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Translation and Polarity in the Histidine Operon

III. The Isolation of Prototrophic Polar Mutations

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Mutants of the histidine operon of *Salmonella typhimurium* have been isolated which show a strong polarity effect but which do not require histidine for growth. The mutants were isolated from an operator constitutive strain, *his01242*, which produces high levels of the histidine biosynthetic enzymes. The selection of these polar mutants was based on the finding that, in constitutive strains, strongly polar mutations within the histidine operon cause a change in colony morphology and temperature sensitivity. Three of the polar mutations map in a structural gene. Another maps in or near the operator region and is distinct from the *his01242* locus. No reverse mutations of the *his01242* locus were found.

1. Introduction

Polar mutants in the histidine operon of *Salmonella typhimurium* have lost one enzyme activity and have reduced levels of the enzymes coded by all histidine genes which, with reference to the operator, are located distally to the point of mutation. To account for such mutants, Ames & Hartman (1963) proposed that there was a single polycistronic histidine RNA message (Martin 1963) and that ribosomes attached only at the operator end during translation. They further proposed the possible existence of modulatory codons near the beginning of genes in the wild-type organism which would cause some of the ribosomes to dissociate from the polycistronic message and lead to reduced translation of distally located genes. These modulating codons would serve the function of allowing different numbers of protein molecules to be synthesized from a single messenger RNA. Polar mutations were thought to be mutations to new modulatory codons within a gene. Subsequently, polar mutations in the histidine operon were equated with nonsense codons (Martin, Silbert, Smith & Whitfield, Jr., 1966*a*; Whitfield, Jr., Martin & Ames, 1966; Martin, 1967).

As expected from the fact that nonsense codons cause polypeptide chain termination, the polar mutants which have been isolated in a number of systems totally lack one enzyme activity, and thus polar mutants in the histidine operon, for example, are histidine auxotrophs. In this communication, however, the isolation of mutants in the histidine operon which exhibit polar effects but which can grow in the absence of histidine is described. Some preliminary results of this investigation have been reported by Martin, Whitfield, Jr., Berkowitz & Voll (1966*b*).

2. Materials and Methods

(a) *Bacterial strains and phage*

S. typhimurium wild-type LT-2 and many of the histidine deletions in non-constitutive strains derived from it have been described (Loper, Grabnar, Stahl, Hartman & Hartman, 1964). Deletions *hisGDCBH2253* and *hisHAFIE2327*, which are in a strain carrying the *hisT1504* constitutive mutation, and deletion *hisDC2236*, which is in a strain carrying the *his01242* constitutive mutation, were obtained from Drs Fink and Kłopotowski (Fink, Kłopotowski & Ames, 1967). The *hisO* locus is cotransducible with the histidine operon, whereas the *hisT* locus is not (Roth, Antón & Hartman, 1966). *His01242* (Roth *et al.*, 1966) is a prototrophic strain containing the *his01242* mutation. This mutation was isolated in *S. typhimurium* strain SL751 and was re-isolated in a prototroph by transduction into the deletion *hisGDC63* and selection of a P22-sensitive prototrophic recombinant clone (Roth *et al.*, 1966).

Phage stocks were prepared and stored as described by Hartman (1956). Nutrient broth cultures infected at low multiplicity in early log phase were incubated for several hours, after which time unlysed bacteria and debris were removed by low-speed centrifugation. Phage stocks were sterilized with chloroform and, when necessary, were diluted in T2 buffer (Hershey & Chase, 1952). Donor phage used in transduction studies were prepared by infecting the donor strain with P22 phage grown on LT-2 or on the deletion, *hisGDCBHAF644*.

(b) *Media*

Liquid E minimal medium (Vogel & Bonner, 1956) contained 0·5% glucose, and minimal E agar contained 1·0% glucose, and were supplemented when necessary with 0·1 mM-L-histidine. Difco nutrient broth containing 0·4% NaCl was used as enriched medium. Solid medium contained 1·2% Difco agar.

(c) *Transduction technique*

About 2×10^8 bacteria from a fully grown histidine-supplemented minimal culture of the recipient strain were spread directly on the surface of minimal agar plates with enough donor phage to give a multiplicity of 1 to 10. The plates were incubated at 37°C for 48 hr or longer and then scored for recombinant colonies.

(d) *Enzyme assays of extracts*

Bacteria were inoculated into 100 or 200 ml. of minimal or of histidine-supplemented minimal medium and incubated at 37°C with agitation until mid-log phase. Cells were harvested by centrifugation and resuspended in 1·5 ml. of 0·1 M-Tris buffer (pH 7·5). The suspended cells were disrupted in a Branson S-75 Sonifier for two 20-sec periods at 4 A. The extracts were clarified by centrifugation at 31,000 *g* for 20 min and then passed through Sephadex G50 gel. The extraction procedures were carried out at 0 to 4°C. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Assays for the histidine enzymes, PR-ATP synthetase (Voll, Appella & Martin, 1967), dehydrogenase (Ames *et al.*, 1963), aminotransferase (Martin & Goldberger, 1967), phosphatase (Ames, Garry & Herzenberg, 1960), isomerase (Margolies & Goldberger, 1966), and amidotransferase and cyclase (Smith & Ames, 1964) were generally done in duplicate.

