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Histidinol Dehydrogenase (*hisD*) Mutants of *Salmonella typhimurium*[1]

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A multidisciplinary analysis has been applied to over 150 *hisD* mutants of *Salmonella typhimurium* in a study of gene-enzyme relationship. The mutants were examined for production of immunologically cross-reacting material by using antibody to purified histidinol dehydrogenase, and for genetic complementation by using a set of F′ factors bearing *Escherichia coli hisD* complementing mutants. Classifications as to missense, nonsense, frameshift, or deletion mutant are proposed on the basis of mutagenesis and suppression tests. For the suppression tests the mutants were examined both by a simultaneous suppression technique and by testing for response to *E. coli* F′ factors bearing a recessive lethal amber and a recessive lethal ochre suppressor. The data are interpreted in relation to the position of the mutations in the recombination and complementation maps and in relation to the known composition of histidinol dehydrogenase. The gene *hisD* appears to be single cistron for the production of a single biosynthetic polypeptide.

Histidinol dehydrogenase [L-histidinol-nicotinamide adenine dinucleotide (NAD) oxidoreductase, EC 1.1.1.23], which catalyzes the last step in histidine biosynthesis, is coded in *Salmonella typhimurium* by gene *hisD*. This gene displays a complex complementation pattern as determined by abortive transduction analysis (14). In relation to the known orders of transcription and translation in the histidine operon (2), the distal region of *hisD* comprises a unique complementation group. All mutations mapped in this region by recombination tests are of the type termed *Da*. The more proximal region of *hisD* contains both noncomplementing mutations, *Dab*, and many *Db* mutations which form the set complementary to *Da*. Most of the complementation data are thus consistent with a two cistron-one enzyme model. There are additional sets of complementing mutations, however, which are not compatible with the designation of two discrete cistrons (14).

We have further defined the nature of the *hisD* gene by enzyme and genetic studies. Compositional studies of purified histidinol dehydrogenase reported by this laboratory indicate the enzyme exists as a dimer of identical subunits (13). This conclusion is supported by independent analyses from another laboratory (23, 27). In the studies presented here, we examined the protein-forming properties of a variety of *hisD* mutants. Only mutants isolated upon the basis of their auxotrophy for histidine were used, as opposed to mutants directly selected due to their polarity [methods of Fink et al. (8)].

One-hundred-and-fifty-two mutants were examined for the production of immunological cross-reacting material (CRM) as detected by agar-gel immunodiffusion tests of extracts of cultures derepressed for histidine biosynthesis. These same mutants have been tentatively identified as to the nature of their mutation—reversion stable, missense, frameshift, or nonsense. The above properties have been compared, relative to the previously established recombination mapping. Complementation mapping available from previous tests of abortive transduction has been reexamined and extended in partial diploids by using *Escherichia coli*-derived F′ factors. Our data support a single cistron model for the histidinol dehydrogenase *hisD* gene and permit a number of conclusions about this gene-enzyme relationship.

MATERIALS AND METHODS

Bacterial strains and growth media. The *hisD* mutants of *S. typhimurium* were obtained from P. E. Hartman. Their parent strains and mode of induction are indicated in Table 1. In addition, a number of

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TABLE 1. *Characterization of hisD mutants*

Map region[a]	Mutant number[b]	Parent strain, LT^{-}[c]	Source of mutation[d]	Complementation pattern[e]	CRM[h]	Mutation to prototrophy		Mutant characterization[k]
						Spontaneous[i]	Induced[j]	
IA	[396	2	x	ab	–	<10	+	a
	[536	2	x	ab	–	0	+	a
	497	2	x	ab[e, f]	–	<10	(D)	f
	946	2	2-AP	ab[e, f]	–	<10	+	a
	952	2	2-AP	ab[e, f]	–	<10	+	a
IB	984	2	DES	ab[e, f]	–	0	–	s, tp
IIB	133	2	sp	ab	–	<10	–, C	f
	230	7	sp	ab	–	40	–, C	f
IIC	245	7	sp	b	+	>200	N	m
	802	2	x	b	+	0	–	s
	27	2	sp	ab	–	<10	–	f
	[54	2	sp	ab	–	<10	–	f
	[78	7	sp	ab	–	100	+	m
	64	7	sp	ab	–	<10	N	m
	139	2	sp	ab	–	<10	(+)	f
	191	7	sp	ab	–	<10	–	f
	273	7	sp	ab	–	30	(D)	f
	607	7	UV	ab	–	<10	–	f
	623	2	FN	ab	ND	20	+	a
	634	2	FN	ab	–	15	+	m
	960	2	2-AP	ab[e, f]	–	<10	+	a
IIIA	137	2	sp	ab	–	0	–	s
	400	7	sp	ab	–	0	–	s
	563	2	x	ab	–	100	+	a
IIIB	141	2	sp	b	–	10	+	m, ts
	150	2	sp	ab	–	0	(N)	m
	154	7	sp	ab	ND	<10	–	f
	411	2	2-AP	ab	–	50	+	a
	471	2	2-AP	ab	–	10	+	o
	490	2	x	ab	–	10	+	m
	603	7	UV	ab	–	100	+	a
	639	2	FN	ab	–	<10	+	o
	873	2	2-AP	ab	–	<10	+	a
	893	2	2-AP	ab	–	10	+	a
	895	2	2-AP	ab	–	<10	N	m
	896	2	2-AP	ab	–	<10	+	a
	956	2	2-AP	ab[e, f]	–	<10	+	o

[a] Map regions are those depicted in Fig. 2.

