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Physical and Genetic Localization of *ilv* Regulatory Sites in λ *ilv* Bacteriophages

JOHN E. GRAY,[1] D. CLARK BENNETT,[2] H. E. UMBARGER,[2] AND DAVID H. CALHOUN[1]*

Department of Microbiology, Mount Sinai School of Medicine, New York, New York 10029,[1] and Purdue University Biochemistry Program and Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907[2]

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A set of nine λ d*ilv* phages were used to transduce bacterial recipients containing point mutations or deletions in the *ilv* genes located at 84 min on the *Escherichia coli* K-12 chromosome. This genetic analysis indicated that two phages carry the entire *ilvGEDAC* cluster; others carry the complete *ilvC* gene and, in addition, bacterial DNA that extends to a termination point between *ilvA* and *ilvC*, within *ilvD*, within *ilvE*, or within *ilvG*. DNA extracted from the λ d*ilv* phages was digested with *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Sal*I, and *Sma*I. The restriction maps revealed that these phages were generated after insertion at four distinct insertion sites downstream (clockwise) of *ilvC*. The physical relationships between the various phages were further examined by electron microscopic heteroduplex analysis. The physical maps of the phages thus generated were straightforward and in complete accord with the genetic data. No evidence for genetic rearrangements of *ilv* DNA in the phage was obtained, thus validating conclusions based on the use of these phages in previous and ongoing research projects. Bacterial cells with deletions of the *ilv* genes were made lysogenic with λ d*ilv* phage to examine the regulation of *ilv* genes present in the phage. The results confirm previous studies showing that one site for control by repression and derepression is upstream (counterclockwise) of *ilvG*. It was shown, in addition, that the activities of dihydroxy acid dehydrase and threonine deaminase were increased when the prototrophic lysogens were grown with 20 mM leucine. Since this increase was exhibited even when the *ilvG*-linked control region was not carried by the λ d*ilv* phage, additional control sites must be located within the *ilvEDA* region of the *ilvGEDA* transcription unit.

In the past, specialized transducing phages have been valuable in the analysis of biochemical genetics in bacteria. The use of bacteriophage λ derivatives was originally restricted to a few *Escherichia coli* K-12 genes, but more recently methods have been devised that permit the detection of phage λ derivatives formed in vivo that carry a wide range of bacterial genes (26, 27). A set of λ d*ilv* phages have been isolated carrying overlapping segments of the *ilv* genes at 84 min. These phages were previously used in genetic and biochemical analyses to determine the order of *ilv* structural and regulatory genes on the *E. coli* K-12 genome (2, 23). However, the validity of these conclusions is open to question, since rearrangements of genes in specialized transducing phages have been reported (21). Also, since only a limited number of bacterial recipients with mutations in the same gene have been tested, it has not been possible in many instances to differentiate between the presence of all or part of an *ilv* gene or control element in the λ d*ilv* phage.

In the studies reported here, we obtain detailed restriction maps, examine critical DNA heteroduplex structures, and test genetically for the endpoint termini within *ilv* genes, particularly for the first structural gene, *ilvG*, and its contiguous control site, for the λ d*ilv* phage. These studies clearly establish the physical structure of these phages, demonstrate that no rearrangements have occurred in *ilv* DNA (7, 20, 21) present in the phage, and reveal their mode of formation by aberrant excision. This knowledge of the precise context of *ilv* structural genes present in the phage prompted an investigation of the presence of genetic control sites. We confirmed previous studies (4, 5, 16, 17, 19, 31) indicating that at least one control site precedes the first structural gene, *ilvG*. We find clear evidence for regulation in at least one internal site as well, and use the phage to identify its location.

