GENETIC FINE STRUCTURE OF THE LEUCINE OPERON IN SALMONELLA[1]

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Received December 17, 1962

MANY current investigations of cell metabolism and cell function are strongly influenced by present day concepts of gene structure, gene action and the mechanisms, both genetic and non-genetic, which regulate gene function. This report presents evidence concerning genetic components associated with the leucine biosynthetic pathway in *Salmonella typhimurium*.

That the regulation of gene activity is basically determined by genetic controlling elements was first prominently suggested by the work of McCLINTOCK with maize (McCLINTOCK 1956, 1961). In bacteria, the evidence that in many cases the genes associated with a single biochemical pathway were clustered together on the bacterial chromosome (see DEMEREC and HARTMAN 1959) together with the evidence for coordinate control of the synthesis of enzymes involved in the pathway, has allowed the development of a model for the regulation of gene activity (JACOB and MONOD 1961a,b). As in the earlier model described by McCLINTOCK for maize, there are three elements involved. The first is the "structural gene" which determines the identities and order of amino acids in a polypeptide chain, thus determining enzyme structure. The second is the "operator" which, in the model of JACOB and MONOD (1961a,b), is a region of the chromosome closely linked to one end of a cluster of structural genes and may, in fact, be a part of the terminal structural gene which it adjoins. The operator directs the activities of the structural genes associated with it in response to the product of the third genetic element of the control system, the "regulator." The product of the regulator, in conjunction with an inducing substrate or repressing end product, determines whether the operator turns the structural genes on or off. The regulator may or may not be located close to the "operon," which is the term used by JACOB and MONOD to describe the conjoined operator and structural gene (or genes). In the system described in this report, the structural genes and operator responsible for leucine synthesis have been defined, but the regulator has not yet been identified. In a brief preliminary note, the presence of four complementation groups involved in leucine synthesis in *Salmonella typhimurium* had been previously reported (MARGOLIN 1959).

Initial evidence of the existence of operons was derived from genetic and

[1] This investigation was supported by a research grant, GM 07178, from the Division of General Medical Sciences, Public Health Service, and by a research grant, NSF-G 19848, from the National Science Foundation.

biochemical studies of enzyme systems associated with carbohydrate fermentation (JACOB and MONOD 1961a,b; ENGLESBERG 1961). Evidence that the genetic components of a biosynthetic pathway may also be organized as an operon has been presented by AMES and HARTMAN (1962) for the histidine pathway in *Salmonella typhimurium*. This is supported by the present transduction analysis of the genetic components responsible for leucine biosynthesis.

MATERIALS AND METHODS

Media: Difco nutrient broth was used for growing cultures to be used as recipients in transductions and preparation of phage lysates. A variation of the DAVIS and MINGIOLI (1950) medium was used for solid minimal medium: K_2HPO_4, 10.5 g; KH_2PO_4, 4.3 g; Na_3-citrate·$3H_2O$, 0.47 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; H_2O, 1000 ml plus either 0.2 percent dextrose or 0.2 percent L-arabinose. To solidify the medium 1.5 percent Difco Bacto-agar was added.

Minimal agar medium with L-arabinose (0.2 percent) as carbon source is referred to as AM medium. For distinguishing abortive transductions, minimal agar medium with 0.2 percent dextrose as carbon source was supplemented with 10 percent w/v Difco leucine assay medium, or 20γ/ml of all amino acids except leucine (DMY medium). For transductions intended to produce wild-type recombinants from leucine auxotrophs, the minimal agar medium with 0.2 percent dextrose was enriched with 1.25 percent v/v reconstituted Difco nutrient broth (DME medium).

Phage: The phage used in transductions was PLT22 (ZINDER and LEDERBERG 1952). Lysates were prepared by infecting log phase cultures of the donor bacteria at low multiplicities (1:200 to 1:400) and allowing rounds of lysis and infection to occur in an aerated culture for about eight hours.

Bacterial strains: All the leucine auxotrophs of *Salmonella typhimurium* were derived from strain LT-2 (ZINDER and LEDERBERG 1952) or its derivatives, and obtained by penicillin selection. The various agents used to induce mutations are described later. DR. M. DEMEREC kindly supplied 25 of the 156 leucine mutants tested, as well as the *ara-9* mutant described below.

Strain *ara-9*, which is unable to ferment arabinose, was derived from a ultraviolet-treated culture of a leucine auxotroph, *leu-39*. The *leu-39* strain had been selected as a spontaneous mutant of LT-2. The double mutant, *leu-39 ara-9*, was then subjected to transduction in order to obtain the *ara-9* strain (GLANVILLE, personal communication). Most of the leucine auxotrophs utilized in this study were selected in this strain. The genetic marker, *ara-9*, was chosen because it had never been found to back mutate, and the arabinose loci are jointly transduced with the leucine loci (GLANVILLE and DEMEREC 1960).