[b] Bracketed numbers indicate mutants not separated in recombination analysis.

[c] Mutant *510* is *S. typhimurium* 533 of H. Plough.

[d] Abbreviations: x, X-rays; 2-AP,2-aminopurine; sp., spontaneous; UV, ultraviolet light; FN, fast neutrons; EMS, ethylmethyl sulfate; 5-BU and 5-BD, 5-bromodeoxyuracil and 5-bromodeoxyuridine, respectively.

[e] Results presented are for both the episome partial diploid tests and the previously reported abortive transduction tests.

[f] Data from F′ factor studies only.

[g] Data obtained by abortive transduction only.

[h] Positive for CRM is designated by +; ND, not done; all others were CRM^{-}.

[i] Values are numbers of colonies arising on whole minimal plates inoculated as described in text; B, bradytrophic.

[j] All mutants were tested for reversion by NG, DES, and ICR191. +, Positive with both NG and DES; N, positive with NG, negative with DES; D, negative with NG, positive with DES; –, negative with both NG and DES; C, ICR191 positive, all other mutants did not revert with this compound. The use of parentheses indicates a weak response, i.e., fewer than 50 colonies induced per plate in contrast with well in excess of 100 colonies induced per plate.

[k] Entries are composite interpretations from data in Table 1 and Table 3 and other data quoted in the text. a, Amber; o, ochre; n, amber or ochre; m, missense or undetected nonsense; f, frameshift; s, stable to reversion; tp, total polar; ts, temperature sensitive, functional at room temperature but not at 37 C.

TABLE 1—*Continued*

Map region[a]	Mutant number[b]	Parent strain, LT⁻[c]	Source of mutation[d]	Complementation pattern[e]	CRM[h]	Mutation to prototrophy		Mutant characterization[k]
						Spontaneous[i]	Induced[j]	
IV	37	2	UV	b	+	0	N	m
	39	2	sp	b	–	0	+	m
	68	7	sp	b	–	10	+	m
	77	7	sp	b	+	0	+	m
	82	7	sp	b	–	>200	N	m
	84	7	sp	b	+	100	+	m
	111	7	sp	b[e, g]	+	B	N	m
	130	2	sp	b	–	<10	+	m
	170	7	sp	b	–	100	+	m
	171	7	sp	b	–	>200	+	m
	216	7	sp	b	–	>200	+	m
	226	7	sp	b	–	30	+	m
	233	7	sp	b	+	60	+	m
	239	7	sp	b	–	200	+	m
	248	7	sp	b	–	200	+	m
	244	7	sp	b	–	200	+	m
	254	7	sp	b	+	>200	+	m
	271	7	sp	b	–	10	(+)	m
	295	7	sp	b	ND	200	+	m
	302	7	sp	b	+	50	+	m
	381	2	x	b	+	0	N	m
	392	2	x	b	+	0	+	m
	465	2	UV	b	+	<10	+	m
	474	2	sp	b	+	0	+	m
	477	2	sp	b	–	0	–	s
	558	2	sp	b	–	20	+	m
	602	7	UV	b	+	0	–	s
	704	2	EMS	b	–	0	–	s
	815	2	x	b	+	0	–	s
	9	2	UV	ab	–	0	–	s
	18	2	UV	ab	–	10	(+)	f
	60	2	sp	ab	–	0	–	s
	123	7	sp	ab	–	10	+	a
	124	7	sp	ab	–	<10	(N)	m
	142	2	sp	ab	–	<10	–	f
	274	7	sp	ab	–	>200	+	m
	410	2	2-AP	ab	–	<10	+	a
	436	2	2-AP	ab	–	<10	+	a
	443	2	2-AP	ab	–	10	+	a
	511	2	32p	ab	–	0	+	m
	529	2	sp	ab	–	10	+	a
	889	2	2-AP	ab	–	10	+	n
VA	108	7	sp	b	–	0	–	s
	236	7	sp	b	+	>200	N	m
	698	2	5-BD	b	+	0	+	m
	49	2	sp	b	–	0	+	m
	83	7	sp	b	–	100	+	m
	88	7	sp	b[e, g]	–	>200	+	m
	223	7	sp	b	–	10	+	m
	237	7	sp	b	–	50	+	m
	420	2	2-AP	b	–	<10	N	m
	451	2	2-AP	b	+	0	+	m
	833	2	x	b	–	0	–	s
	74	7	sp	ab	–	<10	N	m
	85	7	sp	ab	–	40	(D), C	f
	149	2	sp	ab	–	10	–, C	f
	176	7	sp	ab	–	10	–	f
	494	2	x	ab	–	0	–	s
	518	2	x	ab	–	0	(D)	f
	626	2	FN	ab	–	<10	–	f
	891	2	2-AP	ab	–	<10	+	a
	892	2	2-AP	ab	–	<10	+	a