While this manuscript was in preparation, the DNA sequence of the *ilvG* gene was completed, and the nature of the *ilvO* site was elucidated

(16). Although *ilvO* has many properties of an operator gene (mutations are *cis*-dominant and elevate expression of the linked *ilvEDA* structural genes), and this originally led to its designation (discussed in reference 31), it has now been established that *ilvO* is a 10-base-pair site within *ilvG* (the first structural gene of the *ilvGEDA* transcription unit). Mutations in *ilvO* (actually *ilvG* mutations) are 1-base-pair deletions or 2-base-pair insertions (16, 16a) that restore the translational reading frame of the entire *ilvG* gene. The wild-type *E. coli* K-12 carries a naturally occurring frameshift site within the *ilvG* gene (16). The product of the mutant, but not the wild-type, *ilvG* gene is a catalytically active α-acetohydroxy acid synthase II isozyme that is resistant to inhibition of catalytic activity by isoleucine (13, 31). Thus, the mutant, but not the wild type, is Val^r (resistant to growth inhibition by valine). We feel that it is preferable, in agreement with a previous suggestion (16), to discontinue the use of the misleading *ilvO* mnemonic, although any terminology is uncomfortable for such a circumstance, since ordinarily the wild type is genetically and phenotypically proficient and the mutant is deficient. Nevertheless, the wild type is designated $ilvG^+$ (phenotype, $IlvG^-$ Val^s; formerly $ilvO^+$), and the mutant is designated *ilvG* (phenotype, $IlvG^+$ Val^r; formerly *ilvO*).

It should be emphasized that DNA sequence analysis is an ancillary technique that does not replace conventional genetic analysis of phenotypic determinants. The correlation of *ilv* phenotypes with specific physical DNA segments is the topic of this paper. The experiments reported here provide the essential documentation for conclusions as to the effect of observed DNA sequence changes (16, 16a) on the corresponding *ilv* phenotypes.

MATERIALS AND METHODS

Bacteria and bacteriophages. The bacterial and phage strains used are listed in Table 1. The methods used for the isolation of the λ d*ilv* phages and their initial characterization have been described (2, 23).

Transduction. Techniques used for specialized transduction with the λ d*ilv* phages have been described (2, 23). In all cases, lysogenization by the transducing phages was prevented by selecting recombinants growing at 42°C. Since unusual cross-feeding patterns between pairs of isoleucine and valine auxotrophic mutants have been observed (23), the results reported have been confirmed by cloning and retesting of several transductants from each cross, including both valine-susceptible and valine-resistant types, on nonselective media (presence of leucine, isoleucine, and valine).

Enzyme assays. The colorimetric assays for threonine deaminase and dihydroxy acid dehydrase were as previously described (14, 29). Since the *cI857* allele is present in the helper and defective phage, the cells were grown at 30°C to prevent prophage induction. For this reason, and because of the location and transcriptional orientation of the *ilv* genes in the phage, read-through from phage promoters to *ilv* genes is blocked. Where indicated, the dehydrase was assayed with α,β-dihydroxy-β-methylvalerate instead of α,β-dihydroxyisovalerate. Transaminase B was assayed as described by Duggan and Wechsler (10). Whether multivalent repression was exhibited in prototrophic, valine-susceptible strains was determined by comparing the enzyme levels in cells grown in the presence and absence of 0.6 mM leucine, 0.6 mM isoleucine, and 1.2 mM valine. The repressing medium for the leucine auxotrophic, valine-susceptible lysogens was prepared by supplementing the minimal medium of Davis and Mingioli (9) with 0.4 mM leucine, 0.4 mM isoleucine, and 0.8 mM valine. For limiting isoleucine or limiting leucine, the concentration of isoleucine or leucine, respectively, was reduced to 0.02 mM. In addition, enzyme activities in the prototrophic strains were also examined in cells grown in minimal medium supplemented with 20 mM leucine.

Preparation of phage and extraction of DNA. The methods for growth of heat-inducible, lysis-defective phages and the extraction of DNA have been described (32). λ d*ilv58*, λ d*ilv37*, λ d*ilv26*, and λ d*ilv22* were separated from the helper phage by isopycnic centrifugation in cesium chloride. λ d*ilv62* and λ d*ilv73* failed to separate from the helper during centrifugation and were used without separation.