In order to carry out reciprocal three-point transductions between leucine mutants it was necessary to obtain each mutant both with the *ara-9* marker and with the *ara*$^+$ allele. Since most of the leucine auxotrophs already contained the *ara-9* marker it was merely necessary to obtain recombinants in

which the *ara-9* allele was replaced by *ara*$^+$. The double mutants were transduced with phage grown on the wild strain (LT-2), and the infected cells plated on minimal medium containing arabinose as a carbon source and supplemented with 20γ of L-leucine/ml and 1.25 percent v/v reconstituted nutrient broth. All *ara*$^+$ recombinants gave rise to colonies, and those which still retained the leucine requirement were readily identified by replica-plating onto medium which was not supplemented with L-leucine. ZINDER's V1 (frequently referred to as H4) variant of PLT22 phage (ZINDER 1958) was used because clones lysogenized by this phage spontaneously give rise to sensitive cells with a very high frequency. It was therefore relatively easy to select sensitive lines of the appropriate recombinants, which could then be used for further transductions.

Procedure for transduction and recombination studies: Recipient bacteria were grown to saturation in aerated nutrient broth cultures and mixed with an equal volume of the appropriate phage lysate, diluted so that the ratio of phage to bacteria was in the range 5:1 to 15:1. The mixture was incubated at 37°C for six minutes and 0.1 ml aliquots spread on DME plates. After 48 hours of incubation at 37°C any wild-type colonies, resulting from recombination of the two leucine mutants, were replica-plated (LEDERBERG and LEDERBERG 1952) on AM plates to determine the percentage incorporation of the donor's *ara*$^+$ marker. Two controls were employed: (1) recipient bacteria infected with phage grown on the recipient strain itself; and (2) uninfected recipient bacteria.

Procedure for complementation studies: Reciprocal transductions among leucine mutants were made as described above and then spread in 0.1 ml aliquots on DMY agar. Following 48 to 72 hours incubation at 37°C, the plates were examined at 30× to 60× magnification with a stereo microscope for the presence or absence of abortive transductant colonies (OZEKI 1956). The supplement in DMY of all amino acids except leucine was found to increase the frequency and size of the abortive transductant colonies to an extent which made them much easier to recognize.

RESULTS

The complementation groups: All but four of a total of 156 independently arising leucine auxotrophs were found to fall into four complementation groups when studied by means of the abortive transduction method of OZEKI (1956). The results reported by OZEKI indicate that the abortive transduction test is in essence a test of the *trans* configuration of the *cis-trans* test described by BENZER (1957) for phage. As such, it distinguishes functional relationships among nonidentical alleles. This permits mutant sites to be assigned to cistrons in terms of the ability of the mutants to complement each other. HARTMAN, HARTMAN and SERMAN (1960) have carried out a similar analysis of histidine auxotrophs in *S. typhimurium*.

Complementation between mutants was recognized by the formation of microcolonies resulting from abortive transductions. Tests were performed as reciprocal transductions between pairs of mutants, each mutant being used as donor in one transduction and recipient in the other transduction. The absence of micro-

colonies was scored as a lack of complementation, indicating that the two mutants being tested were members of the same complementation group or cistron. The presence of microcolonies was scored as evidence of complementation indicating that the two mutants tested were members of different cistrons. In this manner the four complementation groups were delineated, all mutants giving negative results in the pair-wise tests being considered to be members of the same group. All members of any one group were found to be capable of complementing all mutants of the other three groups. When the sites of the mutations were explored in detail (see below), the four complementation groups were found to represent four closely linked cistrons. Each cistron was homogenous with respect to the complementing activities of the mutants whose sites form the cistron. This contrasts with complementation studies of the histidine loci of Salmonella (HARTMAN, HARTMAN and SERMAN 1960; AMES and HARTMAN 1962) in which complementing subunits within loci have been demonstrated. Similarly, GROSS (1962) has demonstrated complementing subunits within the *leu-2* locus of *Neurospora crassa*. The apparent lack of such sub-units within the leucine cistrons of Salmonella may therefore merely be due to my inability to detect them under the conditions of the complementation tests which were performed.

Table 1 records the modes of origin of the examined mutants as well as the number of mutations in each cistron. It can be seen that many more mutations occurred within cistrons I and III than within cistrons II and IV. It may also be noted that the distribution of mutations among the cistrons is, to some degree, an expression of the modes of origin. This can be illustrated by examining the frequency of mutations occurring within cistrons I and III among untreated cells compared with that in cells treated with 2-aminopurine. Of all spontaneously occurring mutations, 30.8 percent arose within cistron I and 61.5 percent arose within cistron III. In contrast, among cells treated with 2-aminopurine, 52.6 percent of all mutations to leucine auxotrophy arose within cistron I and only 29.8 percent arose within cistron III.

Analyses of leucine biosynthesis in Salmonella and Neurospora (JUNGWIRTH, GROSS, MARGOLIN and UMBARGER 1963) indicated the existence of similar pathways for both organisms. The first reaction is mediated by a condensing enzyme which is lacking in Salmonella mutants with altered sites in cistron I. The second enzyme is an isomerase whose activity is absent in Salmonella cells having sites of mutation in either cistron III or cistron IV. GROSS (1962) has

TABLE 1

The number of leucine auxotrophs per cistron arranged according to their modes of origin

Cistron	Spontaneous	2-aminopurine treatment	X-ray treatment	5-bromouracil treatment	Nitrous acid treatment	Total
I	12	30	16	5	7	70
II	1	7	3	1	1	13
III	24	17	12	1	2	56
IV	2	3	4	2	2	13
Total	39	57	35	9	12	152

found evidence in Neurospora that the active isomerase results from the association of two different polypeptides which are determined by two of the four leucine loci. By inference, therefore, it seems likely that cistrons III and IV of Salmonella are comparable in their action to the two gene loci in Neurospora. The final steps in leucine biosynthesis—oxidation and decarboxylation—appear to be mediated by a single enzyme whose formation is controlled by cistron II.