(e) *Phosphatase assay in toluenized cells*

Nutrient broth cultures were diluted 1/30 in histidine-supplemented minimal medium and incubated at 37°C with agitation to a density of about 4×10^8/ml. The cells were treated with toleune and assayed for phosphatase activity as described by Ames *et al.* (1963), except that 0·010 ml. of substrate was used. Individual colonies were also assayed by this procedure. Colonies from plates were picked up with sterile applicator sticks and resuspended in buffer for assay.

(f) *Growth rate determinations*

Growth rates of mutants were determined in minimal or histidine-supplemented minimal medium at 37°C in a rotary shaker. Absorbancy of the cultures at 650 mμ was measured

as a function of time of incubation. The wild-type strain, LT-2, which has a generation time of approximately 48 min in minimal and histidine-supplemented minimal medium, was used as a control. *His01242* has approximately the same generation time as LT-2.

(g) *Selection experiments*

His01242 has a wrinkled colony morphology on glucose minimal agar and is inhibited in growth at 43°C. Cultures of *his01242* were plated on minimal agar plates (about 10^8 cells per plate) and the plates were incubated at 43°C. Colonies which grew at 43°C and which had smooth surfaces were picked from the plates, streaked out on minimal agar and incubated at 43°C. Isolates able to form smooth colonies on minimal agar at 43°C are listed in Table 1. A single colony from the 43°C streaks was used to prepare stock cultures for further study. The minimal agar plates used for selection and isolation contained 1·0% glucose, except in experiment (e) (Table 1), where 0·1 or 0·5% glucose was used in the selection plates and 0·1% glucose was used in the isolation plates.

(h) *Chemicals*

Triazolealanine and thiazolealanine were gifts of R. G. Jones, Eli Lilly Co., Indianapolis, Indiana, and the acridine half-mustard, ICR-191, was a gift of Dr Hugh J. Creech. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine was obtained from the Aldrich Chemical Co., Inc. and 2-aminopurine nitrate from the California Corp. for Biochemical Research.

3. Results

(a) *Selection experiments*

Mutations in the histidine operon causing polar effects but not resulting in a histidine requirement were selected by a technique based on differences between cells producing high levels of the histidine enzymes and those producing low levels. Cells producing low (repressed) levels of the histidine enzymes, such as the wild-type LT-2, give colonies with smooth surfaces on minimal agar containing 1·0% glucose as the carbon source and they can grow at temperatures up to 45°C. In contrast, strains de-repressed for the histidine enzymes give colonies with wrinkled surfaces on minimal glucose agar (B. N. Ames, personal communication) and their growth is inhibited at 43°C. Although the bases for "wrinkledness" and temperature sensitivity are not known, these properties are associated with cells producing high levels of the histidine enzymes. Strongly polar mutations in the histidine genes abolish these two characteristics (Fink *et al.*, 1967).

The strain used for selection was an operator constitutive strain, *his01242*, which produces high levels of all the histidine enzymes, forms wrinkled colonies, and fails to grow at 43°C. Mutants of this strain able to grow at 43°C and form smooth colonies on minimal agar were isolated and studied. When about 10^8 cells of *his01242* are plated on minimal agar, many distinct colonies are noted in addition to some residual background growth after two days of incubation at 43°C. The number of mutants able to grow at 43°C, which one obtains, varies with slight variations in the temperature of incubation. Generally about 1 in 10^5 cells form distinct colonies at the high temperature. The great majority of these colonies are still wrinkled, but about 1% are smooth (G. Fink, personal communication).

A total of 53 smooth mutants was isolated at 43°C in five separate selection experiments (Table 1). The mutants chosen for study were picked at random except in selection experiment (b), where several smooth mutants from each plate were first examined for phosphatase activity by the assay procedure using toluenized cells.

One (*his01242 C2353*) had low levels of phosphatase activity compared to the high levels found in *his01242*. One to two smooth mutants from each plate were chosen for further study.

TABLE 1

Smooth temperature, mutants of his01242

Selection experiment	Glucose concn on plate (%)	No. of smooth mutants studied	Mutations outside histidine operon (no.)	Mutations inside histidine operon
(a)	1·0	11	8 (500)†	*H2335, H2336, C2352*
(b)				
Culture 1	1·0	2	2 (1000)	
Culture 2	1·0	1	0	*H2337*
Culture 3	1·0	2	1 (500)	*C2353*
Culture 4	1·0	1	1 (1000)	
Culture 5	1·0	2	1 (1000)	*H2338*
(c)				
Culture 1	1·0	1	0	*H2339*
Culture 2	1·0	5	1 (1000)	*H2340, H2341, H2342, H2343*
Culture 3	1·0	3	2 (1000)	*H2344*
Culture 4	1·0	4	3 (500)	*H2345*
Culture 5	1·0	3	2 (300)	*H2346*
(d) (adenine)				
Culture 1	1·0	1	1 (190)	
Culture 2	1·0	1	1 (1000)	
Culture 3	1·0	2	1 (240)	*H2347*
(e)				
Culture 1	0·1	2	2 (250)	
	0·5	1	1 (400)	
Culture 2	0·1	4	4 (100)	
	0·5	2	0	*H2348, H2349*
Culture 3	0·1	3	2 (170)	*H2350*
	0·5	2	0	*C2354, his2355*

In experiments (b), (c), (d) and (e), small inocula (<3500/ml.) of the same single-colony isolate of the stock *his01242* strain were shaken at 37°C in nutrient broth until fully grown (about 2×10^9/ml.). The cultures were diluted 1 : 20 in minimal medium, and 0·20 to 0·50 ml. of each culture was spread on the surface of a minimal agar plate. The plates were incubated at least 2 days at 43°C, and smooth colonies were isolated as described under Materials and Methods. In experiment (a), a nutrient broth culture of the stock *his01242* strain was diluted and plated. Otherwise the procedure was the same. In selection experiment (d), 0·05 mM-adenine was present in the selection plates. Adenine enhances the inhibitory effect of high temperature on *his01242* and clears the background of residual growth. Colonies numbering 250, 85 and 58 were obtained with cultures 1, 2 and 3, respectively. All four smooth revertants from these selection plates were further examined. In selection experiment (e), selection was done on plates containing 0·1 and 0·5% glucose. Colonies of *his01242* are still perceptibly wrinkled on 0·1% glucose minimal agar.

