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A *cis/trans* Test of the Effect of the First Enzyme for Histidine Biosynthesis on Regulation of the Histidine Operon

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Previous studies showed that when triazolalanine was added to a derepressed culture of a histidine auxotroph, repression of the histidine operon occurred as though histidine had been added (6). However, when triazolalanine was added to a derepressed culture of a strain with a mutation in the first gene of the histidine operon which rendered the first enzyme for histidine biosynthesis resistant to inhibition by histidine, repression did not occur. The studies reported here represent a *cis/trans* test of this effect of mutations to feedback resistance. Using specially constructed merodiploid strains, we were able to show that the wild-type allele is dominant to the mutant (feedback resistant) allele and that the effect operates in *trans*. We conclude that the enzyme encoded by the first gene of the histidine operon exerts its regulatory effect on the operon not by acting locally at its site of synthesis, but by acting as a freely diffusible protein.

Repression of the histidine operon of *Salmonella typhimurium* requires participation of aminoacylated histidine transfer ribonucleic acid (histidyl-tRNA; references 1, 7, 12-15, 17). Although the mechanism by which histidyl-tRNA acts in repression is unknown, it has been assumed that this molecule fulfills its function in the form of a complex with a specific regulatory protein (2, 3).

The histidine analogue, DL-1,2,4-triazolo-3-alanine (triazolalanine), is known to cause repression of the histidine operon by becoming aminoacylated to histidine tRNA (6). We previously reported that this analogue cannot cause repression of the histidine operon when the feedback-sensitive site of the first enzyme for histidine biosynthesis, phosphoribosyltransferase [*N*-1-(5′-phosphoribosyl)adenosine triphosphate:pyrophosphate phosphoribosyltransferase, EC 4.2.1c], is blocked chemically or damaged structurally (5). Because the gene for the first enzyme is adjacent to the operator gene of the histidine operon, we considered the possibility that the effect of the enzyme on repressibility by triazolalanine was due to some action of the enzyme at its site of synthesis. To test this, we designed experiments to determine whether the effect of a mutation to feedback resistance was *cis* dominant, as expected for an action of the enzyme at its site of synthesis, or whether it is the wild-type allele which is dominant and effective in *trans*, as expected for an action of the enzyme as a freely diffusible gene product like other known regulatory proteins.

MATERIALS AND METHODS

Enzyme assays and substrates. Protein was determined by the method of Lowry et al. (8) with insulin standards. Assays for the enzymes specified by the first gene (*hisG*), third gene (*hisC*), and seventh gene (*hisF*) of the histidine operon were performed as previously described (10). Enzyme levels are expressed as the amount of activity per milligram of protein. Substrates were obtained as follows: L-histidinol phosphate and 5-phosphoribosyl-1-pyrophosphate were purchased from Cyclo Chemical Co.; *N*′-(5′-phospho-D-ribosylformimino)-5-amino-1-(5″-phosphoribosyl)-4-imidazolecarboxamide was synthesized enzymically from ATP (Sigma Chemical Co.) and 5-phosphoribosyl-1-pyrophosphate (10).

Bacterial strains. The strains employed in this study are listed in Table 1. The following strains were

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TABLE 1. *Strains employed*

Strain no.[a]	Genotype	Histidine requirement
TA 830	*hisOGDCBHAF644*	Yes
hisIF135[b]	*hisIF135*	Yes
TA929[c]	*hisG1102 hisIF135*	Yes
SB900[d]	*hisOG1302 hisW1824*	Yes
TG5718[d]	*hisOG1302 hisW1824 pur-804*	Yes
TG5720[c, d]	*F'80hisG1929 hisB*$^+$*/ hisOG1302 hisW1824 pur-804*	No
TG5722[b, c]	*F'80hisG1929 hisB*$^+$*/hisIF135*	No
TR75[b]	*F'80hisB2405/hisDCBHAFIE712 arg-501 ser-821*	Yes
TR59[b]	*F'80hisC2389/hisDCBHAFIE712 arg-501 ser-821*	Yes
TG769[b, c]	*F'80hisC2389/hisG1102 hisIF135*	No
TG5705[b, c]	*F'80hisC2389/hisOGDCBHAF644*	Yes
TG5719[b, c]	*F'80hisB2405/hisOG1302 hisW1824 pur-804*	No
TG5721[c, d]	*F'80hisG1929 hisB*$^+$*/ hisOGDCBHAF644*	No

[a] All strains designated TG were constructed as part of the present study (see materials and methods).