In Neurospora the enzymes involved in leucine biosynthesis are associated with distinctly separated gene loci. The parallel between the cistron-enzyme relationships in Salmonella and the gene-enzyme relationships in Neurospora makes it reasonable to use the terms cistron and gene interchangeably in referring to the region of the chromosome containing the mutant sites of any one complementation group in Salmonella.

The operator mutant: Modification of an operator alters its mode of control of action of the conjoined structural gene. Different types of modifications are known. One of them is given the designation o^0 because a mutation of this type results in complete inaction of the associated structural genes, regardless of changes in concentration of the repressor to which the operator normally responds. One leucine auxotroph in our series, *leu-500*, which was obtained following treatment of a culture with 5-bromouracil, has all the attributes of an o^0 mutation. The evidence for this is reviewed below.

In abortive transduction tests of *leu-500* with many mutants from each of the four complementation groups no microcolonies appeared. This was true whether *leu-500* was used as a recipient or a donor. Alone, this inability to complement any of the altered structural genes would allow three possible interpretations of the nature of the alteration which gave rise to *leu-500*: 1. The alteration could consist of a long multisite mutation (perhaps a deletion) involving all four cistrons. 2. The alteration could consist of a mutation of a leucine regulator gene to a super-repressor condition whereby it produces a super-repressor substance to which the operator responds by inactivating all the structural genes, even when leucine is in short supply. This would be the equivalent of the i^s gene in the β-galactosidase system (JACOB and MONOD 1961a, b). 3. The alteration could consist of a mutation of the leucine operator to an o^0 condition so that the four structural genes remain inactive under all conditions of culture.

The first hypothesis, that *leu-500* is a long multisite mutation, was quickly eliminated, since wild-type recombinants were produced in large numbers from reciprocal transductions with many mutants from each of the complementation groups. In addition, three-point transduction tests, to be described later, indicated that the site of the *leu-500* mutation is to the left of the leftmost mutant site in cistron I (see Figure 2).

The second possible interpretation, that *leu-500* resulted from a mutation of the regulator gene to a super-repressor condition has the requirement that a super-repressing substance be present in the cytoplasm. Therefore the super-repression would be effective in the trans configuration of a *cis-trans* test (dominant over wild type). This effect of a cytoplasmic repressor substance in partial heterozygotes has been demonstrated in the β-galactosidase system (PARDEE,

JACOB and MONOD 1959; JACOB and MONOD 1961b). Fortunately, the presence of such a cytoplasmic component would be detectable in the case of *leu-500* by means of the abortive transduction test. If a super-repressing substance were present in the cytoplasm, a chromosome fragment, bearing a normal leucine operon, introduced by phage grown on wild-type cells, would not be detected. The leucine structural genes on the fragment would be inactive because of the response of their operator to the presence of the super-repressing substance in the cells. Therefore, no microcolonies would result from abortive transductions. In fact, however, microcolonies were produced under these conditions indicating that this second possible interpretation of the basis of the auxotrophy of strain *leu-500* is not valid.

It appears reasonable to conclude, therefore, that the third hypothesis that *leu-500* resulted from a modification of the leucine operator to an o^o condition, is correct, since it has all the characteristics of such a mutation. The genetic evidence is reinforced by recent tests which indicate that all three enzymes of the leucine biosynthetic pathway are missing in the *leu-500* mutant (BURNS and MUKAI, unpublished). Indication of the unitary response of the leucine operon to various growth conditions is provided by evidence that the three enzymes in the pathway are coordinately repressed and derepressed (BURNS, unpublished).

Multi-cistron auxotrophs: Only three of the 156 independently arising leucine auxotrophs carried genetic alterations involving more than one cistron. Two of these, *leu-39* and *leu-485*, were spontaneous in origin. Tests on minimal agar of more than 5×10^{11} cells of each of these two mutants have never produced a revertant colony. Neither mutant complements any mutant with single site modifications in either cistron II, III or IV, although complementation does occur with mutants of cistron I.

In order to define the limits of the multisite alterations of these two mutants, transduction tests were performed with mutants having single site alterations in one or another of each of the four cistrons. In all cases the doubly mutant strains, *leu-39 ara-9* and *leu-485 ara-9*, were used as recipients and the donor leucine mutants all carried the ara^+ allele. Aliquots of each transduction mixture were spread onto DME medium to select for leu^+ recombinants and onto AM medium supplemented with 20 γ of L-leucine/ml to select for ara^+ recombinants. In the former case, since dextrose is the sugar present in the medium, all leu^+ recombinants are selected, regardless of whether or not recombination for the arabinose markers occurred. In the latter case, on leucine supplemented AM medium, all ara^+ recombinants were selected, regardless of what recombination occurred for the leucine markers. Since all the transductions involved the same *ara-9* recipient and ara^+ donor alleles, variations in the number of ara^+ recombinant colonies obtained served primarily as a measure of differences in the general transduction efficiencies. These differences in efficiency appear to be due to uncontrolled variables in the procedures. The simplest way, therefore, to correct the leu^+ recombinant data for these variables is to express them in terms of their ratio to the number of ara^+ recombinants in each transduction.