† Minimum number of transductants examined with *hisG203* as recipient.

(b) *Mapping of the smooth mutations with respect to the histidine operon*

The smooth mutants were screened to determine whether the mutations causing smoothness were cotransducible with the histidine operator region. Transductions were performed with the mutants as donors and with the deletion, *hisG203*, as recipient, and prototrophic recombinants were selected. *HisG203* lacks the initial segment of the *G* gene, the operator region, and part of the chromosome normally present to the left of the operator region (Fig. 1). Transductants were selected on minimal agar and cotransducibility was scored on the basis of the colony morphology of the transductants. To grow on minimal medium such transductants must incorporate the operator constitutive locus of the donor and hence will be either smooth or wrinkled, depending upon whether or not they also incorporate the mutation causing smoothness.

The smooth mutants fell into three classes on the basis of the transduction test. Thirty-three of the mutants gave wrinkled transductants only, indicating that the mutations to smoothness in these mutants were not cotransducible with the histidine operator region. Nineteen of the mutants gave both wrinkled and smooth transductants, indicating that in these mutants the mutations to smoothness were cotransducible with the operator region but not covered by the *hisG203* deletion. One mutant gave only smooth transductants, indicating that the mutation to smoothness in this case was in the region covered by *hisG203* deletion.

The distribution of the smooth mutants falling in the first class is shown in Table 1, under the heading "Mutations outside the histidine operon". The number in parenthesis is the minimum number of transductants examined for colony morphology. Eleven of these mutants (five from selection (a), all from selection (b) and one from selection (d)) were grown in histidine-supplemented minimal medium and were tested for phosphatase activity by the assay using toluenized cells. All had de-repressed levels of phosphatase activity. Mutants of this class were not further examined, but their occurrence indicates that there are one or more sites outside of the histidine operon which can mutate to produce smooth-colony morphology and growth at 43°C in operator constitutive strains without affecting the histidine enzyme levels.

Those mutants in which the mutations to smoothness were cotransducible with the histidine operator region were further characterized by genetic mapping and by enzymic analyses. All had mutations in the histidine operon. Three were defective in *C* gene activity, 16 had mutations in the *H* gene, and one had a mutation in or near the operator region. The distribution of these mutants among the various selection experiments is shown in the last column of Table 1.

(c) *Mutations in the* C *gene*

The *C* gene is the third gene from the operator end of the histidine operon and codes for an aminotransferase. Three of the smooth mutants had mutations in the *C* gene based on enzymic analyses (Table 2) and genetic tests (Table 3).

As reported previously, *his01242 C2352* and *his01242 C2353* (mutants s-2 and s-16, Martin, Whitfield, Jr., Berkowitz & Voll, 1966*b*) have abnormally low aminotransferase activity and are strongly polar. The activity of the enzymes specified by the genes distal to the *C* gene is about 10% of that found in *his01242* (Table 2). *His01242 C2354* is deficient in *C* gene activity and is also strongly polar.

His01242 C2352 shows a reduction in the activity of the *D* gene. A reduction in activity of the enzyme specified by the gene located operator proximal to a gene