TABLE 1—*Continued*

Map region[a]	Mutant number[b]	Parent strain, LT$^-$ [c]	Source of mutation[d]	Complementation pattern[e]	CRM[h]	Mutation to prototrophy		Mutant characterization[k]
						Spontaneous[i]	Induced[j]	
VB	214	7	sp	b	+	>200	+	m
VI	697	2	5-BD	b	–	<10	+	m
	466	2	UV	ab	–	<10	–	f
	113	7	sp	b	+	10	+	m
	182	7	sp	b	+	100	+	m
	472	2	2-AP	b	+	10	(+)	m
	492	2	x	b	+	<10, B	+	m
	604	7	UV	b	+	<10	+	m
	26	2	UV	ab	ND	10	+	m
	127	7	sp	ab	–	0	–	s
	348	2	5-BD	ab	–	<10	+	m
	351	2	5-BD	ab	ND	<10	+	m
	397	2	x	ab	–	0	–, C	f
VII	1	2	UV	b	–	<10	(+)	m
	36	2	UV	b	–	0	(+)	m
	66	7	sp	b	–	<10	(+)	m
	90	7	sp	b	–	<10	(+)	m
	506	2	32p	b	–	<10	(+)	m
	126	7	sp	a	–	0	+	m
VIII	10	7	UV	a	–	10	N	m
	23	2		a	–	<10	+	o
	112	7	sp	a	–	50	–	f
	166	7	sp	a	–	B	+	m
	208	2	x	a	–	0	–	s
	220	7	sp	a	+	>200	+	m
	450	2	2-AP	a	–	B	+	m
	476	2	sp	a	–	<10	(D)	f
	545	2	x	a	–	0	–	s
	600	7	UV	a	–	40	+	a
	601	7	UV	a	+	20	N	m
	619	2	FN	a	+	<10	+	m
	986	2	2-AP	a	–	0	+	m
	989	2	2-AP	a	+	0	–	s
Partially mapped	92	7	sp	b	+	10	(+)	m
IV-VA	215	7	sp	b	+	>200	+	m
	291	7	sp	b[e, g]	–	150	+	m
	363	2	2-AP	b	–	30, B	+	m
	412	2	2-AP	b	–	<10	+	m
	635	2	FN	b	+	<10	(+)	m
	710	2	EMS	b	–	<10	(N)	m
	924	2	2-AP	b	–	<10	+	m
	941	2	2-AP	b	–	B	N	m
	362	2	2-AP	ab	–	200	+	m
	422	2	2-AP	ab	–	20	+	a
	442	2	2-AP	ab	–	<10	+	a
	461	2	UV	ab	–	<10	–	o
	510	Strain 533		ab	–	<10	+	f
	880	2	2-AP	ab	–	<10	+	a
	883	2	2-AP	ab	–	20	+	a
	894	2	2-AP	ab	–	<10	+	a
	962	2	2-AP	ab	–	<10	+	o
	992	2	2-AP	ab	–	<10	+	a
	996	2	2-AP	ab	–	<10	+	a

strains donating *E. coli* F' factors were employed. Their genotype and immediate source are listed in Table 2.

Nutrient broth (Difco) was used for complex medium; for minimal culture, the E medium of Vogel and Bonner (24) was routinely supplemented with glucose (0.5%) for use. For lactose plates, E medium was modified to eliminate citrate as described by Berkowitz et al. (6) and was supplemented with lactose (1%). Working stocks of *his* mutants were held on slants of Dorset egg medium (Baltimore Biological Laboratories) at 4 C. Donor strains TR869 and LS323 were maintained as suspensions frozen in appropriately supplemented E medium.

Preparation of extracts. *HisD* mutants were grown under conditions known to produce derepression of the histidine operon (1) using *N*-α-formyl-L-histidine (Cyclo Chemical Corp.) as described earlier (12). Volumes of up to 2 liters in appropriately sized Erlenmeyer flasks were shaken at 37 C on a rotary shaker for 18 hr. Grown cultures were sampled to determine per cent of prototrophs by dilution plating, and the cells were collected by centrifugation. Pellets were suspended in saline and repelleted in tared tubes to permit determination of the wet weight of cells. Final pellets were frozen in 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (*p*H 7.5). Thawed cells were suspended in the same buffer at 0.25 or 0.33 g (wet weight) per 1 ml of total volume. Extracts of the more dilute suspensions were prepared by 10 min of exposure to full power of a Raytheon 10-kc sonic oscillator. Other suspensions were exposed intermittently for a total of 60 sec to the full-power oscillations of a microtip S-75 Bronson Sonifier. Extracts were centrifuged 10 min at 27,000 × *g*. The supernatant solutions were tested for CRM before storage at −20 C. Any further manipulations made use of a standard buffer consisting of 50 mM Tris-hydrochloride (*p*H 6.5) and 0.1 mM $MnCl_2$. Protein concentrations in samples filtered through G-25 Sephadex (Pharmacia) were determined by the method of Lowry et al. (15) using amorphous insulin (Lilly) as standard.

Preparation of antibody. Recrystallized histidinol dehydrogenase purified from *S. typhimurium* LT7 *hisG72* cells (13) was used as antigen for the production of immune sera in rabbits. Immunization over a 6-week period was conducted by injection into the toe pads by the procedure of Leskowitz and Waksman (11). Immune sera from one rabbit were pooled, and the immunoglobulin was precipitated in 0.33 saturated ammonium sulfate. The precipitate was concentrated twofold by resuspension and was dialyzed against standard buffer. The resultant antibody was then absorbed against an extract of multisite mutant *hisOGD63* by reaction of 1.7 mg of mutant extract per ml of antibody for 10 min at 45 C followed by 72 hr at 4 C. Three repetitions of this procedure yielded monospecific antibody as judged by Ouchterlony (19) tests with crude and pure enzyme protein (Fig. 1).