The use of the *ara*$^+$ transduction as a correction factor may be subject to one further error. As will be indicated later, the different *leu* mutants appeared to have individual and different effects upon the frequency of crossing-over in their vicinity. These effects might have, in turn, affected the recombination frequency of the closely linked arabinose marker so that the numbers obtained are not strictly an expression of general transduction efficiency alone. However, the effects of the *leu* mutations upon crossing-over appear to be relatively small compared to the differences in transduction efficiencies due to the uncontrolled variables in the procedures. Therefore, the corrected data should approximate the true recombination values reflecting distances between *leu* sites. An additional marker, unlinked by transduction to the leucine operon, would have provided a better measure of general transduction efficiency. Unfortunately, no such marker was available.

The results obtained for the sets of transductions with both *leu-39* and *leu-485* are shown in Table 2. The listing of donor mutants is arranged in essentially the same order as their sites on the bacterial chromosome (see Figure 2). The first column of data in each case presents the actual total numbers of *leu*$^+$ recombinants which were obtained. To indicate the extent of testing which these figures represent, the second column records the number of *ara*$^+$ recombinants calcu-

TABLE 2

Frequencies of leu$^+$ *recombinants in transductions between two multisite mutants used as recipients and various single-site mutants used as donors*

Donors		Recipients					
		leu-39 ara-9			*leu-485 ara-9*		
Cistron	Mutant	Total *leu*$^+$ recombinants	*ara*$^+$ equivalent*	Number *leu*$^+$ per 100 *ara*$^+$	Total *leu*$^+$ recombinants	*ara*$^+$ equivalent*	Number *leu*$^+$ per 100 *ara*$^+$
I	*leu-124*	1,459	13,250	11.010	1,480	10,741	13.780
	leu-121	951	9,485	10.026	1,402	12,432	11.277
	leu-125	233	4,090	5.700	200	2,926	6.840
II	*leu-129*	236	8,620	2.740	232	8,827	2.628
	leu-455	155	13,780	1.120	195	16,587	1.176
	leu-140	38	24,752	0.150	27	22,933	0.118
III	*leu-130*	1	14,615	0.0068	0	18,871	0
	leu-157	0	35,350	0	0	45,090	0
	leu-126	0	12,857	0	0	9,790	0
	leu-122	0	17,305	0	0	11,220	0
	leu-123	1	13,947	0.0072	0	22,210	0
	leu-131	1	9,495	0.0105	0	10,080	0
	leu-156	0	27,380	0	0	29,467	0
IV	*leu-128*	0	43,308	0	0	30,820	0
	leu-466	0	26,630	0	0	26,610	0

* These figures indicate the extent of the testing for *leu*$^+$ in each transduction and were derived as follows: number ml tested for *leu*$^+$ times number *ara*$^+$ recombinants per ml. Further discussion in the text.

lated to be present in the quantity of each transduction mixture which was tested for *leu*$^+$ recombinants. This was determined by multiplying the volume (in ml) tested for *leu*$^+$ recombinants by the number of *ara*$^+$ recombinants per ml found for each mixture. The column is therefore labeled "*ara*$^+$ equivalent."

The resultant figures are especially important in interpreting the meaning of the transductions which produced zero *leu*$^+$ recombinants. They are needed for determining whether *leu*$^+$ recombinants are not able to be formed, or are merely not detected because of insufficient sampling. The number of recipient cells tested would not suffice as a measure of the extent of the sampling, due to the great variations in general transduction efficiencies. What is needed is a value which reflects the number of recipient cells tested which have undergone successful transductions, and this is what is represented by the recorded "*ara*$^+$ equivalent" values.

The third column presents the number of *leu*$^+$ recombinants per 100 *ara*$^+$ recombinants for each transduction mixture. Stated in this way, the relative frequencies of recombination for *leu*$^+$ can be seen, with the variations due to differences in general transduction efficiencies eliminated. Two features of these results are particularly striking. The first is that any *leu*$^+$ recombinants obtained in transductions between *leu-39* and mutants whose sites are to the right of the *leu-140* site were very rare and of a frequency distinctly lower in magnitude than the frequencies obtained from transductions with auxotrophs having sites of mutation to the left of that of *leu-130*. It should be mentioned that the three rare *leu*$^+$ recombinants in Table 2, resulting from transductions between *leu-39* and cistron III mutants, do not represent the actual total which have been obtained. Indeed, about 25 *leu*$^+$ recombinants have been produced from transductions between *leu-39* and *leu-130*, *leu-126*, *leu-122*, *leu-123*, *leu-131*, and *leu-128*. However, in most of the experiments the *ara*$^+$ transduction frequency was not determined, and therefore the data are not presented in the table. In the case of *leu-485* no *leu*$^+$ recombinants were obtained with mutants having sites to the right of that of *leu-140*. The second feature to be noted is that, in general, the further to the left the position of the mutational site of the donor, the higher the frequency of *leu*$^+$ recombinants obtained with both *leu-39 ara-9* and *leu-485 ara-9*. This is consistent with the concept that the greater the distance between two sites, the higher the frequency of recombination.