Restriction endonuclease analysis. The DNA prepared from the phages was digested with *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Sal*I, and *Sma*I, obtained from New England BioLabs. The cleaved fragments were separated by electrophoresis in horizontal agarose gels as described previously (19).

Heteroduplex analysis. The procedures for forming heteroduplexes and analyzing them by electron microscopic examination have been described (19).

RESULTS

Genetic analysis of the λ d*ilv* phages. The presence of *ilv* genes in the λ d*ilv* phages has previously been examined in preliminary genetic experiments by using only a few representative recipient strains defective in specific structural and regulatory genes (2, 23). The presence of a complete and functional copy of the *ilvA*, *ilvD*, and *ilvE* genes was previously determined by measuring increased enzyme levels after thermal induction of appropriate λ d*ilv* lysogens (2). The analysis in Tables 2 and 3 includes strains with additional mutant alleles that permit a genetic determination of the termination point of bacterial DNA present in the λ d*ilv* phage within *ilvD* for λ d*ilv22*, within *ilvE* for λ d*ilv62* and λ d*ilv73*, and within *ilvG* for λ d*ilv26*, λ d*ilv37*, and λ d*ilv43*. Phage λ d*ilv29* failed to generate prototrophic transductants with any *ilvA* auxotrophs tested, including one bearing the *ilvA601* lesion (data not shown), which is the most *ilvC* proximal of the known *ilvA* mutations. Thus, *ilv* DNA carried by λ d*ilv29* probably extends only to a point between *ilvA* and *ilvC*. Phages λ d*ilv58* and

TABLE 1. Bacterial and bacteriophage strains used

Strain	Genotype	Source or reference
Bacterial[a]		
AB3590	F⁻ Δ(*ilvDAC*)*115 thi mtl malA rpsL his trpC tsx lacZ* λ⁻	B. Bachmann, *E. coli* Genetic Stock Center, Yale University School of Medicine
CU4	F⁻ *galT12* λ⁻	Pledger and Umbarger (20)
CU449	F⁺ *ilvC462 rbs-224*	Spontaneous Rbs⁻ derivative of CU1010 isolated by E. L. Kline
CU504	F⁻ *rbs-221 leu-455 galT12* λ⁻	Smith et al. (28)
CU505	F⁻ *ilv2049 leu-455 galT12* λ⁻	Watson et al. (33)
CU519	F⁻ Δ(*ilvDAC115*) *metE201 leu-455 galT12* λ⁻	Smith et al. (28)
CU595	F⁻ *ilvG468 ilvA2058 rbs-215*	Smith et al. (28)
CU655	F⁻ *ilv2076 rbs-221 leu-455 galT12* λ⁻	Smith et al. (28)
CU692	F⁻ *ilvG2095 ilvE2104 leu455 rbs-221 galT12* λ⁻	Smith et al. (28)
CU693	F⁻ *ilvG2096 ilvE2105 leu-455 rbs-221 galT12* λ⁻	Smith et al. (28)
CU853	F⁻ *ilvG2113 ilvG2096 rbs-221 leu-455 galT12* λ⁻	Smith et al. (28)
CU856	F⁻ *ilvG2111 ilvG2095 rbs-221 leu-455 galT12* λ⁻	Smith et al. (28)
CU859	F⁻ *ilvG2095 ilvG2111 leu-455 rbs-221 galT12* λ⁻	Smith et al. (28)
CU860	F⁻ *ilvG2096 ilvG2116 leu-455 rbs-221 galT12* λ⁻	Smith et al. (28)
CU1010	*ilvC462*	Smith et al. (28)
MSR168	F⁻ Δ(*ilvDAC*)*115 thi mtl malA rpsL his trpC tsx lacZ* λ d*ilvGEDAC58*	Lysogenization of AB3590
MSR169	F⁻ Δ(*ilvDAC*)*115 thi mtl mal rpsL his trpC tsx lacZ* λ d*ilv'GEDAC26*	Lysogenization of AB3590
MSR170	F⁻ Δ(*ilvDAC*)*115 thi mtl mal rpsL his trpC tsx lacZ* λ d*ilv'EDAC75*	Lysogenization of AB3590
MSR114	F⁻ Δ(*ilvDAC*)*115 thi mtl mal rbsL his trpC tsx lacZ* λ d*ilv'GEDAC37*	Lysogenization of AB3590
Phage		
λ d*ilv22*	Δ(*T-attP*) [*ilv'DAYC-rho*] *c*I857 *S*am7	Baez et al. (2)
λ d*ilv26*	Δ(*T-attP*) [*ilv'GEDAYC-rho'*] *c*I857 *S*am7	Baez et al. (2)
λ d*ilv37*	Δ(*FI-attP*) [*ilv'GEDAYC-rho*] *c*I857 *S*am7	Baez et al. (2)
λ d*ilv58*	Δ(*Z-attP*) [*rrfC-ilvGEDAYC*] *c*I857 *S*am7	Baez et al. (2)
λ d*ilv62*	Δ(*A-attP*) [ilvDAYC-rho] *c*I857 *S*am7	Baez et al. (2)
λ d*ilv73*	Δ (*A-attP*) [*ilvDAYC-rho*] *c*I857 *S*am7	Baez et al. (2)