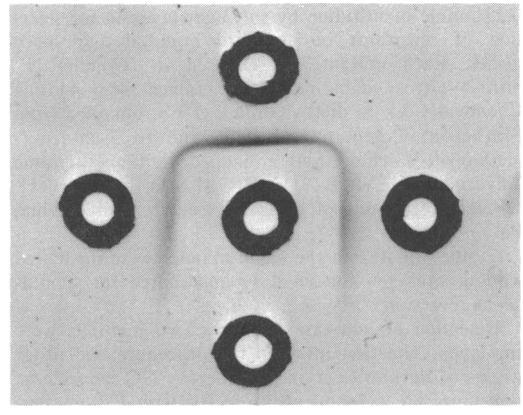

FIG. 1. *Reaction of identity between extracts of CRM⁺ and hisD⁺ strains as seen by agar gel immunodiffusion. The center well was filled with adsorbed antibody to purified histidinol dehydrogenase. Surrounding wells were charged with: left, extract of hisD⁺ cells; top, extract of hisDb113 (CRM⁺); right, purified histidinol dehydrogenase; bottom, extract of hisDab558 (CRM⁻).*

TABLE 2. *Genotype and source of donor strains*

Strain no.	Genotype	Source
Salmonella typhimurium		
SB391	*hisGDCBHAF644*/F' *lac X-82*	D. Berkowitz
SB392	*hisGDCBHAF644*/F' *lac U-281*	D. Berkowitz
SC23	*hisGDCBHAF644*/F' *lac*⁺	Constructed
TR47	*trpA8 purE801 hisBHAFIE612*/F' *hisD2377*	J. Roth
TR48	*trpA8 purE801 hisBHAFIE612*/F' *hisD2378*	J. Roth
TR49	*trpA8 purE801 hisBHAFIE612*/F' *hisD2379*	J. Roth
TR50	*trpA8 purE801 hisBHAFIE612*/F' *hisD2380*	J. Roth
TR51	*trpA8 purE801 hisBHAFIE612*/F' *hisD2381*	J. Roth
TR52	*trpA8 purE801 hisBHAFIE612*/F' *hisD2382*	J. Roth
TR35	*ser-821 arg-501 hisDCBHAFIE712*/F' *his*⁺	J. Roth
SC54	*trpABE 130 hisGDCBHAF644*/F' *hisD3686*	Constructed
SC25	*trpABE 130 hisGDCBHAF644*/F' *his*⁺	Constructed
TR869	*hisC434 metE338 ilvC401 pur-847 arg-544 ilv-545*/F'14 *sup* amber	J. Roth
Escherichia coli W3110		
LS323	*trpR*⁻ *trpA9605 his-29 ilv-1 pro-2*/F'14 *su8*⁺	L. Soll

Detection of CRM. The CRM character for each mutant was determined in Ouchterlony tests on microscope slides. Gels were prepared with 2.7 ml of a solution of 2% Oxoid Ionagar no. 2 (Consolidated Laboratories) in buffered saline (5 mM Tris-hydrochloride, *p*H 7.5) containing 0.2% sodium azide as the preservative. Wells were cut at distances of 3 mm with a tube fashioned from a 14-gauge needle. Each extract was placed adjacent to an extract of *hisD*$^+$ cells for examination of total or partial identity. Precipitin bands were usually visible within 3 hr. Reactions were scored after 6 hr; no new indications of reaction were observed after additional overnight development. Assays were in duplicate and were calibrated to be sensitive for the detection of CRM present at 7% of the derepressed level.

Mutagenesis. Induced reversion studies were conducted as described by Whitfield et al. (25). By that procedure approximately 2×10^8 cells were plated with 0.25 μmole of histidine by soft agar layering for selection of revertants on standard minimal agar petri dishes. Each mutant was exposed to *N*-methyl-N'-nitro-*N*-nitrosoguanidine (NG, obtained from Aldrich Chemical Co.); to diethyl sulfate (DES, obtained from Matheson, Coleman and Bell) and to 3-chloro-7-methoxy-9-[(3-chlorethyl) aminopropylamino] acridine dihydrochloride (ICR191). ICR191 was the gift of H. J. Creech, Institute of Cancer Research, Philadelphia, Pa.

Cultures plated in the same manner were incubated without mutagen for six days and scored for spontaneous reversion.

Detection of nonsense mutants. Two methods were employed. The first involved the procedure and tester strains of Berkowitz et al. (6), whereby NG-induced *his* revertants are subsequently infected with F' *lac*$^-$ episomes. Growth of these recipient cells occurs on lactose minimal plates when the revertants contain suppressors capable of suppressing both the *his*$^-$ and the *lac*$^-$ mutations. Known F' *lac*$^-$ amber mutations *X-82* and *U-281* (see reference 6) were used in the donor strains. These were spread to form bacterial lawns on lactose minimal plates, and mutation plates of the *hisD* strains showing NG-induced revertants were printed onto the lawns by replica plating. Strains with known amber and ochre mutations, *hisC527* and *hisC117*, respectively, were used as positive controls in suppression. Strain SC23, isolated as a spontaneous *lac*$^+$ revertant in SB391, was used to demonstrate the capacity of the recipients to accept an F' *lac* episome.