The differences in recombination frequencies exhibited by the various donors are most probably not strictly proportional to differences in distance from the recipient sites. This is indicated by the fact that the results of transductions involving *leu-124* differ from those involving *leu-121*. In Figure 2 the mutant alterations of these two strains are shown as occupying the same site, since recombination between them has never been detected. However, they are not identical, since they differ with respect to back mutation characteristics. The differences in recombination frequencies are therefore probably due to the sort of individual effects of each mutant alteration which were mentioned above.

These results would indicate that both *leu-39* and *leu-485* represent alterations of the chromosome involving all of the leucine operon to the right of the *leu-140*

site. The complementation studies indicate that the lesions must extend slightly into cistron II although they do not appear to cover the position occupied by the site of *leu-140*. This is indicated diagrammatically in Figure 2. Because *leu*$^+$ recombinants are so rare from transductions between *leu-39* and mutants having altered sites in cistrons III and IV, the earlier experiments, which had tested only about 5×10^8 recipient cells in each case, produced no wild-type colonies. Therefore *leu-39* and *leu-485* had both been initially considered to represent probable deletions.

Although the rarity of *leu*$^+$ colonies which were obtained in transductions between *leu-39* and mutants with sites in cistrons III and IV, makes it possible that these represent contaminants, it should be noted that an equal number of cells tested as controls did not produce any wild-type colonies. Another possible trivial mode of origin exists, since all the donor strains used are known to revert to *leu*$^+$ spontaneously. It is therefore possible that phage lysates derived from the donor cultures included some transducing phages carrying *leu*$^+$ alleles. This is an unlikely explanation, however, since most of the donor strains exhibit very low back mutation frequencies. Furthermore, no *leu*$^+$ recombinants were produced when *leu-485* was used as a recipient with the same donors.

Whatever the explanation, the appearance of the rare leucine-independent colonies casts doubt upon the deletion interpretation of the nature of the multisite alteration which gave rise to *leu-39*. It seems possible that *leu-39* and *leu-485* are the results of essentially identical breaks in the chromosome. In the case of *leu-39* an inversion could have resulted, while in *leu-485* the result was a deletion. This would be consistent with the data obtained. However, no further supporting evidence is presently available for this interpretation.

The third multi-cistron leucine auxotroph, *leu-447*, was obtained from a culture which had been subjected to X-ray treatment. It does not complement mutants of any of the four cistrons. Since *leu-447* does not produce any *leu*$^+$ recombinants in reciprocal transductions with any leucine auxotrophs (including the o^0 mutant, *leu-500*) it is probably the result of a deletion of the entire leucine operon.

Three-point recombination mapping: Recombination mapping by three-point transduction tests has been used by several investigators to ascertain the linear order of a series of very closely linked mutant sites (DEMEREC and HARTMAN 1956; GROSS and ENGLESBERG 1959; BALBINDER 1962a). The tests determine right-left orientation of sites of mutation of mutants having the same functional aberration (leucine auxotrophy in this case). They require the availability of a nonselective outside marker, closely enough linked so as to be jointly transduced with the region being mapped. The fortunate close linkage of the *ara-9* marker to the leucine operon (averaging about 50 percent joint transductions with various *leu* sites) permitted such an analysis of the leucine auxotrophs.

Each three-point test consisted of a pair of reciprocal transductions between two leucine auxotrophs which differed with respect to their arabinose alleles. In the tests described here the donor always carried the *ara*$^+$ allele and the recipient the *ara-9* allele. The transduction mixtures were plated on DME to select for

leu+ recombinants. The *leu*+ colonies were then replica-plated upon AM medium to determine the percentage which had also incorporated the *ara*+ allele of the donor.

As can be seen in Figure 1, when the recipient's *leu* site is to the left of the donor's *leu* site, four "crossovers" are required to produce a *leu*+ recombinant which has also incorporated the *ara*+ allele of the donor. In contrast, only two "crossovers" are required to produce similar *leu*+ *ara*+ recombinants in the reciprocal transduction in which the recipient's *leu* site is to the right of the donor's *leu* site. The frequency of occurrence of four "crossovers" should be considerably less than that of two "crossovers." Assuming the sites of *leu* mutations are arranged in a linear order, in each pair of reciprocal transductions between two mutants, one transduction should include as recipient the auxotroph having its site of mutation to the left, while in the other transduction the second auxotroph with the mutant site to the right, would be the recipient. Therefore, that transduction of a reciprocal pair in which the percentage of *leu*+ recombinants which have incorporated *ara*+ is lower should be the transduction in which the recipient *leu* site is to the left of the donor *leu* site.

Variations introduced by differences in efficiency of transduction between the two transductions of a reciprocal pair do not affect this determination. This is because the comparison is not made between absolute numbers of recombinant colonies, but rather is made between percentages of *leu*+ *ara*+ colonies among the total number of *leu*+ recombinants. In practice, therefore, when two *leu* mutants were tested by such a pair of reciprocal transductions, the transduction which produced the lower percentage of *ara*+ incorporation was noted. The site of mutation of the recipient auxotroph in that transduction was recorded as being to the left of the site of the donor.