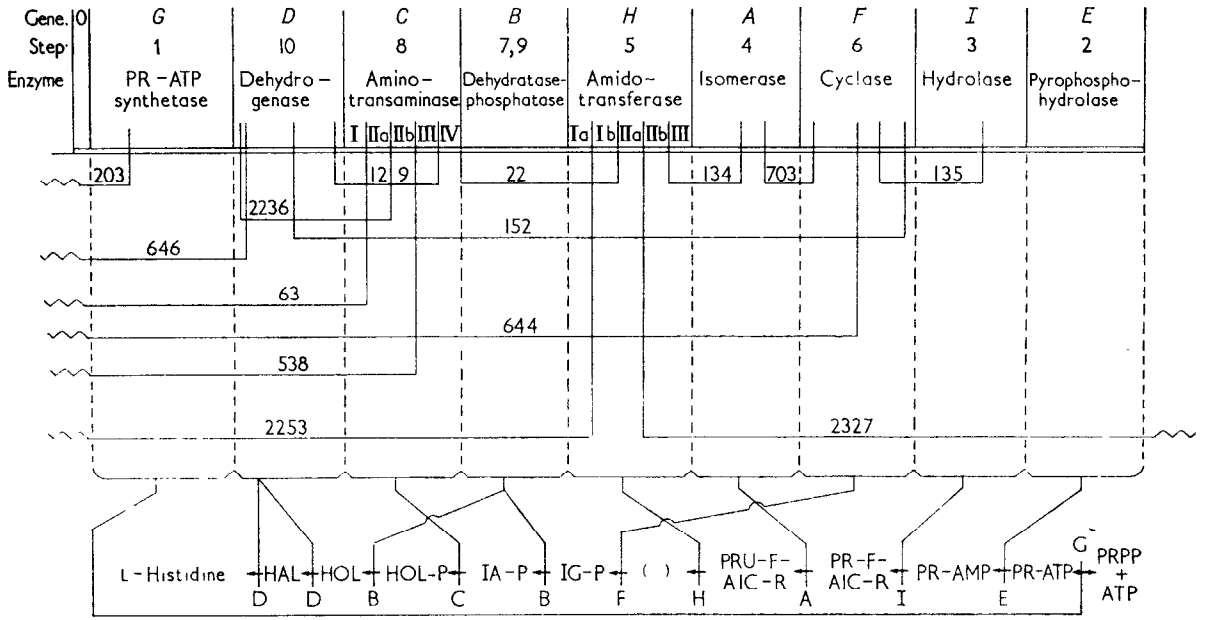

FIG. 1. Map of the histidine operon

The order of the genes, the enzymes specified by the genes, and the order of the biochemical steps controlled by the genes are given at the top of the diagram. The extent of various histidine deletions is shown in the middle. The biosynthetic sequence is shown at the bottom. HAL, histidinal; HOL, histidinol; HOL-P, histidinol phosphate; IAP, imidazole acetol phosphate; IGP, imidazole glycerol phosphate; PRU-F AIC-R and PR-F AIC-R, phosphoribulosyl-formimino- and phospho-ribosyl-formimino- aminoimidazole-carboxamide ribotide, respectively.

TABLE 2

Enzyme levels in C *mutants*

Strain	Relative specific activity				
	G enzyme	*D* enzyme	*C* enzyme	*B* enzyme	*A* enzyme
his01242	1·0	1·0	1·0	1·0	1·0
LT-2	0·1	0·1	0·1	0·1	0·1
his01242 C2352, exp. 1	1·20 (1·73)	0·72 (0·67)	0·02 (0·014)	0·14	0·08 (0·061)
his01242 C2352, exp. 2	0·98 (1·44)	0·37 (0·39)	— (<0·02)	0·12 (0·082)	0·11 (0·074)
his01242 C2353	1·51 (1·50)	—	0·02 (0·025)	0·08 (0·079)	0·08 (0·094)
his01242 C2354	0·89	0·75	<0·10	0·16	0·12

Extracts were prepared and assayed for enzyme activities as described under Materials and Methods. In each experiment, an extract of the parental strain *his01242*, and, in some cases, also the wild-type strain, LT-2, served as controls. Specific activities are expressed relative to those found in *his01242*, which are taken as 1·0. Figures in parenthesis are specific activities relative to those found in LT-2, which are taken as 0·1. The levels of the histidine enzymes found in *his01242* are 10- to 12-fold greater than those found in LT-2. All mutants were grown in minimal medium for assay except *his01242 C2354*, which was grown in minimal medium supplemented with histidine. The relative specific activities given with respect to *G* enzyme activity should be considered as approximations. Although the assay for *G* enzyme activity gives consistent results with any one extract, the amount of *G* enzyme activity extractable from any given strain varies somewhat from preparation to preparation.

having a polar mutation has been reported in the tryptophan operon (Ito & Crawford, 1965). No similar antipolar effect has been found with polar nonsense and frameshift mutations in the histidine genes (Fink & Martin, 1967).

Both *his01242 C2352* and *his01242 C2353* grow well with and without histidine in minimal medium. In minimal medium at 37°C, generation times of 78 and 58 minutes were obtained for *his01242 C2352* and *his01242 C2353*, respectively, compared to a generation time of 50 minutes for LT-2. In minimal plus histidine, both mutants had a generation time of approximately 58 minutes. *His01242 C2354* grows on minimal medium, but extremely slowly. To test the possibility that this mutant might be a histidine auxotroph and that growth might be due to a high reversion rate to prototrophy, a nutrient broth culture of *his01242 C2354* was diluted 10^{-6} in minimal medium and plated on minimal agar. Small but easily visible colonies were produced in about six days of incubation in the expected number and before any fast-growing revertants were noted. In minimal medium plus histidine, the generation time of this strain was approximately that of wild-type.

The *C* gene mutants were mapped by deletion mapping using the deletions shown in Fig. 1. Phage prepared on the mutants were transduced into various histidine deletions and transductants were selected on minimal medium. A mutation to "smoothness" was considered to map within the region covered by a given deletion if (1) all transductants produced were smooth, a minimum of 1000 transductants being examined, and if (2) when transduced into the closest deletions on either side of the given deletion, some wrinkled as well as smooth transductants were produced.

The genetic analysis of the *C* gene mutants is presented in Table 3. *His01242 C2352* maps under *hisGDC63* and *hisDC129*, confining it to region I of the *C* gene and the last segment of the *D* gene. Since this mutant has somewhat reduced *D* gene activity,

as well as a marked reduction in *C* gene activity, the possibility exists that the site of mutation is at the end of the *D* gene. *His01242 C2354* maps in region IIb or region III of the *C* gene. The *C* gene codes for two polypeptide chains and the division point appears to be in region IIb (Martin *et al.*, 1966*a*). The results obtained with *his01242 C2353* are ambiguous. This mutant fails to give wrinkled recombinants with any of the deletions lacking operator-proximal segments of the *C* gene including *hisGDG63*. However, it also fails to give wrinkled recombinants with *hisBH22*, which deletes the gene immediately operator-distal to the *C* gene. It appears that the mutation in the *C* gene of *his01242 C2353* interferes with recombination in its vicinity.

