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Histidine regulation in *Salmonella typhimurium:* An activator-attenuator model of gene regulation*

(positive control/coupled *in vitro* transcription-translation system/guanosine 5′-diphosphate 3′-diphosphate/histidine G enzyme/restoration of repression)

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ABSTRACT **An activator-attenuator model of positive control, as opposed to the classic repressor-operator model of negative control, is proposed for the major operon-specific mechanism governing expression of the histidine gene cluster of *Salmonella typhimurium*. Evidence for this mechanism is derived from experiments performed with a coupled *in vitro* transcription-translation system, as well as with a minimal *in vitro* transcription system [Kasai, T. (1974) *Nature* 249, 523–527]. The product (G enzyme, or *N*-1-[5′-phosphoribosyl]adenosine triphosphate:pyrophosphate phosphoribosyltransferase; EC 2.4.2.17) of the first structural gene (*hisG*) of the histidine operon is not involved in the positive control mechanism. However, a possible role for G enzyme as an accessory negative control element interacting at the attenuator can be accommodated in our model. The operon-specific mechanism works in conjunction with an independent mechanism involving guanosine 5′-diphosphate 3′-diphosphate (ppGpp) which appears to be a positive effector involved in regulating amino-acid-producing systems, in general [Stephens, J. C., Artz, S. W. & Ames, B. N. (1975) *Proc. Nat. Acad. Sci. USA,* in press].**

The likelihood that tRNA and tRNA modifications are involved in regulating gene expression for all cells has accentuated the importance of understanding the mechanism of regulation of *his* operon expression in *Salmonella typhimurium*. It has been well established that charged $tRNA^{His}$ is a negative effector in this mechanism (1), and that a particular modified base—pseudouridine (Ψ)—is necessary for the regulatory function of $tRNA^{His}$ (2), as well as other tRNAs (3).

A regulatory region (*hisO*) with some properties of a Jacob–Monod type of operator locus (4) has been characterized in the *his* operon (5), and this region has been referred to as the *his* "operator," or more currently (6), as the *his* "operator-promoter" locus. However, despite detailed studies of *his* operon regulation, direct evidence for a repressor-operator mechanism of gene regulation (4) has been lacking.

Evidence implicating the product (G enzyme, or *N*-1-[5′-phosphoribosyl]adenosine triphosphate:pyrophosphate phosphoribosyltransferase; EC 2.4.2.17) of the first structural gene (*hisG*) of the operon in the regulation (7–11) has led to the idea that this protein would interact with charged $tRNA^{His}$ and might fulfill the role of a classical repressor (e.g., discussion in ref. 9 and ref. 11). This simple model for regulating *his* operon expression has been unsatisfying for a variety of reasons and is, in fact, eliminated by evidence considered in this paper.

Recently, two investigators have introduced new concepts to explain the mechanism of *his* operon regulation. Based on genetic and physiological studies, Ely (12) proposed a model, similar to that earlier suggested by Gierer (13), in which DNA in the *hisO* region varies between a linear duplex structure competent in binding of RNA polymerase, and a transcriptionally inactive "loop" structure. Kasai (14), using evidence obtained with a minimal *in vitro* transcription system (containing RNA polymerase, DNA, and small molecules necessary for transcription), proposed a novel "attenuator" type of regulation in which a site in the *hisO* region acts as a "barrier" to transcription by RNA polymerase. Evidence from this laboratory was provided in support of the general concept of an attenuator mechanism (14).

We have developed a coupled *in vitro* protein-synthesizing system (containing a crude cellular protein fraction, DNA, and small molecules necessary for transcription and translation), using strains of *S. typhimurium*.† This *in vitro* system mimics regulatory alterations observed *in vivo* with respect to mutations in the *hisO* region, the Ψ modification in tRNA, and the effect of guanosine 5′-diphosphate 3′-diphosphate (ppGpp) (15).