[a] The *ilvG468*, *-2095* and *-2096* alleles are activating *ilvG* mutations that confer the IlvG⁺ and Val^r phenotype (previously *ilvO*); the *ilvG2111*, *-2112* and *-2113* alleles inactivate the catalytic activity of the activated *ilvG* gene product. See text for discussion.

λ d*ilv42* transduce all *ilv* auxotrophs tested and presumably carry the entire *ilv* cluster (Fig. 1).

The rationale for determining the presence of *ilvE*, *ilvD*, *ilvA*, and *ilvC* structural genes from the experiment described in Table 2 is straightforward. Since selection was made at 42°C, the prototrophs selected in each case were the result of recombination between host- and phage-carried genes. Thus, the formation of prototrophic transductants at frequencies in excess of reversion frequencies is evidence for the presence of wild-type DNA in the λ d*ilv* phage corresponding to the site of the altered DNA in the recipient genomes. The rationale for determining the presence of *ilvG* in the λ d*ilv* phage depends upon the Val^r or Val^s phenotype as a selected (Val^r) or unselected (Val^r or Val^s) marker. The Val^r phenotype is dependent on *ilvG* activator mutations (e.g., *ilvG264*, *ilvG671*, *ilvG468*, *ilvG2095*, and *ilvG2096*; previously called *ilvO* mutations) that lead to the synthesis of a catalytically active α-acetohydroxy acid synthase II (16). Additional mutations in *ilvG* (e.g., *ilvG2111*, *ilvG2113*, *ilvG605*, and *ilvG2112*) abolish α-acetohydroxy acid synthase II activity (present in strains with an activated *ilvG*), presumably due to second-site mutations in essential coding regions of *ilvG*. The Val^r transductants of strains CU853 and CU856 (Table 2) resulted, therefore, from repair of the inactivating *ilvG2113* and *ilvG2111* lesions, but retention of the respective activating mutations *ilvG2096* and *ilvG2095*. Note that the *ilvG2111* allele was mapped both by the direct selection procedure (Table 2, strain CU856) and without selection (Table 3, strain CU859); in each case, phages λ d*ilv58* and λ d*ilv42* were found to carry *ilvG2111*$^+$, but λ d*ilv37*, λ d*ilv43*, and λ d*ilv26* did not. Whereas the Val^r pheno-