(d) *Transfer of the* C2352 *mutation into a* hisO$^+$ *strain*

The *C2352* mutation was separated from the *his01242* operator locus by transducing *his01242 C2352* into the (*hisO*$^+$)*hisDC129* deletion and selecting prototrophic transductants on minimal medium. Most of the smooth transductants from such a cross receive both the *C2352* mutation and the operator region of the donor strain. Some, however, receive only the *C2352* mutant locus and are *hisO*$^+$ *C2352*. These clones, being defective in *C* gene activity, should grow poorly in the presence of triazolealanine, a compound which prevents de-repression of the histidine operon in non-constitutive strains (Levin & Hartman, 1963).

HisO$^+$ *C2352* was identified by its sensitivity to triazolealanine. This strain, when grown in the presence of histidine, has approximately the same level of *G* and *D* gene activity as LT-2, while the activity of genes distal to the *C* gene is about 20% of the activity found in LT-2 (Table 4). When grown in the absence of histidine, the levels of enzymes coded by the genes proximal and distal to *C* markedly increases, with polarity being maintained. Since *hisO*$^+$ *C2352* has less than normal *C* gene activity due to mutation and also less than normal amounts of activity of the genes distal to *C* due to polarity, it would be expected to de-repress, as observed, when grown in the absence of an external supply of histidine. Like *his01242 C2352*, *hisO*$^+$ *C2352* also has somewhat reduced levels of *D* gene activity.

HisO$^+$ *C2352* has a generation time of 140 minutes in minimal medium at 37°C. In minimal medium plus histidine, it grows at the same rate as wild-type.

(e) *Revertibility of the* C *polar mutants*

The non-constitutive *hisO*$^+$ *C2352* strain and the constitutive *his01242 C2354* strain grow considerably more slowly than wild-type in minimal medium. The occurrence of mutations to faster growth in the absence of histidine in these strains was tested in experiments similar to those described by Whitfield, Jr. *et al.* (1966).

About 2×10^6 bacteria were spread on the surface of minimal agar plates. In the case of *his01242 C2354*, crystals of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG), 2-aminopurine nitrate, and ICR-191, an acridine half-mustard which induces frameshifts (Ames & Whitfield, Jr., 1966; Martin, 1967), were placed in the center of the plates. After four days of incubation, a circle of fast growers surrounded the zone of inhibition produced by NG. The number of fast growers in the areas of aminopurine and ICR-191 was no greater than the number of spontaneous revertants occurring near the periphery of the plate.

It was only feasible to test *hisO*$^+$ *C2352* for reversion by NG. Whereas LT-2 produces good growth on minimal agar within one day of incubation, comparable growth with *hisO*$^+$ *C2352* requires two days of incubation. When *hisO*$^+$ *C2352* was plated with

TABLE 3

Deletion mapping of C *and* O *mutants*

Donor	Number wrinkled transductants over total number of transductants Recipients									
	203	646	63	*538*	*2236*	*129*	*22*	*134*	*135*	*152*
his01242 C2352	30/219	4/121	0/>2800	0/793		0/>1500	8/208			0/>1000
his01242 C2353	98/1051	8/98	0/>3500	0/>1486	0/2378	0/1384	0/>3000	11/919		0/1204
his01242 C2354			50/>400		2/>800	0/>1300	27/>1800			0/>1000
his01242 his2355	0/1991					7/649	3/588	6/641	7/619	

TABLE 4

Enzyme levels in hisO$^+$ C2352 *and* his01242 his2355

Strain	Media†	Relative specific activity						Inhibition‡ of *G* enzyme by histidine (%)
		G enzyme	*D* enzyme	*C* enzyme	*B* enzyme	*A* enzyme	*F* enzyme	
LT-2		1·0	1·0	1·0	1·0	1·0	1·0	48, 52
hisO$^+$ C2352	min	20·1	3·1	ca0·1	1·23	1·38		
	his	0·95	0·66	<0·1	0·17	0·26		
his01242 his2355								
Experiment 1	min	0·62			0·51		0·62	45
	his	0·51			0·44		0·73	35
Experiment 2	min	0·26			0·42		0·44	48
	his	0·30				0·54	0·42	55

Experiments were performed as described under Table 2. Specific activities are given relative to those found in LT-2, which in this Table are taken as 1·0.

† Min is minimal medium and his is minimal medium plus histidine.

‡ The percentage inhibition of *G* enzyme activity by 7×10^{-5} M-histidine is given.

crystals of NG, after two days of incubation numerous isolated colonies were present between the area of generalized growth and the zone of inhibition. Sixty-eight of the largest colonies were streaked on minimal agar. After 24 hours of incubation, the majority showed good growth. As a control, six single colonies were isolated from the edge of growth at the periphery of the plate. After 24 hours of incubation, the control streaks showed only slight growth.