In this paper we present evidence that has led us to propose a positive mechanism for *his* operon regulation, and an "activator-attenuator" type of regulation, as opposed to the classic repressor-operator mechanism.

MATERIALS AND METHODS

Preparation of *In Vitro* Protein-Synthesizing Extracts. *S. typhimurium* strain TA471 (*his*Δ*OGDCBH2253 hisT1504*) (16) was used as source of DNA-dependent protein-synthesizing extracts. Preparation of S-30 extracts from strains of *S. typhimurium* was similar to procedures published for *Escherichia coli* (17), with several important modifications.†

Conditions for *In Vitro* Protein Synthesis. Except where indicated, reaction mixtures contained, in a final volume of 50 μl: Tris-acetate, 53 mM (pH 8.0); potassium acetate, 55 mM; ammonium acetate, 30 mM; magnesium acetate, 10 mM; calcium acetate, 5 mM; dithiothreitol, 1.3 mM; 20 amino acids, 0.2 mM each; bulk tRNA, 0.5 mg/ml; ATP, 2 mM; CTP, GTP, UTP, 0.5 mM each; ppGpp, 0.1 mM; phosphoenolpyruvate, 20 mM; folinic acid, 30 μg/ml; polyethylene glycol 6000, 35 mg/ml; *p*-toluenesulfonyl fluoride, 30 μg/ml; template DNA, 150 μg/ml; S-30 protein, 3.5 mg/ml. Concentrations given for magnesium acetate, calcium acetate, tRNA and S-30 protein are only approximate and are

Abbreviations: ppGpp, guanosine 5′-diphosphate 3′-diphosphate; Ψ, pseudouridine.

* This is paper XVII in a series; paper XVI is ref. 18.

† S. W. Artz, J. R. Broach, and B. N. Ames, in preparation.

Table 1. *In vitro* regulation of the histidine operon under conditions of coupled protein synthesis*

Template	tRNA	D enzyme (cpm/ hr per 0.15 ml)†
hisO$^+$	*hisT*$^+$	1335 (≡1.0)
hisO$^+$	*hisT*$^-$	2943 (2.2)
hisO1242	*hisT*$^+$	5442 (4.1)
hisO1242	*hisT*$^-$	5260 (3.9)

* The procedure was as described under *Materials and Methods*, except that amino acids (0.2 mM), and tRNA (0.8 mg/ml), were deleted from initial reaction mixtures and then added (in a volume of 2 μl) just prior to addition of S-30 protein. This experiment was performed at the same time as the uncoupling experiment described in Fig. 2 and serves, in addition, as the coupled control for that experiment.

† Each value is the average of duplicate determinations. A background of 120 cpm was subtracted from each value. Numbers in parentheses indicate relative activity.

determined for each protein-synthesizing extract prepared.† Reaction mixtures, minus S-30 protein, were incubated 3 min at 37° before the protein was added to initiate synthesis. Incubation at 37° was continued for 70 min and stopped by addition to the D enzyme assay mixture.

D Enzyme (L-Histidinol:NAD Oxidoreductase; EC 1.1.1.23) Assay. Assay conditions and determination of [^{14}C]histidine from [^{14}C]histidinol have been described (18).

Template DNA. Transducing phage templates were extracted from the purified phages following heat induction of the respective *E. coli* double lysogens†: TA1933 (*his-6607* streptomycin-resistant [*φ80 h imm*$^\lambda$ *cI857 susS7*, *φ80 h dhis*$^+$ *imm*$^\lambda$ *cI857 susS7*]—ref. 19); TA1940 (*his-6607* streptomycin-resistant [*φ80 h imm*$^\lambda$ *cI857 susS7*, *φ80 h dhisO1242 imm*$^\lambda$ *cI857 susS7*]—ref. 19); SB3132 (*eda-1 edd*$^-$ *his-gndΔ* streptomycin-resistant [*φ80 h imm*$^\lambda$ *cI857 susS7*, *φ80 h dhisO3148 imm*$^\lambda$ *cI857 susS7*]—ref. 20). The intact *his* operon contained in each of the transducing phage templates originated from *S. typhimurium* genetic material (21).

