DNA Sequence Changes of Mutations Altering Attenuation Control of the Histidine Operon of *Salmonella typhimurium*

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The DNA sequence changes of 31 mutations altering the attenuation control mechanism of the histidine operon are presented. These mutations are discussed in terms of a model for operon regulation that involves a *his* leader peptide gene whose translation regulates formation of alternative stem-loop structures in the *his* leader messenger RNA. Three suppressible mutations generate nonsense codons (ochre and UGA) in the *his* leader peptide gene, demonstrating that translation of this gene is essential for operon expression. Eight mutations presumably reduce the efficiency of translation initiation of the *his* leader peptide gene, causing reduced levels of operon expression. Five of these mutations directly alter the leader peptide gene initiator codon (AUG). Three mutations alter sequences just in front of the initiator codon and presumably alter the ribosome recognition site. Fourteen mutations reduce the stability of the *his* leader mRNA stem-loop structures that are alternatives to the attenuator stem. The properties of these mutations provide support for the role of these stem-loop structures in preventing formation of the attenuator stem. Finally, we show that mutations that alter the attenuator stem suppress *hisO* mutations. This lends support to the proposal that these *hisO* mutations cause reduced levels of operon expression due to excessive attenuator stem formation. The properties of these 31 mutations provide substantial support for the model of *his* operon regulation described in this paper.

1. Introduction

Gene expression in prokaryotes seems to be regulated in most cases at the level of transcription. One major regulatory mechanism, for which the *lac* operon is the prototype, involves the regulation of the frequency of transcription initiation by repressor or activity proteins (see Miller & Reznikoff, 1978 for reviews). A fundamentally different regulatory mechanism, involving the regulation of transcription terminations, has recently been revealed (Kasai, 1974; Bertrand...
et al., 1975; see Adhya & Gottesman, 1978 for review). This type of mechanism is responsible for the regulation of expression of several bacterial operons encoding amino acid biosynthetic enzymes (Bertrand et al., 1975; Zurawski et al., 1978a; Oxender et al., 1979; Keller & Calvo, 1979; Gardiner, 1979; Gemmill et al., 1979; Lawther & Hatfield, 1980) including the histidine operon of Salmonella typhimurium (Kasai, 1974; Barnes, 1978b).

Under conditions of excess histidine availability, his operon transcription terminates at a site in the his control region called the attenuator. When cells are starved for histidine, transcription proceeds through this attenuator site and into the his structural genes (Kasai, 1974). This event is due to low intracellular levels of histidyl-tRNA (Lewis & Ames, 1972), and requires translation of the his control region (Artz & Broach, 1975). The DNA sequence of the his operon control region reveals two major features important to this regulatory mechanism: (1) a G+C-rich region of dyad symmetry followed by an A+T-rich region that is thought to be the attenuator site where his transcription normally terminates, and (2) proximal to the attenuator site, a small gene 51 bases in length containing seven histidine codons in tandem (Barnes, 1978b; DiNocera et al., 1978). The rate of translation of this small gene is sensitive to histidyl-tRNA levels in the cell, and is thought to regulate termination of transcription at the attenuator site (Barnes, 1978b; DiNocera et al., 1978).

We have proposed a detailed model for the mechanism of his operon regulation (Johnston et al., 1980). Supporting evidence for this model comes primarily from the characterization of a large number of mutations in the his control region (hisO) causing reduced operon expression (Johnston & Roth, 1980). To identify the region in the DNA sequence affected by these mutations, and to understand the basis of the effects of these mutations on his operon regulation, we determined the DNA sequence changes in many of these hisO mutants. In this paper the DNA sequence changes of these mutations are presented and their implications for the mechanism of his operon regulation are discussed in detail.

2. Material and Methods

(a) Genetic techniques using the M13-hisOGD hybrid phage

The M13 hisOGD hybrid phage constructed by Barnes (1979; referred to as M13H076), was the source of hisO DNA for sequencing. This phage carries a 3300 base-pair insert in the M13 intergenic region and contains the hisO, hisG and hisD genes (Barnes, 1979). This phage is able to infect F'-containing strains of S. typhimurium, and can confer on them the ability to grow on histidinol (hisD+). To introduce hisO mutations onto the phage for sequencing, we developed the following in vivo technique to detect M13 phage that have undergone recombination with the chromosome (Bossi & Johnston, unpublished results). M13H076 carrying a hisD- mutation (Bossi & Roth, 1980), was used to infect a hisO mutant strain carrying an F'. Infected cells carrying the M13H076 (replicative form) are His+, presumably due to complementation. Many His+ colonies were picked, pooled, and the M13H076 phage they released collected by centrifugation of the bacterial suspension, followed by heating the supernatant to 70°C for 20 minutes to kill any remaining bacteria (M13 is relatively stable to this treatment). These phage were used to infect a his deletion mutant lacking the hisD gene, selecting growth on histidinol (hisD+). Since the input phage are hisD-, only recombinant or revertant (hisD+) phage should transduce this recipient to
SEQUENCE CHANGES IN his MUTANTS

Hol+. Among the Hol+ transductants, those that carry an M13Hol76 containing the linked hisO mutation can be identified. In most cases, 50 to 100% of the hisD+ M13 phage carry the linked hisO mutation. It should be noted that the level of hisD expressed by the His− hisO mutants is essential to this procedure. Since the His− hisO mutations impair expression of hisD, only conditional His− hisO mutations have been placed on the M13 phage.

To identify those M13Hol76 phage that contain a hisO mutation, we tested for M13 phage that exhibit the phenotype expected of the given hisO mutation. For example, to introduce a hisO amber mutation onto the phage, M13Hol76hisD− phage grown on this mutant were used to infect a his deletion mutant carrying an amber suppressor, selecting Hol+ transductants. Individual transductants were purified and the M13Hol76 phage they release tested for the presence of the hisO amber mutation. Mutant (hisO amber) M13Hol76 should only transduce to Hol+ a his deletion mutant carrying an amber suppressor; wild type (hisO+) should transduce recipients carrying no suppressor to Hol+. All of the hisO mutations that were crossed onto M13Hol76 have some type of conditional phenotype (amber, ochre, UGA, heat or cold-sensitive), and were verified to be present on the phage by demonstrating that M13Hol76 acquired the expected conditional hisD phenotype. Mutations causing a His+ but amino-triazole-sensitive (AT8) phenotype were verified to be on M13Hol76 by genetic mapping: M13 phage containing an AT8 mutation did not recombine in the same mutation in the chromosome, but did give amino-triazole-resistant recombinants with other mutations not located at the same site.