All NG revertible *hisD* mutants were also checked for amber suppression by mating with TR869. Strain TR869 carries a strong amber *sup* on the F'14 episome. This *sup* is believed to be the same as *su7*, the recessive lethal shown by Soll and Berg to insert glutamine (L. Soll, *personal communication*). [The strain TR869 requires arginine, since the F'14 has shortened up and lost its *arg* region (J. Roth, *personal communication*).] In our tests using TR869, *hisD18* and *hisD85* were used as negative controls. SC25 was used to demonstrate the capacity of *his*$^-$ strains to serve as recipients of F' factors. Strain SC25 was constructed as *trpABE130 hisGDCBHAF644* containing the F' *his*$^+$ from TR35.

Recognition of ochre mutants was also attempted in studies similar to those involving TR869. These studies employed strain LS323 which carries the recessive lethal ochre suppressor *su8* on F'14 (22).

RESULTS

Composites of our results and interpretations are presented in Table 1 and Fig. 2. From previous studies of Hartman et al. (Advan. Genet., *in press*) the mutations studied are shown positioned relative to 13 regions of the *hisD* gene. The number of each mutant strain is listed according to the mapping region of the corresponding mutation, and mutations in common regions are grouped according to complementation pattern. In Fig. 2, this is achieved by entering complementing ones above the line and noncomplementing ones below it.

Complementation. Six *E. coli* F' *hisD* mutants, which were obtained from an F' *his*$^+$ strain after DES treatment, were acquired from J. Roth. They were identified as *hisD* by interspecies tests with appropriate *his*$^-$ recipients. These were tested for intragenic complementation by streaking over lawns of *Da*, *Db*, and *Dab* strains recently spread onto minimal plates.

In positive tests, growth appeared in regions of coplating after 24 hr of incubation at 37 C. The six strains were characterized by this procedure as three *Dab* (*his-2377*, *2381*, *2382*), two *Da* (*his-2378*, *2379*), and one *Db* (*his-2380*). All 152 *S. typhimurium hisD* mutants were then checked for complementation by using an F' *Db* mutant (strain TR50) and an F' *Da* mutant (strain TR49). Subsequently, an additional *Db* mutant was isolated after DES treatment (strain SC54). A number of the mutants were also tested by using the F' *Db* of strain SC54 and the F' *Dab* of strain TR47.

As is shown in column 5 of Table 1, the resulting patterns agree with the complementation patterns seen by abortive transduction tests. This is particularly notable since all of these episomal *hisD* mutants are of *E. coli* origin. The distribution of complementing mutations within the recombination map as seen earlier is thus corroborated and extended. All *Da* mutations mapped lie in the distal regions VII and VIII. The great majority of *Db* mutations mapped to date lie distal to region III, but their distribution does not overlap with that of the *Da* mutations. Data from abortive transduction tests have shown minor subsets of complementation which complicate the simple *Da-Db-Dab* pattern (14). No such subpatterns were seen with the few F' *hisD* mutants tested here.

Approximately 50% of the strains tested complement: 15 *Da* and 64 *Db*, as opposed to 73 *Dab*.

CRM. All CRM$^+$ extracts gave reactions of

identity with native histidinol dehydrogenase (Fig. 1). As presented in Table 1, 27 of the 131 revertible mutants tested were CRM$^+$. More significantly, none of the noncomplementing (*Dab*) mutants were CRM$^+$; the capacity of complementation appears to be a prerequisite for CRM. This potential is not sufficient, however, since only 27 of the 63 *Db* mutants tested gave the reaction, and only 4 *Da* mutants were CRM$^+$ among the 15 tested. Four stable mutants (*see below*) were CRM$^+$ but again these occurred as a subclass among complementing strains. None of the strains with extended deletions involving *hisD* were CRM$^+$, including *hisDC129*, the mutation of which describes region *D* VIII and which nevertheless shows *Da* complementation.

Apparent dimeric state of CRM$^+$. Since native histidinol dehydrogenase is a relatively heat-stable dimer (13), it is possible that our antibody preparation is directed against determinant groups presented only by the dimer molecule. If this is so, the CRM$^+$ property might appear only among those mutants which produce a dimerized *hisD* protein. The results of the following experiment are consistent with this possibility. Native histidinol dehydrogenase has a molecular weight of 80,000 daltons and gives a K_D = 0.3 on Sephadex G-100 (13). A column (42 cm by 1 cm) of G-100 was prepared in standard buffer at 4 C. In a series of experiments, 1-ml volumes of extracts of five representative CRM$^+$ mutants were filtered through the column at a constant flow rate by using a nonpulsing pump. One-milliliter fractions were collected, and each fraction was then tested for CRM by gel immunodiffusion. The column was calibrated at the time of each experimental run by applying 1 ml of crude histidinol dehydrogenase, and testing each 1-ml fraction by gel immunodiffusion. Antibody precipitating activity was routinely recovered in a three-tube peak. In each case, the mutant CRM eluted in a volume equal, within one tube, to that of the native enzyme. The mutants tested and their map positions were as follows: *245*, IIC; *302*, IV; *214* VB; *182*, VI; and *472*, VI. From these determinations, it appears likely that strains mutant in any map position are observed as CRM$^+$ only if they make dimers of essentially complete *hisD* polypeptide products.

Mutagenesis. The *hisD* mutants were further characterized on the basis of their susceptibility to spontaneous reversion and reversion in the presence of NG, DES, and ICR191. Note was taken of those strains in which NG or DES gave relatively weak reversion (fewer than 50 colonies per plate as contrasted to responses of well in excess of 100 colonies per plate). Results appear in columns 7 and 8 of Table 1.