Reagents. Bulk tRNA was isolated (22) from *S. typhimurium* strains TA265 (*hisT*$^+$) and TA253 (*hisT1504*). ppGpp was prepared and isolated by John Stephens, in this laboratory, by a published procedure (23). Other reagents were of highest commercial quality available.

RESULTS

Regulated *in vitro his* operon expression

Table 1 and Fig. 1 illustrate regulation of the *his* operon observed *in vitro* under conditions of coupled protein synthesis, as a function both of DNA template and species of tRNA added. *De novo* synthesis of histidinol dehydrogenase (product of the second gene [*hisD*] of the *his* operon) serves as a measure of *in vitro his* operon expression. The results are in good qualitative agreement with observations from *in vivo* studies.

First, a mutation in the *hisO* region (*hisO1242*) that leads to constitutively derepressed expression *in vivo* (5, 12, 18) results in elevated synthesis of D enzyme *in vitro* as compared to expression of the wild-type (*hisO*$^+$) template (Table 1, Fig. 1). Conversely, a mutation in the *hisO* region (*hisO3148*) that causes a defect in expression *in vivo* (12) results in diminished expression *in vitro* (Fig. 1).

Second, compatible with *in vivo* observations (5, 16), expression of *hisO*$^+$ DNA is elevated (Table 1) when tRNA lacking the Ψ modification (*hisT*$^-$) is substituted for tRNA with normal modification (*hisT*$^+$). The finding that expression of *hisO1242* DNA is unaffected by the species of tRNA added (Table 1) suggests that the effect of tRNA modification on *hisO*$^+$ expression represents a true regulatory effect.

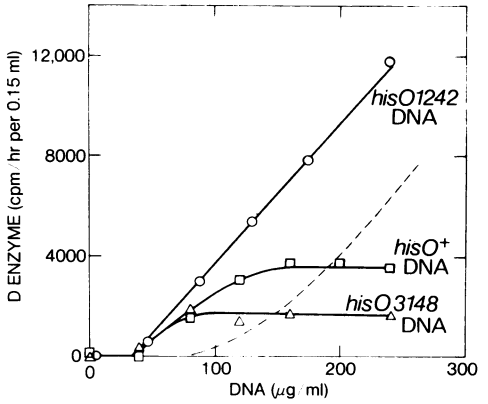

FIG. 1. Coupled *in vitro* expression of the *his* operon as a function of mutations in the *hisO* region. Reaction mixtures contained *hisT*$^-$ tRNA. The broken curve (---) represents the hypothetical result expected for *hisO*$^+$ DNA under control of a simple repressor-operator mechanism.

Indication of positive control from DNA curves

Fig. 1 shows the effects on *his* operon expression of varying concentrations of DNA templates containing different *hisO* regions. The shapes of the curves are most consistent with the concept that it is limitation of a positive control element, rather than repressor action, which is responsible for reduced expression observed with the *hisO*$^+$ and *hisO3148* templates as compared with that of the *hisO1242* template.

If repressor action were responsible we would expect to observe exponentially increasing synthesis as a function of increasing *hisO*$^+$ DNA concentration, until eventually all repressor molecules were bound to *hisO*$^+$ DNA, at which point *hisO*$^+$ expression would approach *hisO1242* expression (represented by hypothetical broken curve in Fig. 1). This type of saturation has been observed, as a consequence of repressor–operator interaction, for the *lac* operon in a coupled system (17). Instead, the experimental curves suggest that it is a limiting amount of positive factor that is eventually bound to *hisO*$^+$ and *hisO3148* templates, at which point addition of more DNA cannot elicit increased synthesis. Thus, *hisO1242* DNA will eventually saturate at a much higher concentration than *hisO*$^+$ DNA because the *hisO1242* mutation leads to independence from the requirement for positive factor, and *hisO3148* DNA saturates at a lower level than *hisO*$^+$ DNA because this mutation leads to a defect in the activation mechanism.