Table 3 records the results of such pairwise three-point transduction tests among nine leucine auxotrophs. Included among the *leu* mutants are two representatives from each structural gene, as well as the o^0 mutant *leu-500*. The mutants are arranged in the table according to the linear order of their sites on the chromosome (which was determined by the information in the table).

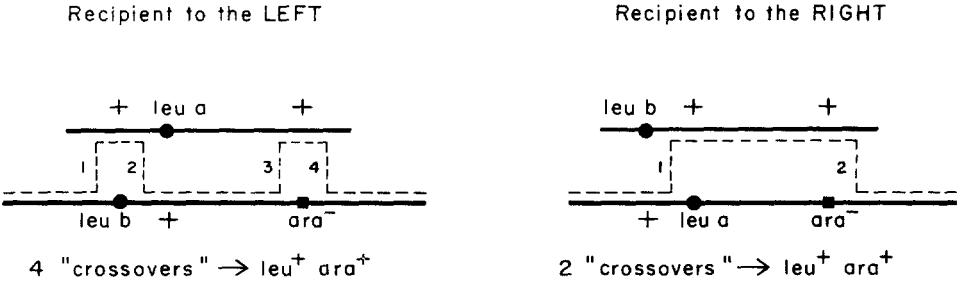

FIGURE 1.—Schematic presentation of the crossovers required to obtain *leu*+ recombinants as well as incorporation of the donor's *ara*+ allele. The recipient chromosome is represented by the lower line in each case and the donor chromosome fragment by the shorter, upper line. When the recipient mutant site is to the left of the donor's mutant site, four crossovers are required to incorporate the *ara*+ allele, while in the reciprocal transduction only two are needed.

TABLE 3

Results from reciprocal three-point transduction tests. The mutants are arranged in the table in the same order as their sites on the chromosome. The data in the body of the table are the percentages of leu$^+$ ara$^+$ *recombinants and, in parentheses, the actual numbers of* leu$^+$ *recombinants tested for* ara$^+$ *incorporation. The table is redundant in that the data in the lower left section are a mirror image of those in the upper right. This permits continuous reading from left to right of results of transductions of each mutant strain with all others*

		leu-500	*leu-124*	*leu-158*	*leu-129*	*leu-455*	*leu-130*	*leu-126*	*leu-128*	*leu-466*
leu-500	(D)		49 (346)	46 (1327)	54 (732)	53 (936)	45 (1159)	53 (997)	62 (1191)	68 (793)
	(R)		10 (527)	20 (236)	10 (260)	10 (767)	12 (390)	9 (590)	9 (480)	8 (990)
leu-124	(D)	10 (527)		39 (632)	39 (1750)	40 (2420)	32 (915)	47 (3688)	51 (3349)	54 (945)
	(R)	49 (346)		23 (218)	18 (2295)	16 (2195)	24 (1383)	14 (882)	15 (1451)	15 (845)
leu-158	(D)	20 (236)	23 (218)		40 (840)	40 (562)	38 (2927)	45 (594)	53 (849)	57 (1922)
	(R)	46 (1327)	39 (632)		29 (1332)	25 (1326)	25 (1948)	21 (342)	18 (573)	12 (1676)
leu-129	(D)	10 (260)	18 (2295)	29 (1332)		42 (330)	40 (607)	54 (501)	50 (593)	64 (1192)
	(R)	54 (732)	39 (1750)	40 (840)		15 (402)	22 (407)	14 (526)	11 (347)	13 (1342)
leu-455	(D)	10 (767)	16 (2195)	25 (1326)	15 (402)		39 (378)	43 (412)	46 (690)	51 (1206)
	(R)	53 (936)	40 (2420)	40 (562)	42 (330)		25 (246)	20 (398)	14 (836)	14 (306)
leu-130	(D)	12 (390)	24 (1383)	25 (1948)	22 (407)	25 (246)		54 (310)	54 (289)	57 (2748)
	(R)	45 (1159)	32 (915)	38 (2927)	40 (607)	39 (378)		14 (396)	13 (234)	10 (2010)
leu-126	(D)	9 (590)	14 (882)	21 (342)	14 (526)	20 (398)	14 (396)		43 (516)	61 (600)
	(R)	53 (997)	47 (3688)	45 (594)	54 (501)	43 (412)	54 (310)		17 (528)	18 (245)
leu-128	(D)	9 (480)	15 (1451)	18 (573)	11 (347)	14 (836)	13 (234)	17 (528)		64 (697)
	(R)	62 (1191)	51 (3349)	53 (849)	50 (593)	46 (690)	54 (289)	43 (516)		8 (273)
leu-466	(D)	8 (990)	15 (845)	12 (1676)	13 (1342)	14 (306)	10 (2010)	18 (245)	8 (273)	
	(R)	68 (793)	54 (945)	57 (1922)	64 (1192)	51 (1206)	57 (2748)	61 (600)	64 (697)	

(D) = Donor (R) = Recipient

The data in the body of the table present the percentage of *leu*$^+$ *ara*$^+$ recombinants resulting from each transduction. The actual number of *leu*$^+$ colonies tested for the presence of *ara*$^+$ is shown in parentheses next to each percentage figure. Table 3 is redundant in that the lower left section is merely a mirror-image arrangement of the data in the upper right section. This arrangement makes it possible to read from left to right when studying the results of reciprocal transductions between any one mutant and all the others listed. For example, note the percentages of *leu*$^+$ *ara*$^+$ resulting from transductions between *leu-455* and all the other mutants. In transductions with *leu-500*, *leu-124*, *leu-158* and *leu-129*—i.e., mutants having sites to the left of the *leu-455* site—the lower percentage was obtained when *leu-455* was the donor. On the other hand, in transductions with *leu-130*, *leu-126*, *leu-128* and *leu-466*, all having sites to the right of the *leu-455* site, the lower percentage appeared when *leu-455* was the recipient. This type of relationship remains consistent throughout the data in Table 3.