[b] Produces normal phosphoribosyltransferase.

[c] Produces feedback-resistant phosphoribosyltransferase.

[d] Produces no phosphoribosyltransferase.

obtained from the collection of P. E. Hartman: *hisOGDCBHAF644*, *hisIF135*, TA929, SB900, TR75, and TR59. The mutation, *hisG1102* (in strains TA929 and TG5769), is a missense mutation which results in the production of a catalytically active feedback-resistant phosphoribosyltransferase (16); the mutation *hisW1824* (in strains SB900, TG5718, and TG5720) is not linked to the histidine operon and results in constitutive expression of the operon (1). Strain TG5769 was prepared by transfer of the episome from strain TR59 into strain TA929, selecting for histidine prototrophy; strain TG5705 was prepared by transfer of the episome from strain TR59 into strain TA830, selecting for growth on histidinol. In both cases, the donor, strain TR59, was counter-selected by omission of arginine and serine from the mating plates; strain TG5718 was derived from strain SB900 by penicillin selection for a spontaneously arising adenine requirement (*pur-804*).

Isolation of a strain carrying an episome with a mutation to feedback resistance was accomplished by the following procedure, which is a modification of the method of Sheppard (16). Strain TG5719 was prepared by transferring the episome of strain TR75 into strain TG5718. This transfer was identified by selecting for histidine prototrophy. The merodiploid strain was used for selection of feedback-resistant mutations. Since it contained no chromosomal *hisG* gene, only episomal mutations could be obtained by selection for feedback resistance. It had been shown by Sheppard (16) that a mutation to feedback resistance causes the organism to excrete histidine. This excretion of histidine can be detected by growth on minimal plates of a histidine auxotroph in the immediate vicinity of a colony of the feedback-resistant strain. We found, in keeping with the findings of J. R. Roth (unpublished data), that the presence of an additional (constitutive) mutation causing derepression of the histidine operon enhances this "feeding" by a feedback-resistant strain. Therefore, we started selection for a feedback-resistance episome by mutagenesis of strain TG5719 which contains the chromosomal constitutive mutation, *hisW1824*. Mutagenesis was carried out by the addition of a few crystals of *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co.) to each of 20 mid-log-phase cultures, each in 10 ml of medium E of Vogel and Bonner (18), supplemented with glucose (0.5%) and adenine (20 μg/ml). After the cultures had been shaken at 37 C for 10 min, the cells were centrifuged, washed, and suspended in fresh medium containing D,L-2-thiazolalanine (thiazolalanine, 10 mM; Cyclo Chemical Co.). Thiazolalanine is an analogue of histidine that inhibits phosphoribosyltransferase by the same mechanism as does histidine (9, 11). By inhibiting the biosynthesis of histidine, it inhibits growth of all strains prototrophic for histidine except those that contain a mutation affecting the ability of phosphoribosyltransferase to be inhibited (feedback-resistant mutant) or a mutation affecting the ability of the cell to transport the analogue (permease mutant). Thus, growth of the mutagenized cultures in the presence of thiazolalanine enriched the cultures for such mutants. Feedback-resistant mutants could then be distinguished from permease mutants by their ability to "feed." After growth to saturation in thiazolalanine, approximately 10^8 mutagenized cells were mixed with approximately 10^8 cells of the histidine auxotroph, strain TA830, in 2 ml of 0.6% melted agar (45 C) containing thiazolalanine (50 mM) and adenine (15 μg/ml). This mixture was poured onto a medium E plate. The presence of thiazolalanine in the pour plate further enhanced selection for feedback-resistant mutants and permease mutants. After incubating the plates for 48 h at 37 C, feeder colonies were easily recognized and picked. Twenty-five such colonies were purified by repeated single-colony isolation on minimal medium. Each of these strains was found to contain a phosphoribosyltransferase highly resistant to inhibition by histidine. One of them, strain TG5720, was chosen for further studies. The phosphoribosyltransferase of this strain was not inhibited at all by histidine at a concentration (5×10^{-2} M), 1,000-fold higher than the K_i of the wild-type enzyme. Transfer of the episome to strain *hisOGDCBHAF644* yielded strain TG5721 which, as expected, was highly feedback resistant but which, in addition, was prototrophic, indicating that a reversion of the episomal *hisB* missense mutation had occurred. This episome was also transferred to strain *hisIF135* to yield strain TG5722.