A technical point concerning these results is the observation that relatively high concentrations of DNA are required to obtain activity. This phenomenon is independent of the template used and is not observed when *E. coli* S-30's are used. Degradation of some of the DNA by *Salmonella* nucleases seems a likely explanation. Specifically, since the *S. typhimurium his* operon is contained in *φ80* transducing phages which are grown in *E. coli*, restriction of the DNA by *Salmonella* S-30's may be partly responsible.

The degradation phenomenon is apparently also responsible for the finding that saturating levels of *hisO1242* DNA cannot be achieved, making the increased amount of expression observed with this template a minimum effect.

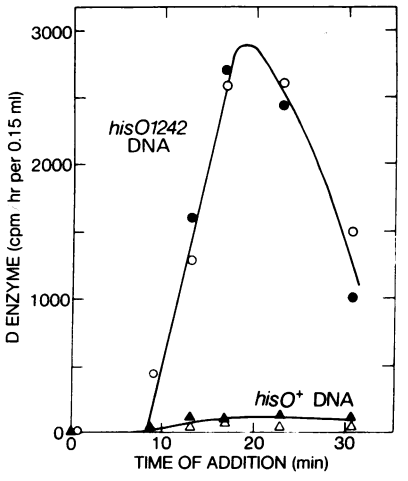

FIG. 2. Uncoupled *in vitro* expression of the *his* operon. The procedure was as described under *Materials and Methods*, except that amino acids (0.2 mM) and tRNA (0.8 mg/ml) were deleted from initial reaction mixtures and then added (in a volume of 2 μl), together with rifamycin (20 μg/ml), at different time-points after the start of the reaction. These results and those shown in Table 1 were obtained in the same experiment. Closed and open symbols specify addition of *hisT*$^-$ tRNA and *hisT*$^+$ tRNA, respectively.

Uncoupling experiments and the activation mechanism

In Fig. 2 are shown the results of uncoupling transcription and translation. This is achieved by performing the reaction with the complete assay mixture but lacking either amino acids, tRNA, or, as indicated in this experiment, both amino acids and tRNA. As we show elsewhere†, enzyme synthesis (i.e., translation) is completely prevented by these conditions; however, transcription may proceed and mRNA accumulates. At different times after the start of the reaction, amino acids and tRNA are added to start translation, together with rifamycin to prevent further transcription initiations, and the amount of D enzyme synthesized serves as a measure of the amount of *his* operon-specific mRNA initiated during the uncoupled portion of the reaction.

With the *hisO1242* template, *his* operon-specific mRNA is synthesized starting at about 8 min. By 20–22 min the rate of synthesis of mRNA has slowed sufficiently to allow the rate of degradation to become significant and a decline in the amount of functional, or translatable, message is observed. The maximum amount of mRNA made supports the synthesis of D enzyme corresponding to 3000 cpm in our assay (Fig. 2). In this particular experiment, the control for coupled synthesis, that is addition of amino acids and tRNA at zero time, without rifamycin, yields about 5000 cpm (Table 1). Thus, about 60% of the amount of coupled synthesis is obtained upon uncoupling with the *hisO1242* template. In different experiments this value varies from 60 to 90%. That is, there is little effect of the uncoupling procedure on expression of the *hisO1242* template. In addition, as with coupled expression of this template, uncoupled expression is unaffected by the species of tRNA added.