Unfortunately, when studying such data it is necessary to limit the interpretation to right-left orientation of sites of mutation. The percentages obtained from the three-point transduction tests do not seem to provide any accurate indications of relative distances between sites. As mentioned previously, each mutation appears to have an individual and characteristic effect upon the frequency of "crossing-over" in the region of the leucine operon. Such individual effects of mutations have been noted by HARTMAN, LOPER and SERMAN (1960) during transduction studies of recombination between histidine auxotrophs of *S. typhimurium*. Similar allele specific effects have been noted by DEMEREC, GOLDMAN and LAHR (1958), and BALBINDER (1962b), studying recombination of tryptophan mutants by transduction.

Some of the effects of individual mutants can be noted in Table 3. For example, both *leu-158* and *leu-130* seemed to have the effect of sharply increasing the frequency of recombinants resulting from quadruple crossovers. This is indicated by the unusually high percentage of *leu*$^+$ *ara*$^+$ recombinants which were obtained from transductions in which the site of mutation in the recipient is to the left of that in the donor. This effect is especially noticeable in those data which involve *leu-158*.

The allele specific effects upon recombination can be intensive enough with certain combinations of mutants to make it difficult to determine the linear order of sites upon the basis of the three-point tests alone. A mild example of this is evident in Table 3 from the data concerning the reciprocal transductions between *leu-130* and *leu-124*. In this case, when the recipient site was to the left (*leu-124* used as recipent) the frequency of *leu*$^+$ *ara*$^+$ recombinants was 24 percent, resulting from quadruple crossovers. When the recipient site was to the right (*leu-124* used as donor) the frequency of *leu*$^+$ *ara*$^+$ recombinants, resulting from double crossovers, was 32 percent. It is evident that any further decrease in the difference would begin to make it very difficult to determine in which transduction of a reciprocal pair the *ara*$^+$ incorporation resulted from four crossovers as opposed to two crossovers.

A more extreme example of this type of problem is available from the results

of reciprocal transductions between *leu-130* and *leu-140* (not in Table 3). In this instance the percentage of *leu*$^+$ *ara*$^+$ recombinants obtained when *leu-130* was the recipient was 36.8 percent, while 34.4 percent was obtained when *leu-140* was the recipient. Apparently with this combination of mutants almost the same frequency of *ara*$^+$ incorporations into the recipient chromosome occur, whether two crossovers are required or four crossovers are required. Fortunately, in this case it was possible to determine more definitely that the site of the *leu-140* mutation was to the left of the *leu-130* mutation by two other criteria. The first was that, by means of complementation tests, it could be ascertained that the site of *leu-140* is located in cistron II and that of *leu-130* in cistron III. Further confirmation was provided by the evidence (mentioned previously) that the site of *leu-130* is in the region of the chromosome involved in the multisite aberrations of *leu-39* and *leu-485*, whereas that of *leu-140* is not. The diagrammatic representation of these relationships can be seen in Figure 2.

Despite the difficulties just described, the three-point transduction test allows a remarkably consistent linear ordering of most mutant sites. Figure 2 presents a map of the order of the sites of mutation in the leucine operon of a number of leucine auxotrophs which have been studied. The map is based upon tests such as those described above, and is not intended to indicate anything about relative distances between sites. The auxotrophs, *leu-39*, *leu-485*, and *leu-447* are indicated as resulting from multisite mutations extending over lengths of the leucine operon determined by the previously described tests. Spontaneous mutants *leu-130*, *leu-127*, and *leu-20* arose independently but appear to be identical mutations of the same site. This interpretation was initially suggested because they produced no *leu*$^+$ recombinants in all combinations of reciprocal transductions between them. It was further supported by the evidence that all three resulted from the same type of purine-pyrimidine base pair transition and that all three respond to approximately the same extent to several different mutagenic agents (MARGOLIN and MUKAI 1961). This is similar to cases reported by BALBINDER (1962a) for tryptophan auxotrophs in Salmonella. In comparison to the "hot spots" de-

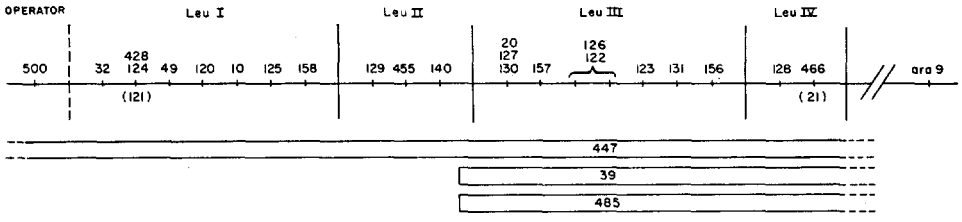

FIGURE 2.—The linear order of leucine mutant sites and relative positions of cistrons, as determined by recombination and complementation tests. Mutants which share the same site and appear to be identical are listed above that site. Those which appear to share a site but show different reversion patterns are listed below the line in parentheses. Mutant sites whose precise right-left orientation with respect to each other cannot be determined are enclosed in a brace. The large multisite mutants are shown below the single site map with the extent of their lesions indicated. Dotted lines at the ends indicate that the precise endpoints of the genetic lesions cannot be determined.

scribed by BENZER (1957) for phage, this site in the leucine operon might be referred to as a "warm spot."