Nonsense mutants. All mutants revertible by NG were examined by using the F′ *lac*$^-$ and the

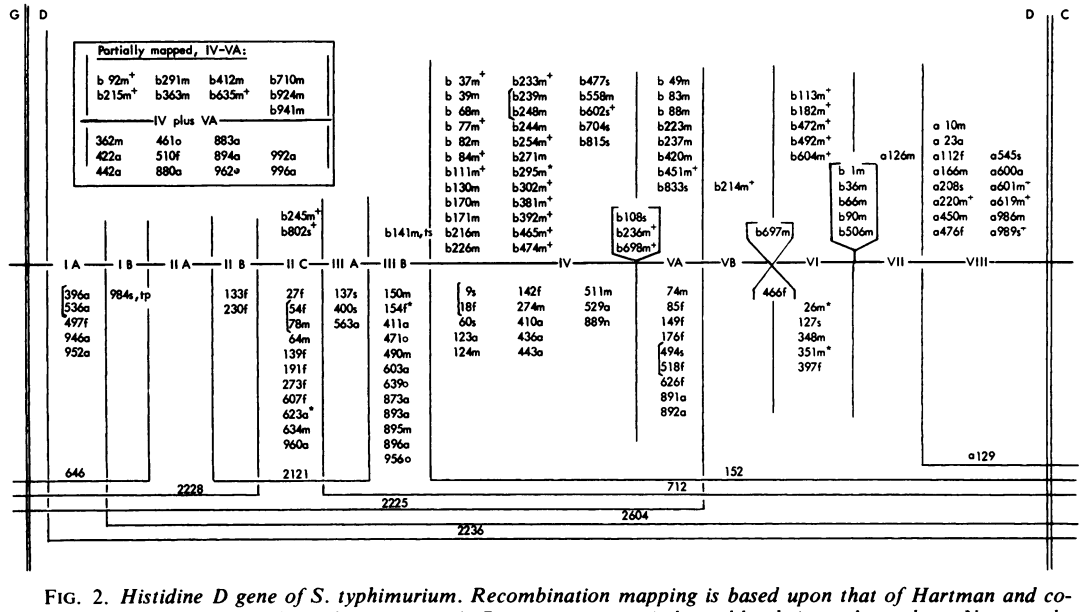

FIG. 2. *Histidine D gene of S. typhimurium. Recombination mapping is based upon that of Hartman and coworkers (Hartman et al., Advan. Gene., in press). Point mutants are indicated by their stock numbers. Noncomplementing mutants are entered below the major horizontal line; mutants entered above the line are designated as to complementation pattern a or b by the appropriate prefix. Most mutants were examined for cross-reacting material, CRM. CRM$^+$ mutants are indicated by +, and untested mutants, by an asterisk; all others are CRM$^-$. Letters after the numbers indicate the mutant characterization as described in Table 1. Mutants shown in brackets were not separated by recombination.*

F' *sup* amber procedures. Four strains, *600*, *601*, *603*, and *604*, were each shown to carry the *lactose*$^-$ trait which was mutable by NG to *lac*$^+$, preventing use of the F' *lac*$^-$ nonsense testers. The nature of such a *lac* genotype in these *Salmonella* strains was not determined. Each of the strains readily accepted the F' *his*$^+$ factor, and none was cured of the trait after growth in acridine orange. Two of the four strains were identified as nonsense by the F' amber suppressor. Thirty-two nonsense mutants were identified by the F' *lac*$^-$ procedure; 26 of these were suppressed by the F'14 amber suppressor. Although F' ochre *su8* did suppress a majority of these amber mutants, the level of suppression did not allow unambiguous recognition of additional nonsense as ochre *hisD* mutants. Thus, in column 9 of Table 1, nonsense mutants are designated as amber only if they respond to the F' amber suppressor. All but one of the six nonsense mutants not suppressed by F'14 amber suppressor did respond to the F'14 ochre suppressor *su8*. On this basis, these five mutants are suggested to be ochre. These results are summarized in Table 3.

Classification of mutant types. Most of the strains show mutagenesis and suppression properties permitting classification of their mutations as nonreverting, missense, nonsense, or frameshift. The predominant mutagenic effects of NG are to cause any of the possible single nucleotide base pair exchanges, whereas ICR191 is believed to effect almost exclusively the addition or deletion of one or more base pairs (25; see also references 5 and 20). Accordingly, mutants induced to revert by NG (NG$^+$) are predominantly of the missense or nonsense types; revertible mutants which are NG$^-$ are presumably of the frameshift type. Those NG$^-$ mutants which are induced to revert by ICR191 are more positively identified as frameshift. Twenty strains are designated frameshift by these criteria, 5 as NG$^-$ ICR191$^+$ and 15 as NG$^-$ ICR191$^-$.