(f) *A mutation in or near the operator region*

The mutation to smoothness (*his2355*) in one of the smooth mutants of *his01242* (isolated on 0·5% glucose) mapped under *hisG203*, indicating that it might be in the operator region or in the initial segment of the *G* gene. The possibility that the *his2355* mutation was a back mutation of the *his01242* locus was ruled out by the finding that the *his2355* and the *his01242* loci were separable by recombination. When *his01242 2355* was used as a donor to transduce to prototrophy the deletions *hisDC129*, *hisBH22*, *hisHA134* and *hisFI135*, most of the transductants were smooth but about 1% were wrinkled. The presence of some wrinkled transductants in the crosses indicates that the *his01242* locus can be recovered free from the *C2355* mutation; assays of phosphatase activity in the wrinkled transductants substantiate this conclusion. A wrinkled and a smooth transductant from each of three of the crosses was examined for phosphatase activity, using the toluenized cell procedure. The smooth transductants had repressed levels of phosphatase. The wrinkled transductants had de-repressed levels of phosphatase. To determine whether the wrinkled transductants were constitutively de-repressed, three wrinkled transductants from a *hisDC129* cross were isolated, purified and grown in minimal medium in the presence and absence of histidine, with *his01242* and LT-2 as controls. Assay of the toluenized cultures for phosphatase activity showed that the wrinkled transductants had the same level of phosphatase both in the presence and the absence of histidine as did *his01242*.

The growth rate of *his01242 his2355* was determined in minimal medium at 37°C with and without histidine. The generation time was 54 minutes in minimal medium and 48 minutes in histidine-supplemented minimal medium. The LT-2 control had a generation time of 49 minutes.

His01242 his2355 was examined for the level of histidine enzymes (Table 4). The activities of the enzymes in *his01242 his2355* were found to be approximately one-half the levels in LT-2. The presence or absence of histidine in the growth medium appeared to have little effect on enzyme levels.

The activity of the *G* enzyme is normally subject to feedback inhibition by histidine. *G* enzyme extracted from *his01242 his2355* showed the same sensitivity to histidine as did wild-type enzyme (Table 4).

His01242 his2355 can be distinguished from LT-2 not only in having lower enzyme levels but also in not being de-repressible. Wild-type organisms can be de-repressed by addition to the medium of thiazolealanine, a histidine analog which inhibits the *G* enzyme (Moyed & Freedman, 1959; Sheppard, 1964). *His01242 his2355* and wild-type LT-2 were streaked radially on a minimal agar plate and thiazolealanine crystals were placed in the center. After incubation at 37°C, the plates showed a zone of inhibition beyond which the LT-2 streak appeared wrinkled due to de-repression, whereas the *his01242 his2355* streak remained smooth. The inability of *his01242 his2355* to de-repress was further confirmed in the following experiment. Thiazolealanine

(0·2 mM) was added to minimal cultures of LT-2 and *his01242 his2355* after approximately one generation of growth in the absence of the inhibitor. Incubation of the cultures was continued for 220 minutes, during which time the cultures underwent less than a generation of additional growth. Immediately before adding thiazolealanine, and at the end of the incubation, toluenized samples of the cultures were assayed for phosphatase activity. Whereas incubation in the presence of thiazolealanine had increased the phosphatase level of LT-2 at least 3·5-fold, the level of phosphatase activity in *his01242 his2355* did not change significantly during incubation with the inhibitor. Failure of *his01242 his2355* to de-repress is presumably due to the presence of the *his01242* locus. *His01242* appears not to be de-repressible (T. Klopotowski, personal communication). Attempts to obtain *his2355* in a *hisO*$^+$ strain have been unsuccessful.

(g) H *gene mutants*

Of the mutations within the histidine region which cause smoothness and ability to grow at 43°C in *his01242*, the greatest number, 16, were in gene *H* (Fig. 1). The *H* gene is the fifth gene from the operator end and probably codes for an amidotransferase; all known *H* gene mutants are leaky, presumably because of a high intracellular concentration of ammonia which circumvents the genetic block (Smith & Ames, 1964). No intragenic complementation has been found in the *H* gene, indicating that it may be composed of a single cistron (Loper *et al.*, 1964).

As previously noted, auxotrophic nonsense mutants which are strongly polar result in a phenotypic reversion to smoothness and ability to grow at 43°C in constitutively de-repressed strains. Since the *H* gene mutants are leaky, a strongly polar nonsense mutant in the *H* gene, occurring in an operator-constitutive strain, would be expected to grow at least to a limited extent on minimal medium and be smooth. This explanation, however, is insufficient to explain the disproportionately large number of *H* gene mutants which was obtained in this investigation.

The distribution of the *his01242 H* gene mutants in the various selection experiments is given in Table 1. The mutants were classified as *H* gene mutants on the basis of genetic and enzymic analyses. The mutants were located by deletion mapping as described above for the *C* gene mutants. *His* deletions *GDCBH2253 BH22*, *HAFIE2327* and *HA134* define regions I, a and b; II, a and b, and III of the *H* gene (Fig. 1.) *H* gene mutants were crossed by transduction into these deletions to determine the region of the *H* gene in which they were located. The presumed map locations are shown in Table 5. *HisHB22* deletes region I of the *H* gene and all of the *B* gene which codes for a dehydratase–phosphatase. All mutants which mapped under *hisHA22* were assayed for phosphatase activity and had de-repressed levels of this enzyme. *HisHA134* deletes region III of the *H* gene and part of the *A* gene. Two of the mutants in this region were shown to be deficient in *H* gene activity (Table 6). The third mutant, *his01242 H2337*, was not examined for enzyme levels and therefore the possibility that it is an *A* mutant, though unlikely, cannot be excluded. Two mutants, *his01242 H2341* and *his01242 H2344*, failed to give any wrinkled recombinants when transduced into either *hisBH22* or *hisHAFIE2327*, although wrinkled recombinants were obtained with *hisGDCBH2253* and *hisHA134*, indicating that they are located within the middle portion of the gene. Mutant *his01242 H2336*, (designated s-10, Martin *et al.*, 1966*b*), which has been shown enzymically to be an *H* mutant, has proved refractory to mapping because of its high reversion rate.