The spontaneous auxotroph, *leu-124*, has never been found to revert in platings of over 10^{12} cells. It produces no *leu*$^+$ recombinants in reciprocal transductions with the X-ray induced, non-reverting mutant, *leu-428*. On the other hand, the spontaneous mutant, *leu-121*, which does not produce *leu*$^+$ recombinants from transductions with either *leu-124* or *leu-428*, does revert spontaneously. Rather tentatively *leu-124* and *leu-428* are being considered as resulting from small deletions of approximately the same portion of cistron I, which also includes the site of *leu-121*.

DISCUSSION

The dissection, by transduction, of the region of the Salmonella chromosome controlling leucine biosynthesis has led to a classical picture of an operon. Even the position of the operator adjoining cistron I is consistent with the model of JACOB and MONOD (1961a,b). It should be noted, however, that in one recent study (MORSE 1962) concerned with the genes involved in galactose utilization, there is evidence that the operator may not be in a terminal position in the operon.

The position of cistron I, controlling the first enzyme in the pathway, next to cistron II which controls the last enzyme, may be of some general significance. AMES and HARTMAN (1962) have noted a similar juxtaposition of the genes controlling the first and last enzymes in the histidine pathway.

DAWSON and SMITH-KEARY (1960) have reported a suppressor region (*su-leuA*) at the left end of cistron I. Mutations in this *su-leuA* region suppress the leucine auxotrophy of a specific cistron I mutant. The relative positions of this suppressor region and of the operator region are at present unknown. It seems unlikely that the suppressors reported by DAWSON and SMITH-KEARY are in the operator region. One of them is apparently due to a long multisite mutation, and all of them, when isolated from the leucine auxotroph which they suppress, do not by themselves cause any leucine auxotrophy.

Thus far, the mapping by three-point transduction tests has remained remarkably consistent with the complementation grouping. Each cistron consists of a linearly homogeneous group of mutant sites. The map developed by these two tests has been at least partially verified by the transductions involving the multisite mutants *leu-39* and *leu-485*. Their lesions cover a group of mutant sites which had been independently grouped together at the right-hand end of the map by the three-point tests.

The types of mutations occurring in the leucine operon are in general similar to those described by others for other regions of the Salmonella chromosome (see DEMEREC and HARTMAN 1959). Large multisite mutations involving the leucine operon are quite rare. Of the three which have been demonstrated, two are spontaneous in origin and appear to have approximately identical sites of termination of their left ends. This is reminiscent of a high frequency of almost identical large multisite mutants involving the *cysC* region of the Salmonella chromosome, which were reported by DEMEREC, LAHR, BALBINDER, MIYAKE, ISHIDSU,

MIZOBUCHI and MAHLER (1960). Furthermore, two large multisite mutants of the histidine operon have also been reported to have an identical site of termination (AMES and HARTMAN 1962). All this suggests that multisite mutations do not occur randomly over the entire chromosome. Certain regions are more likely than others to become involved in what are probably chromosomal aberrations —perhaps deletions or inversions. It seems likely that the nonrandom multisite aberrations are initiated by some abnormal occurrence at their terminal points. This further suggests that the portions of the chromosome containing these terminal points may differ in some way from the rest of the chromosome.

In the general picture presented by the array of mutations in the four structural genes, two characteristics are noticeable. The first is that cistrons I and III show a significantly higher frequency of mutation to leucine auxotrophy than cistrons II and IV. At present it is not possible to decide whether this difference represents a difference in the sizes of the cistron or in their stability with respect to mutation. The second evident characteristic is that the different cistrons respond to various mutagenic agents differently. This may be at least partially due to differences in their composition with respect to adenine-thymine:guanine-cytosine ratios. Evidence for such effects in the leucine genes have been previously reported (MARGOLIN and MUKAI 1961).

SUMMARY

A total of 156 leucine auxotrophs of *Salmonella typhimurium* were found by complementation tests to result from mutations in an operon consisting of an operator region and four closely linked structural genes. These control the three enzymes in the leucine biosynthetic pathway. Three-point transduction tests produced a recombination map of mutant sites which was consistent with the results obtained by complementation. Some evidence suggested that the locations of spontaneous multisite mutations do not occur randomly, and that repeated occurrences of such aberrations at the same place may result in an inversion on one occasion and a deletion on another occasion. Possible reasons for differences in spontaneous and induced mutation frequencies among the four structural genes are noted.

ACKNOWLEDGMENT

The author wishes to express his gratitude for the expert technical assistance of MISS DORIS A. BLUME and MRS. BERNADINE P. MILLER.

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