Growth conditions. Cells were routinely grown in 2 liters of medium E of Vogel and Bonner (18), with

glucose at 0.5%, in a 4-liter flask. The cultures were aerated vigorously in a New Brunswick rotary shaker at 37 C. The histidine auxotrophs were grown in the presence of a sufficient amount of L-histidine to support growth to an optical density at 700 nm of approximately 0.35 (3.4×10^8 cells/ml). After depletion of histidine from the growth medium, growth was supported by L-histidinol (2.5×10^{-5} M). Derepression of prototrophic merodiploid strains was produced by adding 3-amino-1,2,4-triazole (aminotriazole, 15 mM; Aldrich Chemical Co.). Aminotriazole inhibits imidazole glycerol phosphate dehydratase, the enzyme catalyzing the seventh step of the histidine pathway. Because aminotriazole also inhibits a step of purine biosynthesis (4), adenine (50 µg/ml; Cyclo Chemical Co.) was added to the culture medium.

Experimental design. In a typical experiment, 2 liters of culture was grown in a rotary shaker in a 4-liter flask. Samples (80–100 ml) were withdrawn periodically, so that six samples were collected during derepression and six samples were collected after the addition of histidine (1.6×10^{-3} M) or triazolalanine (3.2×10^{-4} M; Cyclo Chemical Co.). Each sample was immediately mixed with ice and excess histidine. The samples were then centrifuged, and the pelleted cells were washed with 0.05 M tris(hydroxymethyl)aminomethane buffer, pH 8.0, and recentrifuged. The cells were then suspended in 3.5 ml of the same buffer. Extracts were prepared from the suspensions with a French pressure cell (American Instrument Co.) at 6,000 lb/in^2 (ca. 4,218, 600 kg/m^2), clarified by centrifugation, and assayed.

RESULTS

The studies reported here concern the relationship between wild-type and mutant alleles of the *hisG* gene with respect to their effects on repressibility of the histidine operon by triazolalanine. The experimental design was essentially the same as that employed in a previous study (5) which showed that triazolalanine does not cause repression in feedback-resistant mutants. In that study we showed that, when triazolalanine was added to a derepressed culture of a histidine auxotroph, repression of the histidine enzymes occurred as though histidine had been added. Repression failed to occur, however, when triazolalanine was added to a derepressed culture of an isogenic strain which had, in addition, a mutation in the *hisG* gene which rendered the phosphoribosyltransferase resistant to feedback inhibition (5).

In the first series of experiments, we examined the question of whether the response to triazolalanine in a feedback-resistant strain would be altered if an episome, bearing a wild-type *hisG* gene, were present in the cytoplasm (Fig. 1). Figure 1A shows the results of a control experiment in which the episome was in the cytoplasm of a strain from which the histidine operon had been deleted. The histidine operon of the episome was derepressed by limiting the amount of histidine available in the culture medium and was repressed by the addition of triazolalanine. Figure 1B shows the results of another control experiment, in which derepression and repression were studied in the feedback-resistant strain alone. As shown previously (5) and confirmed here, when triazolalanine was added to the derepressed culture, repression of the histidine operon did not occur, despite the fact that the growth rate returned to that characteristic of repressed cells. Figure 1C shows the results obtained in the merodiploid strain: episome with a wild-type *hisG* gene; chromosome with a feedback-resistance mutation in the *hisG* gene. The presence of a mutation in the *hisC* gene of the episome allowed us to use *C*-enzyme (aminotransferase) activity to assess chromosomal expression; the presence of a deletion of the *hisI* and *hisF* genes of the chromosome allowed us to use *I*- and *F*-enzyme activities to assess episomal expression. The addition of triazolalanine to a derepressed culture of this merodiploid strain caused repression of the histidine operons of both chromosome and episome. The same results were obtained in numerous experiments. In all cases, repression of the histidine operon by triazolalanine could be obtained in a feedback-resistant strain only when an episome bearing a wild-type *hisG* gene was present in the cytoplasm. Thus, the normal phosphoribosyltransferase, specified by the wild-type episomal *hisG* gene, was able to restore repressibility to the chromosome, demonstrating a *trans* effect of the wild-type allele.