Revertants (hisD+) of M13Hol76 carrying the ochre mutation his09654 were selected by using this phage to transduce a his deletion strain (without an ochre suppressor) to Hol+.

The strains carrying the M13Hol76 replicative form DNA that contain the sequenced hisO mutations are TT5443 through TT5468, inclusive. Their general genotype is his-644 zee-l::Tn10 leu-414 /Fpro+ lac+ /M13Hol76hisO−. (The chromosomes of some of these strains carry his-9709 hisT1504 pyrB64 metA53.) The strains that carry nonsense hisO mutations on the M13Hol76 DNA also carry the appropriate nonsense suppressors (supC, supF, or supG).

(b) Preparation of DNA for sequencing

(i) Preparation of single-stranded template

M13Hol76 DNA was prepared by a modification of the method of Barnes (1978b; and personal communication). An M13Hol76-containing strain (strains TT5443 to TT5468, see Table 1) was grown to stationary phase in 200 ml of minimal medium containing 2 mM-histidinol. Cells were removed by centrifugation and the supernatant brought to 0·5 M-NaCl, 2% polyethylene glycol and incubated overnight at 4°C to precipitate the M13Hol76 phage (Yamamoto et al., 1970). The phage precipitate was centrifuged and resuspended in 2·5 ml 100 mM-Tris (pH 7·9), 300 mM-NaCl. Proteinase K (Boehringer Mannheim, 0·25 mg) was added and incubated at 37°C for 1 h. The solution was then deproteinized with 3 vol. water-saturated phenol, followed by 2 extractions with a chloroform/isoamyl alcohol (24:1, v/v) mixture. The DNA was precipitated twice with 300 mM-ammonium acetate and 3 vol. ethanol at −20°C overnight, collected by centrifugation, and resuspended in DNA buffer (10 mM-Tris (pH 7·9), 10 mM-NaCl, 0·1 mM-EDTA) to a final concentration of 1 mg/ml. This procedure routinely yielded 15 to 200 μg of DNA.

(ii) Preparation of restriction fragments

Plasmid DNA pWB91 (Barnes, 1977) was purified from a cleared lysate (Katz et al., 1973) using 2 successive CsCl/ethidium bromide gradients by the method of Barnes (1978b; and personal communication). This DNA was cut with restriction enzyme HincII and the resulting fragments were separated by electrophoresis on a 5% to 20% acrylamide gradient gel (Jeppesen, 1974). The fragment bands, visualized under long-wave u.v. light after ethidium bromide staining (0·5 μg/ml), were excised, placed in a 5 ml syringe (plugged with siliconized glass wool), crushed to a fine paste, and soaked overnight at 37°C in 5 ml 500 mM-
ammonium acetate 10 mM-magnesium acetate, 1 mM-EDTA, 0.1% sodium dodecyl sulfate (Maxam & Gilbert, 1977). The solution was collected from the syringe by centrifugation and the DNA precipitated twice with ethanol and resuspended in DNA buffer to a final concentration of 1 mg equivalent/ml. To make smaller fragments suitable for DNA sequencing, fragment R5, which contains all of the his control region (Barnes, personal communication), was recut with HhaI and the above procedure repeated. All enzymes, unless otherwise noted, were obtained from New England Biolabs, Inc.

(c) DNA sequencing

DNA sequencing was done using the dideoxynucleotide chain termination method of Sanger et al. (1977) according to Barnes (1978b). All hisO mutations were sequenced using the restriction fragment RH51 as primer (see Barnes, 1978b, for restriction map).

(d) Enzyme assays

The hisD enzyme, histidinol dehydrogenase, was assayed according to the method of Ciesla et al. (1975), as modified by Tadahiko Kohno (personal communication). The reaction contained, per assay, 10 µl 0.1 M-NAD (pH 6.0), 10 µl 0.5 M-glycine (pH 9.8), 10 µl 5 mM-MnCl₂, 20 µl [¹⁴C]histidinol (approx. 40,000 cts/min), and 50 µl tolenuelized cells. The reaction was stopped after 1 h by the addition of 0.9 ml 0.05 M-sodium citrate (pH 3.25), and loaded onto a 2-cm Dowex 50X8 column. The [¹⁴C]histidine was eluted with 3 ml 0.5 M-sodium citrate (pH 6.0). Specific activity is cts/min per hour per O.D₆₅₀nm unit of cells. [¹⁴C]Histidinol was a generous gift from Tadahiko Kohno. The hisB enzyme, histidinol phosphate phosphatase, was assayed according to Martin et al. (1971).

(e) Construction of hisO double mutants

To construct the double mutants carrying one of the mutations causing reduced operon expression along with the attenuator deletion hisO1242, we selected recombinants that had undergone a recombination event in or near the his control region, and then identified the recombinants that had inherited the 2 desired mutations. The design of these crosses is diagrammed in Fig. 1. To select recombinants in the his control region, we constructed hisO mutant strains carrying a Tn10 (tetracycline resistance) insertion near, but not in the operon, upstream of the hisO mutation of interest, and a Tn5 (kanamycin resistance) insertion in the hisO gene. These strains (TT3602, TT3603, TT3604) were used as donors in transductional crosses with a hisO1242, hisO double mutant as recipient (TR3063). By selecting the tetracycline-resistant transductants that are also kanamycin-sensitive, we can select only those transductants that have undergone a recombination event in or near the his control region. The 3 major types of recombinants arising from this cross are diagrammed in Fig. 1; recombinant type 2 is the desired one.

