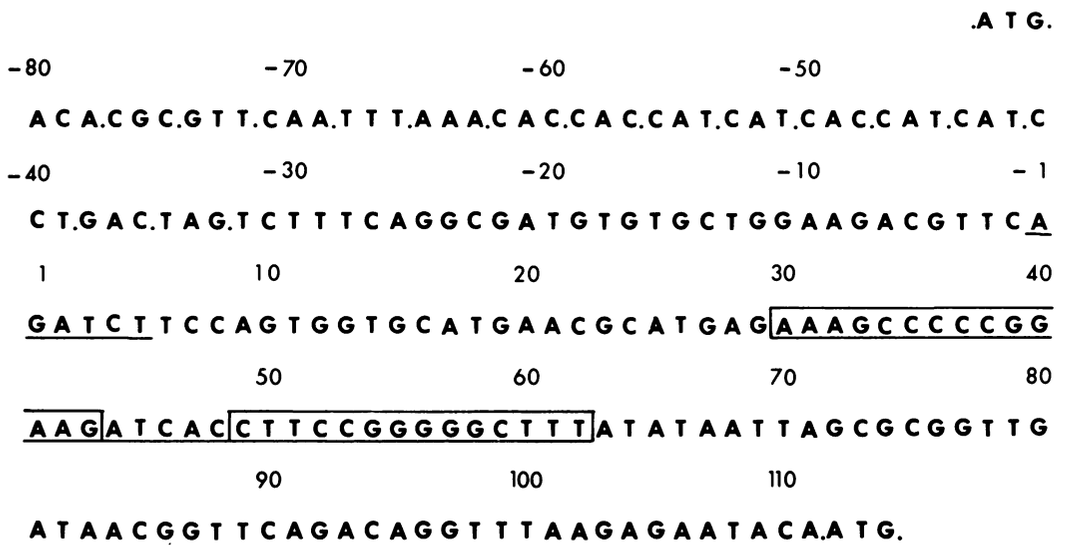

FIG. 4. Nucleotide sequence of the attenuator region of the *his* operon of *E. coli*. Numbering starts at the *Bgl* II site (underlined). In the region coding for the putative "histidine peptide" (−83 to −33) the individual triplets are separated by periods. In the palindromic sequence (+30 to +62) the 14-bp stem regions (+30 to +43 and +49 to +62) have been boxed and the five-nucleotide loop (+44 to +48) has been underlined. The ATG at position +112 may represent the start of the first structural gene, *hisG*.

the intracellular concentration of histidyl-tRNA^{his} is decreased (10)—i.e., under conditions in which the rate of translation of the attenuator peptide would be strongly reduced. However, *his* regulatory mutants show increased levels of *his* mRNA both in *E. coli* (5) and in *S. typhimurium* (12). Therefore, deficient translation of the *his* attenuator peptide could act to prevent transcription termination and thus cause derepression. Coupling of transcription and translation was suggested to play a role in regulation of *his* gene expression (37). It is interesting to note that as early as 1967 Martin, Ames, and Hartman‡ proposed a model of *his* regulation requiring translation of a putative histidine-rich peptide.

The perfect palindrome followed by the A+T-rich region might represent a transcription-termination site. The nucleotide sequence of the *his* attenuator region could be arranged in various possible secondary structures. One can imagine that ribosomes stabilize one or the other of such structures in the nascent mRNA and hence affect transcription termination. However, no such model of *his* regulation can be substantiated at this time by any experimental evidence. Moreover, the available evidence on the physiology and genetics of *his* regulation suggests that the attenuator represents only part of a more complex regulatory system. We think therefore that presentation of a detailed model should await the gathering of more experimental information, particularly on the precise location of the initiation and termination site(s) of transcription, on the translation of the "histidine peptide," and on the direct effect, if any, of this peptide on the regulation of *his* operon expression.

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