Because more than one copy of an episome is present in the cytoplasm, in order to pursue the question of dominance, we carried out a series of experiments which are reciprocal to those described above. For these experiments we studied the effect of a wild-type chromosomal *hisG* gene on repressibility of an episome bearing a feedback-resistance mutation. It was first necessary to isolate a feedback-resistance episome. A procedure was developed by which very highly feedback-resistant episomal mutants could be selected with the same basic principles originally used by Sheppard (16) to select feedback-resistant chromosomal mutants. Assays of extracts of strains carrying these episomes (but with the chromosomal histidine operon deleted) showed that the phosphoribosyltransferase they produced was very highly resistant to inhibition by histidine.

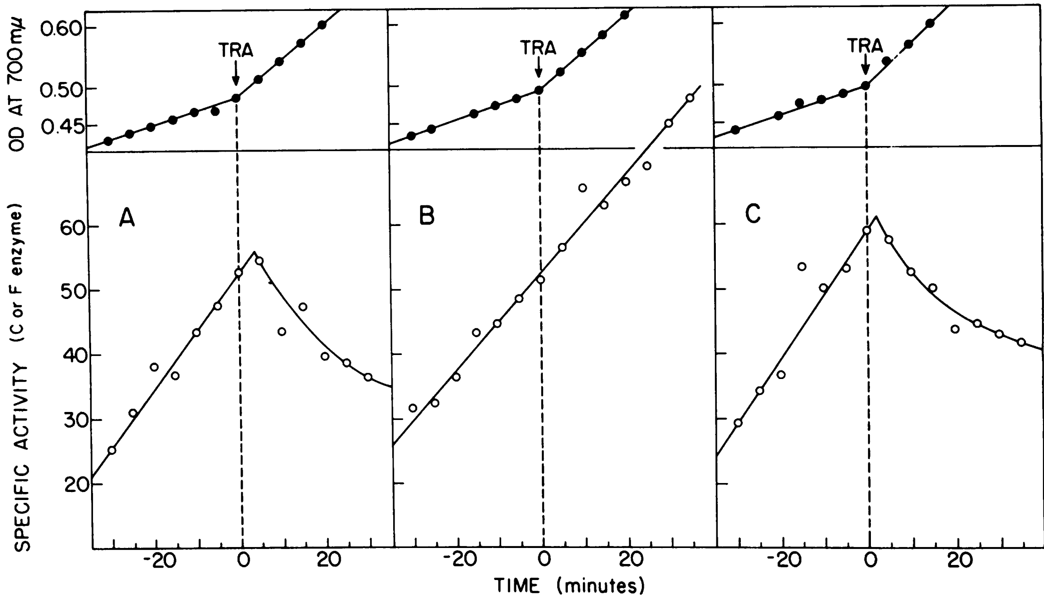

FIG. 1. *Effect of a wild-type hisG gene carried by an episome in the cytoplasm of a strain with a chromosomal mutation to feedback resistance. For each part, the growth curve is shown at the top. During the first portion of the experiments, the organisms were grown on limiting histidine which caused derepression of the histidine operon. When triazolalanine was added to the derepressed cultures (arrows), the growth rates immediately increased to that characteristic of repressed cells. The specific activity of the C enzyme or F enzyme at various times during the experiments is shown at the bottom. (A) F-enzyme activity of strain TG5705 (episome with a mutation in the hisC gene; chromosome with a deletion of almost the entire histidine operon). The data show that the episomal histidine operon is derepressible by histidine limitation and repressible by triazolalanine. (B) C-enzyme activity of strain TA929 (haploid strain in which there is a hisG mutation to feedback resistance and a deletion of the hisI and hisF genes). The data show that the (chromosomal) operon of this feedback-resistant strain is derepressible by histidine limitation but cannot be repressed by triazolalanine. (C) C-enzyme activity of strain TG5769 (episome with a mutation in the hisC gene; chromosome with a mutation to feedback resistance in the hisG gene and a deletion of the hisI and hisF genes). The data show that the episome (carrying a wild-type hisG gene) confers upon the histidine operon of the chromosome the ability to be repressed by triazolalanine.*

The effect of triazolalanine on repressibility was tested by using a feedback-resistance episome in a strain in which the histidine operon was deleted from the chromosome. Surprisingly, when the growth of this merodiploid strain was limited by histidine deprivation, the histidine operon failed to become derepressed at all. An experiment which illustrates this phenomenon is shown in Fig. 2A. Although repressibility of the episome alone could not be studied, the question of whether or not a wild-type *hisG* gene on the chromosome would confer upon the episome the ability to become derepressed and repressed could be studied. Therefore, we introduced the feedback-resistance episome into a strain with a wild-type chromosomal *hisG* gene but with a deletion of the *hisI* and *hisF* genes. Because of the chromosomal deletion in this strain, we could use *I*- and *F*-enzyme activities to assess episomal expression. When the growth of this merodiploid strain was limited by histidine deprivation, the histidine operon of the episome did become derepressed; when triazolalanine was then added to the derepressed culture, the histidine operon of the episome became repressed. These findings are illustrated by the experiment shown in Fig. 2B. Numerous similar experiments gave the same results. Thus, it is clear that the episomal operon, which is by itself unresponsive to the availability of histidine, becomes responsive and becomes repressible by triazolalanine when it is in the cytoplasm of a strain which carries a wild-type chromosomal *hisG* gene. These findings demonstrate again the *trans* effect of the wild-type allele. They also demonstrate that the feedback-resistance mutation does not show a position effect due to gene dosage.

Table 2 summarizes the results of more than 50 experiments, each involving duplicate assays for several enzymes and for protein in at