If the hisO1242 mutation suppresses the His⁻ phenotype caused by the hisO mutation, then we can identify the 3 recombinant types using a backcross, diagrammed in Fig. 1(b). The hisO1242 mutation results in a wrinkled colony morphology, due to the high levels of operon expression it causes (Roth et al., 1966; see also Materials and Methods of accompanying paper (Johnston & Roth, 1980)). Wild-type (hisO⁺) colonies have a smooth morphology. If each recombinant type arising from the cross diagrammed in Fig. 1(a) is used as a recipient in a backcross with transducing phage grown on a His⁻ hisO mutant, the colony morphologies of the His⁻ transductants should identify each recombinant class. Recombinant type 1 should yield both wrinkled (hisO⁻) and smooth (hisO⁺) transductants. The desired recombinant, type 2, should yield only one class of transductants: they will either be wrinkled, if the double mutant exhibits levels of operon expression characteristic of hisO1242; or they will be smooth, if the double mutant has reduced levels of operon expression. Colony morphology effects are fully described by Murray & Hartman (1972).
(a) First cross: select Tet\(^R\) (Kan\(^R\))

Transduced fragment

\(\text{zee4::Tn5 his09654} \)  \(\text{his6::Tn5} \)  TT3602

Recipient chromosome

Type I  Type 2  Type 3

\(\text{his01242} \)  \(\text{his09675} \)  \(\text{hisGΔ} \)  \(\text{hisD} \) etc.

(b) Backcross, select His\(^+\)

Type 1

\(\text{9654} \)  \(\text{1242} \)

Smooth  Rough  colonies  colonies

Type 2

\(\text{9654} \)

Rough colonies

Type 3

\(\text{9654} \)

No His\(^+\) transductants

Fig. 1. Crosses to construct his0 mutants containing the attenuator deletion his01242. (a) In the first cross, tetracycline-resistant transductants were picked and scored for absence of Tn5 (kanamycin sensitivity). For each cross, the following donors gave: TT3602 (his09654), 116 Kan\(^R\)/400 Tet\(^R\); TT3063 (his09663), 48 Kan\(^R\)/100 Tet\(^R\); TT3064 (his09675), 27 Kan\(^R\)/100 Tet\(^R\). (b) In the backcross, His\(^+\) was selected, and the colony morphology of the transductants scored. TT3062 as donor yielded 116 Kan\(^R\) recombinants in the first cross; 3 gave only rough transductants in the backcross (type 2); 99 gave both rough and smooth transductants (type 1); 3 yielded no His\(^+\) transductants in the backcross (type 3). TT3063 as donor yielded 48 Kan\(^R\) recombinants in the first cross; 3 were type 2, 45 were type 1. TT3064 as donor yielded 27 Kan\(^R\) recombinants in the first cross: 1 was type 2, 26 were type 1.

When double mutants were prepared with his01242 and either the ochre (his09654) or the UGA (his09675) mutations, both of which block leader peptide gene translation, or with one of the mutations altering the BC stem (his09663), one class of recombinants yielded only rough transductants in the backcross (for exact numbers, see the legend to Fig. 1). To verify that this class contains both the his01242 and the his09654, his09663, or his09675 mutations (and arc therefore type 2 recombinants), the his0 deletion was first crossed out of these strains. Both his0 mutations were then verified to be present in these strains by the following criteria. (1) They produce wrinkled colonies, and therefore must contain the his01242 mutation. (2) The his09634, his09663 and his09675 mutations can be separated from the his01242 mutation in these strains and, when separated, cause the same phenotype (His\(^-\)) as the original mutation. (3) These mutations, when separated from his01242, map in the correct deletion interval, and are also suppressed by the appropriate suppressors (amber, ochre, UGA). Thus, we feel confident that the class of recombinants that yields only rough transductants in the backcross carry both the his01242 and the his09654, his09663, or his09675 mutations.

(f) Other methods

All other methods, including cell growth and genetic techniques, have been described (Johnson & Roth, 1979).

(g) Bacterial and phage strains

All bacterial strains and their sources are listed in Table 1. Unless otherwise noted, all strains were constructed for this study. Some strains used in this study are listed in Table 1 of Johnston & Roth (1980).
Table I

Multiply marked bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<tr>
<td>TT3136</td>
<td>hisY9708 hisT1505 pyrB64 metA53/Fpro+ lac+</td>
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</tr>
<tr>
<td>TT3602</td>
<td>zee:4::Tn10 his09654 his09647::Tn5 hisT1504</td>
<td></td>
</tr>
<tr>
<td>TT3603</td>
<td>zee:4::Tn10 his09663 his09647::Tn5 hisT1505</td>
<td></td>
</tr>
<tr>
<td>TT3604</td>
<td>zee:4::Tn10 his09675 his09647::Tn5 hisT1504</td>
<td></td>
</tr>
<tr>
<td>TT3670</td>
<td>hisA644 zee-1::Tn10/Fpro+ lac+</td>
<td>L. Bossi</td>
</tr>
<tr>
<td>TT3672</td>
<td>hisA644 zee-1::Tn10 leu-414 supF/Fpro+ lac+</td>
<td>L. Bossi</td>
</tr>
<tr>
<td>TT4415</td>
<td>hisA644 zee-1::Tn10 supJ128/Fpro+ lac+ /M13Holt6 L. Bossi</td>
<td></td>
</tr>
<tr>
<td>TT4416</td>
<td>hisA644 zee-1::Tn10 supE leu-414/Fpro+ lac+ /M13Holt6</td>
<td>L. Bossi</td>
</tr>
<tr>
<td>TT5437</td>
<td>zee-4::Tn10 his09663 his01242 hisA0G439</td>
<td></td>
</tr>
<tr>
<td>TT5438</td>
<td>zee-4::Tn10 his09675 his01242 hisA0G439</td>
<td></td>
</tr>
<tr>
<td>TT5439</td>
<td>zee-4::Tn10 his09654 his01242 hisA0G439</td>
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</tr>
<tr>
<td>TT5440</td>
<td>zee-4::Tn10 his09663 his01242</td>
<td></td>
</tr>
<tr>
<td>TT5441</td>
<td>zee-4::Tn10 his09675 his01242</td>
<td></td>
</tr>
<tr>
<td>TT5442</td>
<td>zee-4::Tn10 his09654 his01242</td>
<td></td>
</tr>
<tr>
<td>TT5469</td>
<td>hisA9708 hisT1504 pyrB64 metA53/Fpro+ lac+ /M13Holt6</td>
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</tr>
<tr>
<td>TT5472</td>
<td>hisA644 zee-1::Tn10 leu-414 supC zey-656::Tn5/Fpro+ lac+</td>
<td>Johnston &amp; Roth (1980)</td>
</tr>
<tr>
<td>TT5473</td>
<td>hisA644 zee-1::Tn10 supU1253/Fpro+ lac+</td>
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Unless otherwise noted, all strains were constructed for this study. The nomenclature for Tn10 insertions has been described (Chumley et al., 1979).