TABLE 5

Some properties of the H *mutants*

Mutant	Region†	Polarity‡	Growth rate§ (LT-2=1)
his01242 H2348	Ia	—	3·2
his01242 H2340	Ib	—	1·1
his01242 H2342	Ib	—	1·1
his01242 H2343	Ib	1·0	1·3
his01242 H2347	Ib	0·6	3·1
his01242 H2241	Ib, II	≃1·0	1·4
his01242 H2344	Ib, II	≃1·0	2·0
his01242 H2350	IIa	—	1·4
his01242 H2338	IIb	—	1·0
his01242 H2345	IIb	—	2·1
his01242 H2346	IIb	≃1·0	1·4
his01242 H2349	IIb	0·4	3·3
his01242 H2335	III	0·6	1·0
his01242 H2337	III	—	1·2
his01242 H2339	III	≃1·0	1·9
his01242 H2336	—	0·1	1·1

† The region of the *H* gene in which the *H* mutations mapped is given.

‡ Specific activity of the *F* and/or *A* enzymes relative to the corresponding specific activities in *his01242*. Data taken from Table 6.

§ Generation time of mutant in minimal medium/generation time of LT-2.

A number of the mutants were assayed for several of the histidine enzymes (Table 6). The data indicate that mutants *his01242 H2335*, *his01242 H2336*, *his01242 H2349* and probably *his01242 H2347* are polar. Mutants *his01242 H2339*, *his01242 H2341*, *his01242 H2344* and *his01242 H2346* are either not polar or weakly polar. Mutant *his01242 H2343*, the polarity of which was more rigorously tested by assaying *A* gene activity as well as *F* gene activity, is not polar.

The generation times of the *01242 H* mutants in minimal medium are given in Table 5 and vary from that of wild-type to more than three times that of wild-type.

4. Discussion

Mutations in structural genes which have polarity effects have been described in a number of systems (see review by Ames & Martin, 1964). Polar mutations have been found to be either nonsense mutations (UAA or UAG), which cause premature termination of polypeptide chain synthesis, or to be frame-shift mutations (Newton, Beckwith, Zipser & Brenner, 1965; Yanofsky & Ito, 1966; Whitfield, Jr. *et al.*, 1966). Both types of mutations generally result in complete loss of enzyme activity. However, if a nonsense mutation occurred near the end of a cistron, in some cases enough of the polypeptide chain might by synthesized to have some activity. Similarly, if a nonsense mutation occurred at the beginning of a cistron and was followed within a short distance by an in-phase codon (e.g. AUG) where translation could be re-initiated, a product with some activity might be synthesized. Another type of prototrophic polar mutation might arise from a mutation in a chain initiator. Evidence has been presented suggesting the existence of chain-initiating regions at the beginning of each cistron in the histidine polycistronic message of *S. typhimurium* (Martin *et al.*,

TABLE 6

Enzyme levels in H *mutants*

Strain	Media	Relative specific activity						
		G enzyme	*D* enzyme	*C* enzyme	*B* enzyme	*H* enzyme	*A* enzyme	*F* enzyme
his01242		1·0	1·0	1·0	1·0	1·0	1·0	1·0
LT-2		0·1	0·1	0·1	0·1	0·1	0·1	0·1
his01242 H2335	min	1·33 (1·32)		1·10 (1·36)	0·99 (1·01)	<0·15	0·61 (0·69)	0·58 (0·80)
his01242 H2336	min	1·24 (1·23)		0·94 (1·16)	0·76 (0 78)	<0·15	0·13 (0·15)	0·15 (0·21)
his01242 H2339	his	—	0·83			<0·10		0·76
his01242 H2341	his	—	0·76			<0·10		0·82
his01242 H2344	his	0·85	0·83			<0·10		0·66
his01242 H2346	his	0·76	0·80			<0·10		0·68
his01242 H2343	min	1·26	1·14			0·18	0·97	0·83
his01242 H2343	his	1·02	—			0·10	1·10	0·83
his01242 H2347	his	1·60	1·03	0·93		<0·10		0·61
his01242 H2349	his	0·87		1·12	0 92	†		0·37

For details see legend under Table 2.

† *H* activity in this mutant was found to be low in a qualitative preliminary assay and was not measured in this experiment.

1966*a*). A mutation in a chain-initiating region might affect subsequent translation of the polycistronic message; and, if the chain-initiating region codes for protein, an altered enzyme might be produced.

Polar mutations of the type postulated above would occur at the extremities of a cistron. Two of the prototrophic *C* gene polar mutations described in this communication have been located within defined regions of the histidine operon by deletion mapping. One (*C2352*) is located in the initial region of the *C* gene (or at the end of the *D* gene) and the other (*C2354*) is located in the middle of the *C* gene. The *C* gene appears to be composed of two cistrons. Studies of the *C* enzyme indicate that it is composed of two polypeptide chains (Martin, Voll & Appella, 1967), and the *C* gene can be divided into two parts on the basis of the level of polarity found in *C* nonsense mutants (Martin *et al.*, 1966*a*). Nonsense mutants in the first half of the *C* gene are more strongly polar than nonsense mutants in the second half of the *C* gene. (The prototrophic *C* polar mutants all have polarities similar to those of nonsense mutants in the first half of the *C* gene). The break in polarity occurs in region IIb. One of the *C* prototrophic mutations (*C2354*) is located in region IIb or III. This mutant therefore is near the end of the first cistron or in the first part of the second cistron. The prototrophic *C* polar mutants might therefore be nonsense mutants or chain-initiator mutants of the type postulated above.