In striking contrast, expression of the *hisO*$^+$ template is virtually eliminated in the uncoupled reaction. Thus, the ratio of expression—*hisO1242* DNA/*hisO*$^+$ DNA—is increased to about 30 upon uncoupling (Fig. 2), as compared to coupled ratios (Table 1) of 4 with *hisT*$^+$ tRNA, and 2

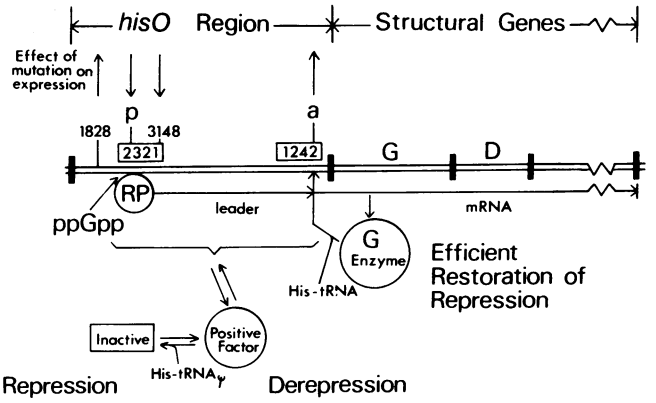

FIG. 3. Activator-attenuator model for regulation of *his* operon expression. Abbreviations: p, promoter; a, attenuator; RP, RNA polymerase. The site of interaction of the positive factor is unspecified, as indicated by the bracket (⌒). Horizontal bars enclosing the numbers specify mutations that are thought to be small deletions. Order of mutations in the *hisO* region is adapted from Ely *et al.* (6). Dimensions of regions in the *his* operon, and factors involved in the regulatory mechanism, are not meant to imply actual physical size relationships.

with *hisT*$^-$ tRNA. The increase in ratio is due entirely to loss of expression of the *hisO*$^+$ template.

We believe these results support the following interpretations:

(*i*) In leaving out amino acids and tRNA during the transcription process, we have eliminated, or greatly depleted, components of the co-regulator, namely, histidine and $tRNA^{His}$. We show elsewhere† that the concentration of $tRNA^{His}$ alone is reduced by about 95%, under conditions of this experiment. By any sort of repression mechanism involving charged $tRNA^{His}$, these conditions should result in increased expression of *hisO*$^+$ DNA, which is clearly not observed. These results, therefore, provide further evidence of the requirement in *hisO*$^+$ expression for an activation mechanism. The *hisO1242* regulatory mutation leads to constitutive expression which is specifically independent of this activation mechanism. In addition, uncoupling the reaction in the presence of tRNA, by deleting only amino acids, yields essentially similar results (data not shown). This is consistent with the idea that $tRNA^{His}$ is not required as an inducer, and is in agreement with results obtained *in vivo* (24).

(*ii*) G enzyme, acting as a transcriptional repressor, could not be responsible for the observed difference between expression of the two templates in this experiment, since no G enzyme is present during the transcription reaction. However, as discussed below, this does not eliminate the possibility that G enzyme could act as an accessory, negative control element.

(*iii*) The finding of decreased *hisO*$^+$ expression in the uncoupled reaction indicates that translation is somehow necessary for *in vitro* expression of the operon. Again, the *hisO1242* mutation specifically eliminates this requirement. The consequences of this observation in the mechanism of *his* operon regulation are considered below, as well.

DISCUSSION

The Activator-Attenuator Concept. Based on evidence presented in this paper, we propose that the major down-

stream regulatory site in the *hisO* region of the *his* operon is regulated in a positive fashion. The *hisO1242* mutation, which defines this site (Fig. 3) and is presumed to be a small deletion (6, 25), obviates an activation mechanism and results in elevated expression of the operon (Figs. 1 and 2). It is apparent, therefore, that the region of DNA deleted by *hisO1242* normally serves to diminish *hisO*$^+$ expression. We have adopted the general term "attenuator" (14) to describe this regulatory site.

Evidence obtained with a minimal *in vitro* transcription system confirms that attenuation occurs at the level of transcription. Kasai (14) observed that, in the absence of specific transcription factors other than RNA polymerase, transcription of the *hisO*$^+$ operon is deficient in comparison with transcription of other bacterial genes on the transducing phage template. The *hisO1242* deletion alleviates this deficiency, specifically enhancing transcription of *his* structural genes.