3. The Model for his Operon Regulation

Transcription initiating at the his promotor produces a leader mRNA that is either terminated at the attenuator site, or is extended through this site to produce his structural gene mRNA (Kasai, 1974). The model for his operon regulation proposes that the leader transcript can assume one of two conformations by base-pairing to form RNA stem-loop structures. Figure 2 diagrams the his leader mRNA sequence, deduced from the S. typhimurium DNA sequence (Barnes, 1978b), and the two conformations it can assume. Figure 2(a) and (c) show the configuration due to pairing to form one set of stems (AB, CD, and EF): an alternative pairing arrangement of these sequences results in the conformation shown in Figure 2(b) (BC and DE stems). The model proposes that the formation of one of these stems, the EF stem, causes transcription to terminate within the run of nine U residues distal to this stem. Transcription has been shown to terminate at sites similar to this in other systems (see Adhya & Gottesman, 1978, for review). For this reason, the EF stem is termed the attenuator stem. The leader RNA conformation shown in Figure 2(a) and (c) includes the attenuator stem and therefore leads to transcription termination. The configuration shown in Figure 2(b) does not include the attenuator stem and hence would allow transcription to proceed into the his structural genes.

Which of the two alternative conformations the his leader RNA assumes is determined by the extent of translation of the leader peptide gene. We assume that a ribosome translating this small gene prevents the formation of secondary
Fig. 2. Alternative secondary structures for the histidine operon leader mRNA. The stems can be drawn in several slightly different ways; the form chosen is predicted to be thermodynamically favored (Borer et al., 1974; Bloomfield et al., 1974). The letters (A to E) denote the stretches of sequence involved in the alternative structures. The length of message presumed to be covered by the translating ribosome is above the double lines. Hyphens have been omitted for clarity.

structure in the 16 ribonucleotides 3' to the codon occupying the aminoacyl site on the ribosome. The effect of varying the extent of leader peptide gene translation on leader RNA secondary structure is shown in Figure 2. Figure 2(a) presents the situation when the cell is growing with high levels of histidyl-tRNA, which allow
the ribosome to proceed rapidly through the run of seven histidine codons. The ribosome has stopped translation at the UAG terminator codon of the leader peptide gene, and in this position prevents formation of the AB stem. As the polymerase continues transcription, leaving the ribosome behind, the CD stem forms, followed by the attenuator (EF) stem, which causes transcription termination. Figure 2(b) shows the situation when the cell becomes limited for histidyl-tRNA due to histidine starvation. The ribosome is stalled in the run of histidine codons and prevents A sequences from forming stem structures. As the polymerase proceeds, the BC stem can form, followed by the DE stem, which prevents attenuator (EF) stem formation and allows transcription to continue through the attenuator site. Figure 2(c) presents the outcome if the leader peptide gene is not translated, or if the ribosome stalls before the run of his codons. A ribosome stopped before the first histidine codon prevents no stem structures from forming. As transcription proceeds, the AB, CD and, finally, the attenuator (EF) stems form, resulting in transcription termination. This sequence of events explains the requirement of hisO translation for his operon expression in vitro (Artz & Broach, 1975).

This model is necessarily a kinetic one, because calculations suggest that the configuration that includes the EF stem is thermodynamically more stable than the alternative conformation. We assume that the relative thermodynamic stabilities will determine which stems are able to form and that, once formed, these stems are stable over the time-scale involved in making the regulatory decision.

In this paper, we present the DNA sequence changes of many of the hisO mutations described by Johnston & Roth (1980) (accompanying paper). These alterations are discussed in relation to the above model for his operon regulation.

4. Results and Discussion

The his operon control region (hisO), and the first two his structural genes (hisG and hisD) have been cloned into the single-stranded DNA phage M13 (Barnes, 1979). The hybrid phage provides a convenient source of DNA for determining DNA sequence by the chain-termination method of Sanger et al. (1977) and Barnes (1978). To determine the DNA sequence changes of hisO mutations, these mutations were placed on the M13-hisOGD hybrid phage by normal recombination in vivo (see Materials and Methods). Each mutation was verified to be present on the M13-hisOGD phage by genetic criteria (see Materials and Methods). Single-stranded DNA was isolated from M13 phages carrying the hisO mutation, and used as template to determine the DNA sequence changes of the mutations. Some representative sequencing data are presented in Figure 3.

(a) Mutations affecting the leader peptide gene

Three mutations lie in the leader peptide gene: their sequence changes are presented in Figure 4. Two of these mutations (hisO9634 and hisO9675) prevent operon expression (cause a His− phenotype), and one (hisO9876) causes low levels of operon expression that do not increase in response to histidine starvation.
Fig. 3. Representative sequence data of selected hisO mutants. The sequence was generated using restriction fragment RH51 as primer (see Barnes, 1978b, for restriction map) on M13Ho76 carrying one of the hisO mutations as template (see Barnes, 1979, and Materials and Methods). The sequence around each mutation is labelled in the Figure. The mutated bases are circled.
resulting in a His\(^+\) strain that is sensitive to the histidine analog amino-triazole (see Johnston & Roth, 1980). Our interpretation of the effects of these mutations follows.

(i) The his leader peptide gene is translated into protein

The mutation \textit{his09654} is a single base change that generates an ochre (UAA) chain-terminator codon preceding the run of seven histidine codons in the leader peptide gene. This mutation is suppressed by ochre suppressor tRNAs (see Johnston & Roth, 1980). Similarly, \textit{his09675} is a duplication of eight bases within the leader peptide gene that generates a chain-terminating UGA codon in front of the histidine codons. This mutation results in a His\(^-\) phenotype that is suppressed by a UGA suppressor (\textit{supC7}).