A nonsense mutation in a strain containing a suppressor for it would cause a polarity effect without complete loss of enzyme activity. That the *C* mutants described in this report might be the result of such a combination seems unlikely. The hypothetical suppressor must be indigenous to LT-2 and its derivatives since the mutations, when transduced into histidine deletions, are still prototophic. Since a large number of nonsense (amber and ochre) mutants have been found throughout the histidine operon (Whitfield, Jr. *et al.*, 1966; Margolies & Goldberger, manuscript in preparation; Fink *et al.*, 1967; Fink & Martin, 1967; Z. Hartman, personal communication) which are completely histidine dependent, the hypothetical suppressor in LT-2 could be either (a) one that reads a nonsense codon which is neither an amber nor an ochre codon (e.g. UGA)†, or (b) a suppressor which reads an amber or ochre codon but inserts an amino acid which, except in a very few cases, completely inactivates the gene product.

Ames & Hartman (1963) have postulated the existence of modulator codons which serve the function of allowing different numbers of molecules of enzymes to be translated from a polycistronic message by causing reduced translation beyond the point of their occurrence (see Introduction). The *C* gene mutations described in this report cause such a modulatory effect without completely inactivating the gene product, and thus it is clear that modulation can exist although the mechanism is not clear. In the normal histidine operon, the ratio of the number of molecules of enzymes produced by individual genes is still not known with certainty, although the best estimate (Margolies, Voll, Loper & Martin, manuscript in preparation) indicates equal numbers of molecules of the *C* and *A* enzymes and perhaps greater numbers of molecules of the *G* and *D* enzymes than of *C* and *A* enzymes.

One of the smooth polar mutants isolated in this study, *his01242 his2355*, appears to contain a mutation in the operator region (or the adjacent promoter region pro-

† Some evidence that the wild-type does not contain a UGA suppressor is provided by experiments mixing it with a F′ lac containing a UGA mutation (obtained from D. Zipser) on a lactose plate (B. Ames, personal communication).

posed by Jacob & Monod (1965)) or at the beginning of the *G* gene. It is clear that this strain is a double mutant, since the *his01242* locus can be separated from the *his2355* locus by recombination. Unfortunately, whereas the *his01242* locus can be recognized by its wrinkled phenotype, the phenotype of a strain carrying *his2355* alone is unknown and we have not yet been able to isolate it. *His01242 his2355* has about one-twenty-fourth the level of the histidine enzymes of the *his01242* strain and about one-half the wild-type level; it does not de-repress under conditions which induce an internal shortage of histidine. The enzyme coded for by the first gene in the operon (*G* gene) is present in amounts comparable to enzymes produced by more distal genes and exhibits normal histidine sensitivity. *His01242 his2355* thus appears to be a constitutively repressed strain. It cannot be said at present whether the *his2355* mutation is actually in the operator locus. It is conceivable that *his01242 his2355* is similar to the *C* prototrophic polar mutants and carries a mutation at the beginning of the *G* gene which does not alter to any extent the catalytic function of the *G* enzyme.

The *01242 H* gene mutants obtained in this investigation represent a heterogeneous class. They map throughout the *H* gene, are both polar and non-polar, and they exhibit a wide range in generation time in minimal medium. It appears that a number of seemingly unrelated mutations in the *H* gene cause phenotypic reversion to a smooth-colony morphology and ability to grow at 43°C in an operator-constitutive strain. A simple explanation would be that an overproduction of the product of the *H* enzyme is necessary to produce wrinkledness. However, non-polar, non-leaky mutants in the *G* gene and in the *A* gene are wrinkled when de-repressed. These mutants cannot synthesize the product of the *H* enzyme because they are blocked in prior enzymic steps. At present no satisfactory explanation for the *H* mutants is available.

No true revertants of the *his01242* locus were obtained in this investigation. A revertant to the wild-type operator locus would presumably fall among the class of smooth mutants which were examined. The number of smooth mutants obtained when approximately 10^8 cells were plated was generally less than the number (53) of smooth mutants which were isolated and studied in this investigation. It is concluded that true reversions of the operator locus, *his01242*, occur at a spontaneous frequency of less than one in 10^8 cells. Since the *his01242* cells probably go through several divisions of growth at 43°C during the selection period, the reversion rate of the operator locus is most likely well under one per 10^9 cells per generation. In another study (Fink *et al.*, 1967) in which smooth mutants of *his01242* were selected at 43°C on minimal medium plus histidine, 21 of the mutants isolated grew normally in the absence of histidine. None of these mutants represented reverse mutations of the *his01242* locus, since in each case the mutation to smoothness was outside the histidine operon. When these mutants were tested for the presence of amber and ochre suppressors, none was found, indicating that the *his01242* mutation is not suppressible.

In the lactose operon of *Escherichia coli*, no true revertants of O^c operator constitutive mutants have been found. Moreover, since *lac*O^c mutations are not inducible by base analogues and have never been found to be suppressible, it appears that operator-constitutive mutations in the lactose operon are deletion-type mutations (Jacob & Monod, 1965). The findings on *his01242* presented here, and those of Fink *et al.* (1967), are consistent with the notion that the *his01242* mutation is the result of a deletion.

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