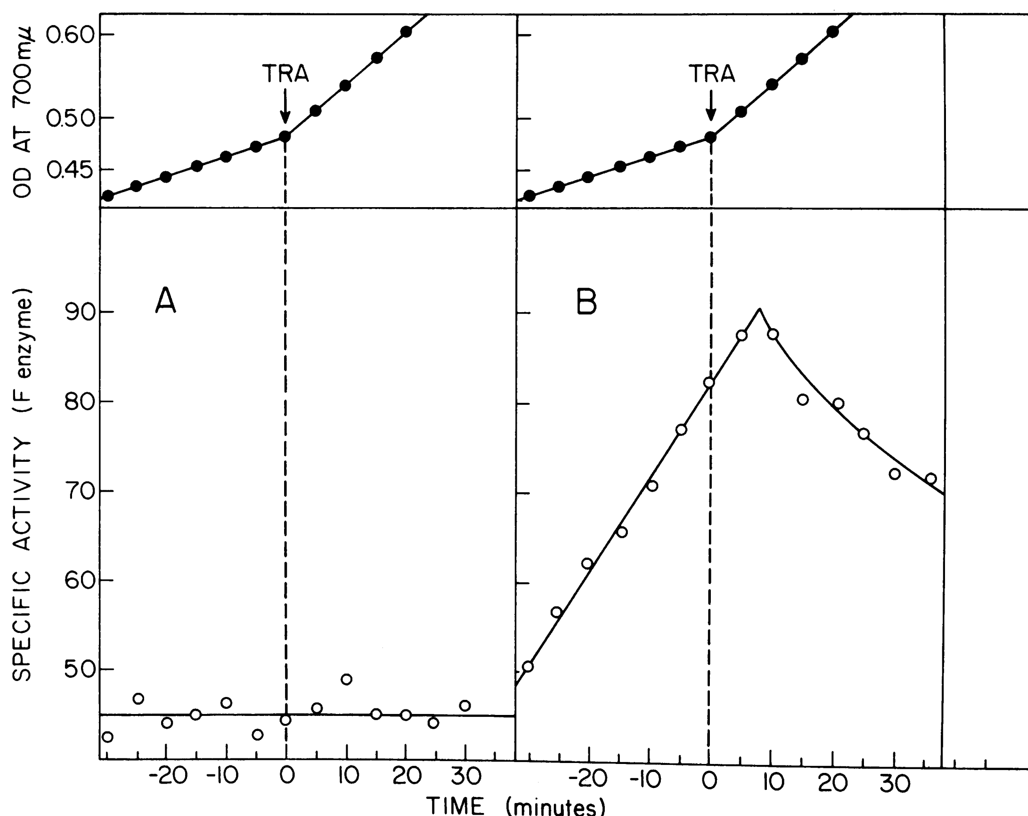

FIG. 2. *Effect of a wild-type hisG gene carried on the chromosome of a strain which has in its cytoplasm a feedback-resistance episome. For both parts, the growth curve is shown at the top. During the first portion of the experiments, the organisms were grown on limiting histidine. When triazolalanine was added to the cultures (arrows), the growth rates immediately increased to that characteristic of repressed cells. The specific activity of the F enzyme at various times during the experiments is shown at the bottom. (A) F-enzyme activity of strain TG5721 (episome with a hisG mutation to feedback resistance; chromosome with a deletion of almost the entire histidine operon). This experiment shows that the episomal histidine operon is not under histidine regulation. (B) F-enzyme activity of strain TG5722 (episome with a hisG mutation to feedback resistance; chromosome with a deletion of the hisI and hisF genes). This experiment shows that, when there is a wild-type hisG gene on the chromosome, the episomal histidine operon is under histinde regulation and is repressible by triazolalanine.*

TABLE 2. *cis trans tests*

Strain number	Genotype	Phosphoribosyltransferase		Repressibility by triazolalanine
		From chromosome	From episome	
hisIF135[a]	*hisIF135*	Normal		+
TA929[a]	*hisG1102 hisIF135*	Feedback resistant		–
TG5705	*F'80hisC2389/hisOGDCBHAF644*	None	Normal	+
TG5769	*F'80hisC2389/hisG1102 hisIF135*	Feedback resistant	Normal	+
TG5721	*F'80hisG1929 hisB⁺/hisOGDCBHAF644*	None	Feedback resistant	[b]
TG5722	*F'80hisG1929 hisB⁺/hisIF135*	Normal	Feedback resistant	+

[a] Previously reported data (5), confirmed in the present study.