The model for operon regulation predicts that a ribosome stopped at either of these two sites in the leader peptide gene should cause the his leader RNA to assume the conformation shown in Figure 4. Since this structure includes the attenuator stem, these mutations should cause transcription termination, and the fact that these mutants are His\(^-\) is consistent with the model. The assumption that these mutants are His\(^-\) due to excessive attenuator stem formation is supported by the observation that \textit{his09654} and \textit{his09675} are both suppressed by deletion of the attenuator (see below). The ability of these two mutations to be suppressed by informational suppressors demonstrates that the leader peptide gene is translated.

(ii) The extent of leader peptide gene translation is central to regulation

The \textit{his09654} and \textit{his09675} mutations cause ribosomes to stop nine and 20 bases, respectively, in front of the first histidine codon of the leader peptide gene. A
dominant.

ribosome halted at these sites results in a His\(^-\) phenotype, presumably due to excessive attenuator stem formation. In contrast, the +1 frameshift mutation, \textit{hisO}9876, causes the ribosome to terminate at an ochre (UAA) codon only four bases before the first histidine codon. This mutation causes a His\(^+\) AT\(^S\) phenotype that is suppressed by ochre suppressors. Apparently, a ribosome stopped this close to the run of histidine codons prevents attenuator stem formation often enough to result in a His\(^+\) phenotype, while a ribosome halted at the other two positions is never able to prevent attenuator stem formation (causing a His\(^-\) phenotype). This result can be explained if we assume that 16 to 20 ribonucleotides 3' to the aminoacyl site on the ribosome are unable to participate in stem-loop structures. This is slightly larger than the length of RNA protected from nuclease digestion by translating ribosomes (Steitz, 1979). However, it is conceivable that the ribosome could disrupt a larger length of RNA secondary structure than it is able to protect from nuclease digestion. A ribosome covering 16 to 20 bases would be unable to disrupt the AB stem when stopped at the \textit{hisO}9654 or \textit{hisO}9675 sites; this would result in excessive attenuator stem formation. However, a ribosome stopped at the ochre codon generated by \textit{hisO}9876 might cause frequent disruption of the AB stem located 14 nucleotides downstream of this ochre codon. This would lower the frequency of attenuator stem formation by allowing the BC and DE stems to form occasionally (Fig. 2(b)) and permit transcription to proceed through the attenuator site. In fact, there is a direct correlation between the distance the ribosome advances in the leader peptide gene, and the level of operon expression: the \textit{hisO}9876 mutation causes a His\(^+\) phenotype, the \textit{hisO}9654 mutant is leaky His\(^-\), and \textit{hisO}9675 is a tight His\(^-\) mutant. The levels of operon expression in these mutants, presented as \textit{hisD} enzyme levels, are shown in Table 2. These results suggest that the extent of ribosome advance in the leader peptide gene is central to the regulatory mechanism.

### Table 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>\textit{hisO} mutation</th>
<th>Relative spec. act.</th>
<th>No. of bases from nonsense codon to base of AB stem</th>
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<tbody>
<tr>
<td>LT2</td>
<td>Wild type</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>TR5758</td>
<td>\textit{hisO}9876</td>
<td>1.4</td>
<td>14</td>
</tr>
<tr>
<td>TR5580</td>
<td>\textit{hisO}9654</td>
<td>0.6</td>
<td>19</td>
</tr>
<tr>
<td>TR5601</td>
<td>\textit{hisO}9675</td>
<td>0.2</td>
<td>30</td>
</tr>
</tbody>
</table>

Cells grown to late log phase (O.D.\(_{650} \sim 1.0\)) in 0.8\% nutrient broth + 0.5\% NaCl were assayed as described in Materials and Methods. Specific activity is the amount of \(^{14}\text{C}\)histidinol converted to \(^{14}\text{C}\)histidine (cts/min) per ml of cells per hour per O.D.\(_{650}\) unit of cells. The specific activity of LT2 was \(1.3 \times 10^5\).

(iii) \textit{The leader peptide has no function}

The \textit{hisO} mutations, including those that lie in the leader peptide gene, are \textit{cis}-dominant. Therefore, either the leader peptide gene produces a non-functional
product, or its product is a cis-acting protein. Consistent with the first possibility is the fact that all three of the leader peptide gene mutations are nonsense or frameshift mutations. The properties of two of the leader peptide gene mutants, discussed below, support the first possibility.

The his09675 mutation, in addition to generating a UGA codon, causes a shift to the −1 reading frame of the leader peptide gene due to the duplication of eight bases. Therefore, when this mutation is suppressed by a UGA suppressor, it causes the production of a leader peptide substantially different from the wild-type peptide. Nonetheless, the suppressed UGA mutant (his09675) has a His+ phenotype. Similarly, the +1 frameshift mutant (his09876) is His+, even though it produces a shortened peptide. These results imply that the leader peptide is not required for at least some transcription to proceed through the attenuator site. Instead, it is probably only the act of translation of the leader peptide that is responsible for readthrough of the attenuator site by RNA polymerase. It is possible, although we believe unlikely, that the leader peptide is required to obtain fully derepressed levels of operon expression.

(iv) Reduced leader peptide gene translation causes reduced operon expression

As diagrammed in Figure 2(c), less translation of the leader peptide gene should result in reduced operon expression due to the increased frequency of attenuator stem formation. Five mutational changes, diagrammed in Figure 5, presumably impair initiation of translation of the leader peptide gene. Three of these changes affect the AUG initiator codon of the leader peptide gene: the other two alter sequences just in front of the initiator codon. The effects of these mutations on operon regulation are discussed below.

The mutations his09709, his09613, his09856, his09866 and his09867 presumably reduce the efficiency of translation initiation of the leader peptide gene.