TABLE 2. Termination point of bacterial DNA carried by λ d*ilv* phages

Bacterial recipient	Selection[a]	Phage donor and *ilv* genes present in phage[b]								
		λ d*ilv42* (*GEDAC*)	λ d*ilv58* (*GEDAC*)	λ d*ilv37* (*'GEDAC*)	λ d*ilv43* (*'GEDAC*)	λ d*ilv26* (*'GEDAC*)	λ d*ilv73* (*'EDAC*)	λ d*ilv62* (*'EDAC*)	λ d*ilv22* (*'EDAC*)	λ d*ilv29* (*C*)
CU449 (*ilvC462*)	*ilvC462*$^+$	5.0×10^6	8.9×10^5	1.6×10^6	3.5×10^6	2.4×10^6	1.8×10^6	8.2×10^5	2.0×15^5	6.3×10^5
CU595 (*ilvG468 ilvA2058*)	*ilvA2058*$^+$	NT[c]	3.6×10^3	3.5×10^3	NT	1.8×10^4	1.9×10^4	3.0×10^4	5.5×10^1	0
CU655 [Δ(*ilvDA*)*2076*]	Δ*ilvDA2076*$^+$	NT	3.0×10^3	2.0×10^3	NT	2.0×10^3	1.0×10^3	4.0×10^2	4.9×10^1	0
CU692 [*ilvG2095* Δ(*ilvED*)*2104*)	Δ*ilvED2104*$^+$	1.0×10^3	4.0×10^2	1.3×10^3	6.9×10^2	2.6×10^2	8.2×10^1	1.8×10^1	0	0
CU609 (*ilvG468 ilvE2061*)	*ilvE2061*$^+$	NT	7.6×10^2	4.5×10^2	NT	2.6×10^3	0	0	0	0
CU693 (*ilvG2096 ilvE2105*)	*ilvE2105*$^+$	2.8×10^3	5.1×10^3	3.2×10^3	2.2×10^3	4.1×10^3	0	0	0	0
CU859 (*ilvG2095 ilvE2109 ilvG2111*)	*ilvE2109*$^+$	1.8×10^4	1.7×10^2	7.8×10^3	5.8×10^4	5.8×10^3	0	0	0	0
CU860 (*ilvG2096 ilvE2110 ilvG2112*)	*ilvE2110*$^+$	4.6×10^4	1.0×10^4	3.6×10^3	3.8×10^4	8.0×10^3	0	0	0	0
CU853 (*ilvG2096 ilvG2113*)	*ilvG2113*$^+$	3.0×10^1	6.5×10^1	2.2×10^1	0	0	0	0	0	0
CU856 (*ilvG2095 ilvG2111*)	*ilvG2111*$^+$	1.2×10^3	1.3×10^2	0	0	0	0	0	0	0

[a] The wild-type markers for genes *C*, *A*, *D*, and *E* were selected by growth in the absence of isoleucine and valine. Selection of the wild-type *ilvG2113*$^+$ and *ilvG2111*$^+$ alleles was made on medium containing valine but no isoleucine. Alleles *ilvG468*, *-2095*, and *-2096* are IlvG$^+$ Val^r, whereas *ilvG2111*, *-2112*, and *-2113* are IlvG$^-$ Val^s. Combinations of the two types are IlvG$^-$ Val^s, and the wild-type *ilvG*$^+$ is IlvG Val^s.

[b] The numbers record the recombinants receiving the selected marker per 0.1 ml of transduction mixture.

[c] NT, Not tested.

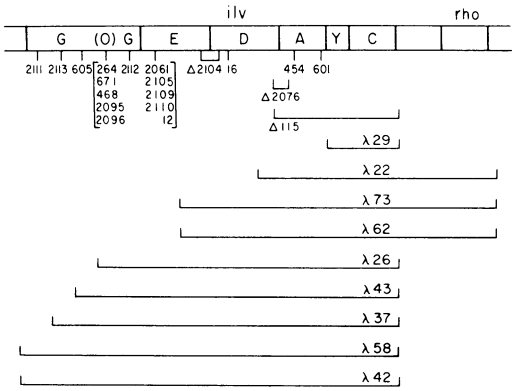

FIG. 1. Genetic map of *ilv* DNA carried by λ *dilv* phages. The genes in the *ilv* cluster are arranged as they occur in the *E. coli* chromosome, with the clockwise direction on the chromosome map as usually represented reading from left to right. The gene arrangement is not drawn to scale. The bars below represent the genetic material carried by the indicated phages. The allele numbers enclosed in brackets (e.g., 264, 2112, 2061, etc.) were not ordered in this analysis. (O) indicates the location of the frameshift site in the *ilvG* gene.