[b] Cannot be derepressed by limitation of histidine.

least 12 samples. The data shown in the first and second lines of the table demonstrate, as reported previously (5), that mutation to feedback resistance in a haploid strain causes loss of repressibility by triazolalanine. The data shown in the third and fourth lines of the table

demonstrate that the presence of an episome bearing a wild-type *hisG* gene in the cytoplasm of a feedback-resistant strain restores to the chromosomal histidine operon the capacity to be repressed by triazolalanine. The data shown in the fifth and sixth lines of the table demonstrate that the histidine operon of an episome which has a mutation to feedback resistance (and has lost the capacity to respond to regulation by histidine) becomes derepressible by limitation of histidine and repressible by triazolalanine, when it is present in the cytoplasm of a strain with a wild-type chromosomal *hisG* gene.

DISCUSSION

Since the gene that specifies the phosphoribosyltransferase of histidine biosynthesis is located next to the operator gene of the histidine operon, we wondered whether the effects of the enzyme on regulation were exerted by the nascent or newly completed protein at its site of synthesis. If that were the case, the effect of a mutation to feedback resistance on repressibility by triazolalanine would be *cis* dominant. The studies reported here rule out this possibility. It is clear that in merodiploid strains it is the wild-type allele which is dominant and that the effect operates in *trans*. We conclude that phosphoribosyltransferase exerts its regulatory effect on the histidine operon as a freely diffusible protein.

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