![Diagram of mutations affecting leader peptide gene translation initiation. The sequence complementary to the 3' end of 16 S rRNA (GGA) is underlined. Also underlined is an AUG codon that is in the −1 reading frame of the leader peptide gene. The leader peptide gene initiator codon is enclosed in the box. Hyphens have been omitted for clarity.](image)
gene, since they change the AUG initiator codon. The fact that these mutants exhibit reduced operon expression is consistent with the model for operon regulation. An analogous mutation in the trp operon leader peptide gene has a similar phenotype (Zurawski et al., 1978b). The model for operon regulation predicts that the absence of translation of the leader peptide gene should cause exclusive attenuator stem formation, and the His- phenotype of the hisO9654 (ochre) and hisO9675 (UGA) mutants confirms this prediction. The hisO9613 mutation results in a His- phenotype, presumably by preventing any initiation of leader peptide gene translation. The fact that hisO9709, hisO9856, hisO9866 and hisO9867 are His+ suggests that there must be some low-level translation of the leader peptide gene in these mutants. We propose two explanations for how this could occur. First, the ACG and AUA codons in these mutants might provide low-level initiation of leader peptide gene translation. A mutant T4 AUA codon is able to serve as an initiator for translation (Belin et al., 1979; Belin, 1979). A mutant T7 phage gene whose initiator codon is changed to ACG produces a low but detectable level of protein, but in this case it is not clear if the ACG codon is serving as the initiator (Dunn et al., 1978). Second, it is possible that ribosomes in these mutants are using a different AUG codon to initiate leader peptide gene translation. The AUG codon that precedes the leader peptide gene by 26 nucleotides (underlined in Fig. 4), could serve to initiate translation. Translation beginning from this site is out of frame with respect to the leader peptide gene and would normally terminate at the UGA codon beginning with the second nucleotide of the leader peptide gene. The hisO9709 mutation changes this codon to CGA and would allow translation to proceed well past the end of the leader peptide gene (to a UGA codon 101 nucleotides downstream from the leader peptide gene initiator). However, the hisO9856, hisO9866 and hisO9867 mutations generate an ochre (UAA) chain- terminator codon at this position, in frame with the alternative AUG initiator codon. Therefore, ribosomes initiating at this alternative AUG cannot be expected to permit leader peptide gene translation in these mutants. Since there is no other AUG (or GUG) codon that could serve to initiate leader peptide gene translation (Barnes, personal communication), it is probable that translation initiates at the AUA codon in mutants hisO9856, hisO9866 and hisO9867.

Furthermore, it seems unlikely that the alternative AUG codon is used to initiate translation in the hisO9709 (ACG) mutant. The hisO9613 mutation alters the UGA codon that is in phase with the alternative AUG initiator, and would allow ribosomes initiating from this site to proceed to an ochre (UAA) codon four nucleotides in front of the first histidine codon of the leader peptide gene. We know from the phenotype of the hisO9706 mutant (discussed above) that ribosomes proceeding to this point cause a His+ AT<sup>S</sup> phenotype. The fact that the hisO9613 mutant is His- suggests that translation does not initiate from the alternative AUG codon.

Two other mutational changes affect nucleotides just in front of the leader peptide gene initiator codon, and presumably reduce translation initiation by altering the ribosome binding site. One pair of mutations (hisO9873 and hisO9889) result in low levels of operon expression (His<sup>+</sup> AT<sup>S</sup> phenotype); the other mutation (hisO9674) completely blocks operon expression (His- phenotype). Since neither of
these mutations directly affect the sequence complementary to the 3' and of 16S rRNA (Shine & Dalgarno, 1974), it is not immediately clear how they could affect ribosome binding. Examination of the surrounding sequence suggests a possible mechanism. In wild type, a stem-loop structure in the leader RNA can be drawn in this region (Fig. 5). This stem includes two of the three nucleotides complementary to the 3' end of 16S rRNA (GGA), and the first two bases of the AUG initiator codon. These sites, if paired in such a stem, might not be available to bind ribosomes. Since this stem-loop structure is relatively unstable in wild type (see Table 3), it may not form significantly and should have little effect on ribosome binding. However, in the mutants, this stem is much more stable, and could cause a reduction of translation initiations. There is a direct correlation between the strength of this stem in the mutants and the level of operon expression (Table 3).

Models similar to this have been suggested to explain reduced translation initiation in mutants of phage ſ2 (Atkins et al., 1979), and in phage lambda (Iserentant & Fiers, 1980).

Table 3

<table>
<thead>
<tr>
<th>Mutation</th>
<th>ΔG of stem</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>−3.4</td>
<td>His⁺ AT⁺</td>
</tr>
<tr>
<td>his8783</td>
<td>−7.2</td>
<td>His⁺ AT⁵</td>
</tr>
<tr>
<td>his8889</td>
<td>−14.4</td>
<td>His⁻</td>
</tr>
</tbody>
</table>

Calculated stabilities (ΔG) of a stem-loop structure including the leader peptide gene initiator region. The ΔG values of the stem were calculated according to Bloomfield et al. (1974). Also noted are the phenotypes caused by the 2 mutation types.

(b) Mutations altering the RNA stem-loop structures

Nineteen hisO mutations probably affect the stability of the leader mRNA stem-loop structures that prevent formation of the attenuator stem (Fig. 2(b)). Some of them prevent operon expression (causing a His⁻ phenotype) and some of them cause only reduced levels of operon expression (His⁺ AT⁵ phenotype). Their sequence changes are presented in Figure 6 and are discussed below.

(i) All mutations weaken one set of alternative stems

All mutations are predicted to reduce the stability of one of the two stems (BC and DE) that are alternatives to the attenuator (EF) stem. Presumably, the BC and DE stems form less frequently because of these mutations, resulting in excessive formation of the attenuator stem. The calculated stabilities of each of the stems for wild type, and for each mutant are presented in Table 4. In general, the mutations causing the greatest reduction in BC or DE stem stabilities result in a His⁻ phenotype. Apparently the BC and DE stems never form in these mutants,
SEQUENCE CHANGES IN *his* MUTANTS

(a) Point mutations

(b) Deletion mutations

Fig. 6. Mutations affecting the mRNA stem-loop structures. The base changes and the phenotype caused by each mutation is presented. The first codon in the Figure is the leader peptide gene initiator codon. Hyphens have been omitted for clarity.

resulting in exclusive attenuator stem formation, thus preventing operon expression. This is supported by the fact that a deletion of the attenuator stem serves to suppress one of these mutants (*his*09863, see below). Seven mutations that cause the least reduction in the stability of the DE stem (*his*09712, *his*09713, *his*09854, *his*09864, *his*09892, *his*09896 and *his*09897) result in a His⁺ AT₅S phenotype. One mutation (*his*09863) that reduces the stability of the BC stem also causes a His⁺ AT₅S phenotype. Presumably, the BC and DE stems are able to form in these mutants at some low frequency, occasionally preventing attenuator stem formation and allowing transcription to proceed into the structural genes. The sequence changes and phenotypes of these mutants are consistent with a role for
the BC and DE stems in preventing attenuator stem formation.