TABLE 3. *Response of NG$^+$ mutants to nonsense testers*[a]

No. of mutants	SB391	SB392	TR869
26	+	+	+
6	+	+	−
2	ND[b]	ND	+

[a] All NG-revertible mutants were tested as described in the text. Plates were examined after 1, 2, 4, 6, and 10 days. Positive tests usually appeared with SB391 at 4 days, with SB392 at 2 days, and with TR869 after 24 hr. Members in the three groupings are: (26 mutants) *396, 536, 946, 952, 623, 960, 563, 411, 873, 893, 896, 123, 410, 436, 443, 529, 891, 892, 23, 422, 442, 880, 883, 894, 992, 996;* (6 mutants) *471, 639, 956, 889, 461, 962;* (2 mutants) *603, 600*. Of the set of six mutants, five responded to the ochre *sup* donated from LS323 within 3 days, whereas one mutant, *889*, was still negative at 10 days.

[b] Not done.

Two additional mutants, *18* and *139*, are classified frameshift, although they show a limited positive response to NG (and DES). They were shown to be polar in an earlier study by Ames and Hartman (3), a property consistent with frameshift mutations (25).

DES was included in our survey as a potent mutagen shown to induce a variety of single base pair exchanges. In examining a number of *S. typhimurium hisC* mutants, Whitfield et al. observed DES-induced reversion in 19 of 19 nonsense, 14 of 21 missense, and in none of 16 frameshift mutants (25). That this compound might also revert frameshift mutations on occasion is indicated by other studies (7). Seven of the *hisD* mutants classified as frameshift did respond to DES, including mutant *85* which is highly revertible by ICR191. As with the *hisC* mutants, all nonsense mutations were highly sensitive to reversion by DES as well as by NG.

The remaining mutants are nonreverting or are classified as missense. A few of these latter which are highly susceptible to NG-induced reversion could be unresolved ochre. UGA nonsense mutations are rare in the *his* operon (20); none were observed in fairly extensive examination of these mutants (Loper, *unpublished data*).

The data in Table 1 permit a comparison of the spectrum of mutation arising relative to the type of mutagenic treatment. For example, as has been noted in similar studies (25), by far the predominant class of 2-amino-purine induced mutations are nonsense (22 of 32 *hisD* mutants). Refer to Hartman et al. (Advan. Genet., *in press*) for a more extensive treatment of this type, where *hisD* data are contained in a comparison based upon over 1,000 *his* operon mutants.

DISCUSSION

Profile of the hisD gene. Inspection of Fig. 2 permits ready recognition of a number of properties of the gene for histidinol dehydrogenase. CRM$^+$ is only observed among complementing mutants and appears to be a property of complete polypeptide dimers. This conclusion is also supported by the nature of the *Da* mutants. Strain *hisD129*, the deletion of which defines region VIII, is itself complementing but is CRM$^-$; the CRM$^+$ strains mutant in this region, three missense and one stable, probably produce completed polypeptides. The CRM$^+$ stable mutant *Da989* presumably resulted from a short in-phase deletion.

Regarding the rest of the gene, *Db* comple-

menting mutations are clustered in regions IV through VI, whereas the *Dab* mutations are more evenly distributed throughout. Thus, 37 mutations, comprising about 24% of those studied map in the proximal regions I through IIIB. Of these, only 3 mutations are *Db*, and the remaining 34 mutations are *Dab*. In regions IV through VI, which together contain 100 of the mutations used in this study, 61 are *Db* and 39 are *Dab*.

All of the *Db* mutants are classified as either missense (57 mutants) or stable (7 mutants). These stable mutants are presumed to contain short in-phase deletions. In fact, although not indicated in Fig. 2, *b802s*$^+$ has been mapped as a short deletion overlapping *139* (Hartman et al., Advan. Genet., *in press*).

As noted above, the noncomplementing *Dab* mutations are distributed as follows: 34 in regions I to IIIB and 39 in regions IV to VI. The mutations are quite evenly distributed as to class type as well. Fifteen nonsense, 10 frameshift, 6 missense, and 3 stable mutants comprise the 34 mutations of regions I to IIIB; the 39 mutations of regions IV to VI are made up from 17 nonsense, 10 frameshift, 8 missense, and 4 stable mutants. Thus, the majority of *Dab* mutations are of types causing drastic change in the protein product—nonsense termination itself or altered coding arising from shifts out of the normal reading frame. Additionally, some of the missense class of *Dab* mutations may be undetected nonsense, and many of the stable *Dab* mutations may represent deletions which also effect a frameshift.

This occurrence of predominantly nonsense or frameshift mutations within certain regions of genes has been observed in other cases (10; Hartman et al., Advan. Genet., *in press*). Here, it is taken to indicate that many single base pair exchanges in the proximal *hisD* regions I to IIIB fail to prevent active enzyme production, which indicates a relatively nonspecific function for the N-terminal portion of the completed polypeptide. In fact, of the three *Db* strains mutant in proximal *hisD*, two are CRM$^+$, and the third, *141*, is temperature sensitive, producing a functional enzyme at 24 C.

Conversely, we interpret the relatively high incidence of missense mutations in regions IV to VI to indicate a large number of critical amino acid positions in the polypeptide product. At these positions, the specific amino acids encoded are important in determining the final protein configuration and function.

We suggested that only polymerized mutant polypeptides can react with antihistidinol dehydrogenase antibody. An alternative possibility of course is that prematurely terminated and structurally altered polypeptides incapable of polmerization are quickly degraded in the cell. Unfortunately, neither CRM$^-$ or CRM$^+$ *hisD* complementing mutant products were shown to complement in vitro (14). This is quite possibly related to the complex sulfhydryl content of the enzyme. It was previously shown that 12 half-cystines can be recovered per dimer. Only eight of the half-cystines are titratable in the enzyme in urea solution as free sulfhydryls, suggesting the presence of two disulfides per enzyme (13). Purified native enzyme may be 90% renatured after inactivation in 4 M urea (26), but no restoration of activity has been observed after denaturation in urea plus reducing agents (Loper, *unpublished data*). It is possible that dimerization normally occurs only during biosynthesis of the *hisD* polypeptide, and establishing the correct configuration in in vitro experiments is unfavored thermodynamically.