In vivo, the *hisO1242* mutation elicits essentially maximal, constitutive *his* operon expression (1, 5, 12). Thus, predominant operon-specific regulation can be accommodated by an activation mechanism, the effects of which are exerted at an attenuator.

A Working Model for Regulation of the Histidine Operon. Major features of our model for regulation of *his* operon expression are outlined in Fig. 3. Important points may be summarized as follows.

Under conditions of histidine limitation, derepression is accomplished by a positive factor which interacts in the *hisO* region, allowing RNA polymerase (RP) to bypass an attenuator (a) and transcribe the histidine structural genes. Repression is maintained, under conditions of histidine excess, by inactivation of the positive factor. Repression and derepression are under negative control of the well-established co-regulator of the operon, His-tRNA. In order for His-tRNA to perform this regulatory function, it must contain the Ψ modification in the anticodon loop.

The site of interaction of the positive factor within the *hisO* region is unspecified (—). The factor could interact with RNA polymerase, the promoter, or directly at the attenuator. Mutations such as *hisO3148*, which lead to defects in the activation process (Fig. 1), but which apparently do not affect RNA polymerase binding (12, 14), might define the site of interaction of the positive factor. However, as indicated in the following section, such mutations might also be explained as causing defects in the positive factor itself.

Mutations such as *hisO2321* presumably define the promoter (p) (12, 14); that is, the region of DNA encompassing the site(s) at which RNA polymerase binds and initiates transcription. A role for the regulatory site located at the upstream end of the *hisO* region, defined by *hisO1828* and similar mutations (6), cannot yet be specified.

In another paper (15), an independent mechanism of *his* operon regulation involving ppGpp as a positive effector will be described. ppGpp appears to regulate amino-acid-producing systems, in general. The site at which ppGpp exerts its effect is unknown, but it is *not* the attenuator. The promoter seems a likely possibility. The state of Ψ modification in tRNA appears to be still another regulatory input that affects amino-acid biosynthetic systems, in addition to histidine (2, 3).

Although we consider the central concept of an activator-attenuator mechanism of *his* operon regulation to be strongly supported, other aspects of the model are attractive, but less firmly grounded. In following sections, we consider the possible existence and function of a transcribed and translated "leader" in the *hisO* region, and an accessory regulatory function of G enzyme.

Nature of the Activation Mechanism. The requirement for translation in expression of the *hisO*$^+$ template, but not the *hisO1242* template, observed in uncoupling experiments provides an important clue as to the mechanism of activation, or to the identity of the positive factor, or both. Two interpretations are most apparent.

One possibility is that the requirement for translation is an inherent property of the activation mechanism. That is, transcription of structural genes on the *hisO*$^+$ template is somehow coupled with translation. This cannot be related to a general feature of the bacterial transcription-translation process, but must be intimately related to the regulatory process, since a mutation in the *hisO* region (*hisO1242*) specifically obviates the translation requirement. A second possibility is that a factor necessary for transcription of the *hisO*$^+$ template, but not the *hisO1242* template, is absent from the crude S-30 extract and must be synthesized in the coupled reaction, as a product of a gene on the histidine transducing phage template.

Several recently uncovered aspects of regulation of the *E. coli* tryptophan operon bear directly on the nature of the activation mechanism of the *S. typhimurium* histidine operon. Evidence has been obtained for a very analogous attenuator site located just prior to the first structural gene of the *trp* operon (26). Regulation exerted at the *trp* attenuator is independent of the previously characterized repressor-operator mode of regulation of this operon (26, 27). Of particular interest is the observation that the *trp* operon structural genes are preceded by a very long transcribed "leader" region of at least 160 base pairs (27, 28). A function has not yet been defined for the *trp* leader region. In addition, Imamoto (29, 30) has previously reported a translational requirement for expression of the *trp* operon, *in vivo*, and that this requirement is exerted within, or near, the *trpO* region.