Four mutations (his09712, his09685, his09892 and his09897) reduce the stability of both the DE and the attenuator (EF) stems (Table 4). Since these mutations cause reduced operon expression, we believe that the relatively minor changes near the top of the attenuator stem do not appreciably impair its ability to form and cause transcription termination.

(ii) Amber suppressor tRNAs suppress BC and DE stem mutants

Genetic characterization of the His− hisO mutants revealed that many of them are suppressed by amber suppressors (see Johnston & Roth, 1980). Unlike the ochre and UGA mutations, the amber-suppressible mutations do not generate nonsense codons in the leader peptide gene (no codon in this gene can be converted to UAG by a single base change). We discovered that all of the His− mutations that weaken the BC or DE stems are suppressed by amber suppressors, even though none of them generate an amber codon, nor do they even lie in a region of hisO thought to be translated.

We believe that suppression of these mutations by amber suppressor tRNAs is due to ribosomes that read through the normal terminator codon (UAG) of the leader peptide gene. A ribosome allowed to read through this terminator codon would proceed to a UGA codon at the base of the attenuator stem (five nucleotides in front of the base of the EF stem). A ribosome following polymerase closely up to this point would prevent attenuator stem formation and allow transcription to proceed through the attenuator site. Since very little operon expression is required for a His+ phenotype (about 0·1 of basal levels), relatively few ribosomes would
SEQUENCE CHANGES IN his MUTANTS

need to read through the leader peptide gene terminator to elicit suppression. The fact that all of the His− stem mutants are suppressed by amber suppressors, even though they all result in different sequence changes, is consistent with this model for the mechanism of suppression.

This explanation for suppression of these mutations predicts that amber suppressors should cause increased operon expression in wild-type cells. In fact, strains wild-type for hisO containing various amber or ochre suppressors (both of which are able to read UAG) have two- to threefold increased levels of his operon expression (Johnston et al., 1980; Chumley, 1980).

(iii) Only the EF stem is responsible for attenuation

The model proposes that the EF (attenuator) stem is responsible for the termination of transcription at the attenuator site. However, since both of the leader RNA structures that include the attenuator stem also include the CD stem (Fig. 2(a) and (c)), it is possible that CD is also required for transcription termination. The four mutations that delete D sequences (hisO9653, hisO9676, hisO9679 and hisO9689) rule out this possibility. None of these mutants should be able to form the CD stem, yet they are all His−, presumably due to excessive attenuator stem formation. Therefore, transcription termination is complete even in the absence of the CD stem. The role of the CD stem, then, must simply be to sequester D sequences, thus preventing DE stem formation, and allowing formation of the attenuator (EF) stem.

(c) Mutations altering the attenuator stem

The DNA sequence changes of two mutations that alter the attenuator stem are presented in Figure 7. One of these mutations, hisO1242, was sequenced by Wayne Barnes and has been described (Johnston et al., 1980). It is discussed here in relation to the other hisO mutations, and in the context of the model for his operon regulation. The other attenuator mutation, hisO9922, is a new mutation isolated in this study. These mutations are discussed below.

(i) The attenuator stem causes transcription termination

The hisO1242 mutation causes constitutively high levels of operon expression (Roth et al., 1966), and prevents transcription termination at the attenuator site (Kasai, 1974). The fact that this mutation deletes E and F sequences, and thus prevents formation of the attenuator stem strongly suggests that this stem is responsible for the termination of transcription (Barnes, 1978b). The observation that hisO1242 suppresses mutants that are His− presumably due to excessive attenuator stem formation strengthens this conclusion (see below).

(ii) Revertants of hisO mutants have attenuator stem lesions

All of the His− mutations that map between the his promotor and the attenuator, including those described here, revert to His+ at a very high
spontaneous frequency ($10^{-5}$ to $10^{-6}$): this frequency is increased by both base substitution and frameshift inducing mutagens (see Johnston & Roth, 1980). This suggests that the $\text{his}O$ mutations are being suppressed by second-site mutations in these revertants, as was shown to be the case by Johnston & Roth (1980). The model for operon regulation explains these reversion properties, and predicts the sequence changes of some of the second-site suppressor mutations.

According to the model, all of the $\text{His}^-$ mutants defective in leader peptide gene translation or in alternative stem formation are unable to express the operon due to excessive formation of the attenuator stem. Any mutation that reduces the stability or frequency of formation of the attenuator stem should therefore suppress these mutations. The large number of ways of impairing the attenuator stem or its formation could explain the high reversion frequency of these mutations.

The ochre mutation $\text{his}\,0\,9\,6\,5\,4$, when present on the M13-$\text{his}O\,G\,D$ hybrid phage, prevents expression of the $\text{his}G$ and $\text{his}D$ genes present on this phage. We isolated a revertant phage able to express the $\text{his}D$ gene (see Materials and Methods), and sequenced the second-site suppressor mutation. As shown in Figure 7, the new mutation ($\text{his}0\,9\,9\,2\,2$) is a deletion of one base from the attenuator stem, which apparently destabilizes the attenuator stem sufficiently (about 8 kcal, see Table 4) to allow some operon expression. That such a minor destabilization of the attenuator stem allows operon expression is consistent with the fact that only low levels of expression are required for the $\text{his}D^+$ phenotype. However, since the M13-$\text{his}O\,G\,D$ hybrid phage replicative form may exist in the cell in many copies (Hohn et al., 1971; Barnes, 1979), such minor attenuator stem defects could cause the $\text{his}D^+$ phenotype when present on the phage, but not when present in the chromosome. Nevertheless, this result suggests that many revertants of the $\text{His}^-$ $\text{his}O$ mutants carry mutations altering the attenuator stem, and further indicates that the attenuator stem is responsible for transcription termination.
(d) **Deletion of the attenuator stem suppresses hisO mutations**

We have argued that the hisO mutations that alter the RNA stem-loop structures or affect leader peptide gene translation cause reduced operon expression due to excessive attenuator stem formation. If this is correct, then these mutations should be suppressed by deletion of attenuator stem sequences. We have constructed double mutants carrying the attenuator deletion hisO1242 and one of three mutations (hisO9663, hisO9654, hisO9675) affecting leader peptide gene translation or alternative stem formation. The fact that these double mutants have the wrinkled colony morphology characteristic of hisO1242 indicates that their levels of operon expression are similar to that in a hisO1242 mutant. This is demonstrated by measurement of hisB enzyme levels in these strains, presented in Table 5. Therefore, the hisO mutants that are His− due to excessive attenuator stem formation are completely suppressed by deletion of the attenuator. The construction of these double mutants is described in Materials and Methods.