There is no evidence from our studies to support a two-cistron model of complementation in *hisD*, the second cistron coding for a discrete *Da* polypeptide. Such a system should be expected to reveal nonsense or otherwise chain terminator mutants which nevertheless produce the *Da* gene product and appear as *Db* complementing. Although terminator mutants are often polar, their polarity usually is less when their distance from the next translation initiator is decreased; terminator mutants mapping near the end of a cistron may be nonpolar with respect to subsequent cistrons in the operon (16, 28). Thus, one would expect an increased frequency of such *Db*-complementing types among termination mutations in the map regions approaching DVII. We find only missense *Db* mutations in the regions immediately preceding DVII to VIII, and all other *Db* mutations are either missense or are stable, presumably in-phase deletions. This is true although a number of nonsense or potentially terminating mutations were observed in these regions: 17 nonsense and 10 frameshift mutations among 39 noncomplementing *Dab* mutations in regions IV through VI. Our combined mutagenesis and complementation data are thus consistent with the chemical evidence that the *hisD* gene is transcribed as one cistron to yield a single biosynthetic polypeptide. The significance of the discrete mapping of all *Da* mutations remains as an unresolved aspect of structure and function in the dimeric enzyme.

There is the possibility that mutational events in *hisD* might occur which could then permit internal reinitiation of translation. In the *E. coli lac z* gene, termination by nonsense mutation was observed to result in reinitiation and translation of the distal portion (9). We have no evidence for this, although it should be noted that the sta-

tistical chance of finding one or more internal translation initiators may be low for *hisD*. Michels and Zipser observed two restarts in *lac z* although this gene codes for 24 methionines in the normal polypeptide (17). They suggested that a terminator event followed within a short distance by an internal methionine AUG codon is a necessary but not sufficient condition for reinitiation (17). In the *hisD* gene there are only four internally coded methionines (13).

Interspecies complementation. Several studies by ourselves and others failed to reveal any recombination within the *his* region for *S. typhimurium* bearing *E. coli* F' *his* factors (4; L. Garrick-Silversmith and P. E. Hartman, Genetics, *in press*). This is interpreted to indicate considerable heterogeneity in nucleotide sequence. Nevertheless, the respective *hisD* products of these organisms must be highly similar in overall structure as attested by the extensive F' factor complementation data presented here. All of the major complementation mapping data available from abortive transduction in intraspecies tests were reproduced in *S. typhimurium-E. coli* interspecies tests. Some subsets of incompatibility as seen in the intraspecies studies (14) might be expected by using a greater variety of *E. coli hisD* mutants.

To what extent the nucleotide heterogeneity between the *E. coli* and *S. typhimurium hisD* genes is reflected in different amino acid sequence is not known. Purified histidinol dehydrogenase from *E. coli* shows reproducible differences in total amino acid content compared to its *Salmonella* counterpart, but in general the composition is quite similar (Firca and Loper, *unpublished data*). As might be expected considering the complementation homology, *E. coli* histidinol dehydrogenase cross-reacts with antibody prepared to the *S. typhimurium* enzyme (Firca and Loper, *unpublished data*).

Nonsense suppression. The technique of simultaneous suppression of known F' *lac* nonsense mutants for the identification of nonsense mutations in other pathways was developed by Berkowitz et al. (6). The recent availability of F'14 amber suppressor and F'14 ochre suppressor permitted us to compare these two techniques with respect to detection of *hisD* nonsense mutants. A high percentage of *hisD* nonsense mutants as identified by using F' *lac*$^-$ were suppressible by the F'14 amber *sup*, which is believed to insert glutamine. It would appear that many of these are noncritical sites in terms of the actual amino acid replacement. Since the F'14 *sup* is a recessive lethal mutation, suppression in the F' *lac*$^-$ procedure would have had to involve some amino acid other than the glutamine of this amber *sup*.

The F' ochre *sup* suppressed known *hisD* nonsense mutants in most cases with growth in 3 to 7 days. Spontaneous mutation on plates during such weaker suppression made detection of additional ochre mutants among the remaining "missense" class ambiguous, and their distinction was not attempted. Of the six nonsense mutants which failed to respond to the recessive lethal amber *sup*, however, five mutants did grow after mating in the ochre suppressor F'14 *su8*, suggesting these five may be ochre mutants. The actual amino acid replacement specificity of these and of the remaining nonsense mutant *889* may be of value in relating protein structure and enzymatic activity.

Polarity. No quantitation of polar effects in *hisD* mutants by measurement of enzyme activities from neighboring *his* genes was attempted in this survey, although use was made of data available from other studies (3). We surveyed all available *hisD* mutants for totally polar effects, however. Such tests are possible by using F' factors bearing mutants of the *E. coli his* operon which are inactive for *hisC*, the gene distally adjacent to *hisD* (14). These tests are quite sensitive and allowed recognition of one mutant, *hisD984*, as totally polar (S. A. McIntire and J. C. Loper, *unpublished data*).

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