The important point is that if we extend the analogy to include a transcribed leader in the *hisO* region of the *his* operon (Fig. 3), then these observations fit nicely with our finding that translation is necessary for the activation mechanism, *in vitro*. Thus, translation of the hypothetical *his* leader could be required for transcription beyond the attenuator in a kind of coupling mechanism, or translation of the leader could produce a positive factor participating directly in the activation mechanism. Based on suggestive evidence, Wyche *et al.* (31) have proposed histidyl-tRNA synthetase as a positive factor in *his* operon regulation.

G Enzyme Is Not an Essential Component of the Activation Mechanism. Recent evidence has clearly established that G enzyme is unnecessary for repression or derepression of the *his* operon in *S. typhimurium* (J. F. Scott, J. R. Roth, and S. W. Artz, in preparation). This conclusion is derived from studies of mutants in which virtually the entire *hisG* gene is deleted. Such mutants display normal regulation by the following criteria. *hisG* deletion mutants maintain normally repressed enzyme levels when grown in minimal media containing excess histidine, derepress normally upon histidine starvation, and yield expected constitutively derepressed enzyme levels as a consequence of the *hisT1504* (16) regulatory mutation. Since the effect of deleting the *hisG* gene does not resemble the consequences of any regulatory mutation in the *hisO* region, G enzyme cannot be the sole factor interacting with any known site in the *hisO* region. In addition, regulatory effects of the co-regulator, His-tRNA,

could not be a consequence solely of interaction with G enzyme.

The activation mechanism we have described appears to be required for expression of the *his* operon. It therefore seems highly unlikely that G enzyme is the positive factor, itself, or that this protein is an essential component of the activation mechanism.

A Possible Accessory Function for G Enzyme. Biochemical evidence that has been used to implicate G enzyme in regulating expression of the *his* operon includes the following. G enzyme: (*a*) inhibits correct-strand *his* mRNA synthesis in an *in vitro* transcription system (10); and, (*b*) binds specifically to ϕ80d*his*$^+$ DNA, but poorly to ϕ80d *hisO1242* DNA (ref. 11 and R. F. Goldberger, personal communication), that is, DNA from which the attenuator region has been deleted. Assuming these observations to be valid, it is possible to accommodate a specific, accessory function for G enzyme in our model.

Thus, we suggest that G enzyme may be important in obtaining *efficient* restoration of repression of the derepressed *his* operon. The mechanism by which this role of G enzyme would be accomplished is interaction of the protein at, or near, the attenuator to terminate transcription once repressing conditions have been re-established (Fig. 3). This interaction could be facilitated by His-tRNA (9). Loss of the function, as in *hisG* deletion mutants, would not influence the ability of the *his* operon to maintain repression, or to derepress, since these components of the regulatory process depend on the activation mechanism.

At least two situations can be described to illustrate the potential utility of such an accessory factor in our model. First, once the activation mechanism has been triggered, it becomes relatively insensitive to the repression signal (e.g., dissociation of the activation complex is slow). Second, multiple RNA polymerase molecules traverse the proposed leader region, producing several copies of *his* mRNA, even after repression has been signaled. In either case, G enzyme, by terminating transcription at the attenuator, could over-ride the activation mechanism and re-establish repression efficiently.

Implications. It appears likely that the activator-attenuator mechanism will prove to be of widespread significance in cell regulation. Evidence for a similar attenuation phenomenon in regulation of *trp* operon expression (26, 27) has been discussed. Regulation of phage lambda expression by *N* gene product may entail a related mechanism (32). If a positive factor participating in *his* operon regulation is, in fact, coded by the *hisO* region, then the analogy to lambda *N* gene regulation is enhanced.

Finally, it is interesting to note that while regulation of expression of the histidine gene cluster was originally included in the classic paper of Jacob and Monod (4) as a counterpart for a biosynthetic system of the lactose catabolic system, the evidence so far obtained is consistent neither with a specific repressor-operator mechanism of *his* operon regulation, nor with overall negative control. An entirely different type of regulation appears to be in effect.

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