5. Further Discussion

Under conditions of excess histidine availability, the his operon is still expressed at a substantial basal level. Since the His− hisO mutants owe their phenotype to excessive formation of the attenuator stem, this stem is able to block virtually all transcription. Therefore, the basal levels of expression cannot be due to the inability of the attenuator stem to prevent readthrough. Instead, the basal levels must reflect the frequency with which the attenuator stem does not form under repressed conditions, caused by formation of the alternative set of stems (BC and DE, Fig. 2(b)). This could be due to statistical fluctuations in ribosome position under fully repressed conditions. If occasionally the first ribosome is slowed due to transient decreases in concentration of any of the charged tRNAs, or is late in initiating leader peptide gene synthesis, the attenuator stem would not form. The RNA stem-loop structure that includes the initiator region of the leader peptide gene (Fig. 5) could be responsible for setting the basal level of operon expression. If this stem occasionally forms under repressing conditions, it would cause some ribosomes to be late in initiating leader peptide gene synthesis, resulting in occasional disruption of the attenuator stem. Another possible mechanism could involve release of the ribosome from the leader mRNA. A ribosome halted at the terminator codon of the leader peptide gene could slightly disrupt the CD stem, situated 19 nucleotides downstream from the terminator codon. If the ribosome remained at this position while the polymerase synthesized E stem sequences, the DE stem would form, preventing attenuator stem formation. However, if the ribosome was released from the leader mRNA while polymerase was synthesizing D stem sequences, the CD stem would form, allowing formation of the attenuator stem. Thus, the basal levels could reflect random differences in the time of ribosome release at the leader peptide gene terminator codon.

Many of the hisO mutations result in a temperature-dependent phenotype: some hisO mutants are heat sensitive, and some are cold sensitive (see Johnston & Roth, 1980). Although the basis for these effects is not clear, they may reflect the effects
of temperature on mRNA secondary structure. All the point mutations, and one deletion mutation \((\text{hisO}96553)\) affecting the BC and DE stems are heat-sensitive. This could be due to the possibility that the stabilities of these stems, which are already reduced by the mutations, are further reduced at high temperatures, leading to more frequent attenuator stem formation. The ochre mutant \(\text{hisO}96554\) is cold-sensitive. This might be due to less frequent attenuator stem formation due to the decreased stabilities of this stem at high temperatures. The interpretation of the temperature effects is complicated, however, since temperature could effect many different functions, including translation and transcription rates, frequency of translation initiations, and translation and transcription termination.

All of the mutations affecting the alternative stems BC and DE are suppressed by amber suppressors. However, the mutations altering the BC stem seem to be more strongly suppressed than those affecting the DE stem (see Table 5 of Johnston & Roth, 1980): BC stem mutants are suppressed well in a \(\text{hisT}^-\) strain; the DE mutants are suppressed weakly in \(\text{hisT}^-\) strains. Both BC and DE stem mutants are more strongly suppressed in \(\text{hisT}^+\) strains. Reasons for the \(\text{hisT}^+\) effect on suppression are not clear. The explanation of suppression that we favor is that the suppressor causes ribosomes to read through the terminator codon (UAG) of the leader peptide gene. These ribosomes follow polymerase closely and ultimately disrupt the attenuator stem (see Results, section (b)). In \(\text{hisT}^+\) mutants, ribosomes are thought to move more slowly (especially through the run of \(\text{his}\) codons) due to their lack of anticodon loop pseudouridine residues (Johnston et al., 1980). We suggest that when ribosomes are thus delayed they do not follow RNA polymerase closely enough to adequately disrupt the attenuator stem. It is not clear why the DE stem mutations are suppressed less strongly than the BC stem mutations.

It is interesting to note that all of the mutations that disrupt the alternative stems BC and DE alter the bottom part of each stem (see Fig. 6). Apparently, mutations in the upper part of these stems have little or no affect on operon expression. In fact, it is only the upper part of the BC and DE stems that contain unpaired sequences. This suggests that only the bottom halves of these stems are critical to the regulatory mechanism.

Unlike the \(\text{trp}\) operon, histidine specific regulation of the \(\text{his}\) operon appears to occur entirely at the level of transcription termination at the attenuator site; the initiation of transcription at the promoter is not specifically regulated by histidine levels. This conclusion is drawn from the fact that, in the \(\text{hisO}1242\) mutant, operon expression does not increase appreciably in response to histidine starvation (Roth et al., 1966). General metabolic regulation of the operon however, which is sensitive to guanosine tetraphosphate levels, is presumably mediated at the promoter (Stephens et al., 1975: Winkler et al., 1978).

The genetic map of the \(\text{his}\) control region contains a number of mutations that map near the attenuator and cause constitutive high levels of operon expression (Ely et al., 1974: see Johnston & Roth, 1980). We believe that all of these mutations, like \(\text{hisO}1242\), will affect the stability or formation of the attenuator stem. The constitutive mutations that map under \(\text{hisO}1242\) almost certainly affect E or F sequences. The mutations that map just upstream of \(\text{hisO}1242\) might affect C sequences, thus preventing C/D stem formation and causing excessive formation
of the DE stem. Both types of mutations would be expected to form the attenuator stem less frequently and lead to high levels of operon expression. Such C sequence mutations probably account for the interspersion in the hisO genetic map of constitutive mutations and mutations causing reduced operon expression.

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