type can be selected directly, the Val^s phenotype must be identified as an unselected marker. The identification of *ilvG2095*$^+$ and *ilvG2096*$^+$ derivatives of strains CU692 and CU693, respectively (Table 3), was based on the presence of the unselected Val^s phenotype among *ilvE*$^+$ prototrophic transductants. The Val^r recombination would thus have resulted from a crossover event between the *ilvE* auxotrophic marker and the respective *ilvG* lesion.

The order inferred from these and other crosses with the λ d*ilv* phages for these *ilv* loci is *G2111-G2113-G605-(G2112, G264, G671, G468, G2095, G2096, E2061, E2105, E2109, E2110, E12)-ED2104*. The mutant alleles within parentheses have not been ordered with respect to each other by the λ d*ilv* mapping procedure, although four of the (*G671, G468, G2095*, and *G2096*) have been ordered by DNA sequence analysis (16a). Evidence from three-factor crosses presented earlier (28) indicated the order *ilvG2096-ilvG2112-ilvE*; this genetic order is consistent with the results presented here, since all phages tested carry either both or neither of the *ilvG2096*$^+$ and *ilvG2112*$^+$ alleles. The previous interpretation (28) of these three-factor crosses with either that *ilvG2096* was unusual in its location or that the *ilvG*-activating and -inactivating markers were interspersed. The results of the crosses described here support the latter possibility and would indicate that *ilvG2112* (inactivating) is downstream (i.e., on the *ilvE* side) of the *ilvG* activation site, since it was the only one of the three *ilvG* inactivating markers examined that λ d*ilv43* and λ d*ilv26* could repair.

TABLE 3. Detection of *ilvG*$^+$ in λ d*ilv* phage as unselected markers in *ilvE*$^+$ transductants[a]

Bacterial recipient	Unselected marker detected[b]	Phage donor and *ilv* genes present in phage						
		λ d*ilv42* (*GEDAC*)	λ d*ilv58* (*GEDAC*)	λ d*ilv37* (*'GEDAC*)	λ d*ilv43* (*'GEDAC*)	λ d*ilv26* (*'GEDAC*)	λ d*ilv73*[c] (*'EDAC*)	λ d*ilv62*[c] (*'EDAC*)
CU692 [*ilvG2095*, Δ(*ilvED*)*2104*]	*ilvG2095*$^+$	41/78 (53)	45/96 (47)	8/96 (8)	8/63 (13)	6/96 (6)	0/96 (0)	0/66 (0)
CU693 (*ilvG2096 ilvE2105*)	*ilvG2096*$^+$	45/145 (31)	44/95 (46)	24/141 (17)	19/141 (13)	8/137 (6)		
CU859 (*ilvG2095 ilvE2109 ilvG2111*)	*ilvG2111*$^+$	85/98 (87)	221/235 (94)	196/196 (100)	100/100 (100)	190/190 (100)		
CU860 (*ilvG2096 ilvE2110 ilvG2112*)	*ilvG2112*$^+$	92/98 (94)	126/146 (86)	132/146 (90)	85/100 (85)	115/146 (79)		

[a] Prototrophic transductants (Table 2) were tested for their Val^s phenotypes. The ratio given for each represents (number of Val^s transductants)/(number of transductants tested). For each cross, representative transductants (Val^r and Val^r) were streaked for single-colony isolation on nonselective media (leucine, isoleucine, and valine); the phenotype (Val^s or Val^r) was confirmed by transferring several well-isolated colonies to plates with and without valine. Numbers in parentheses are percentages.

[b] The presence of *ilvG*$^+$ alleles in the phage was detected by the formation of Val^s transductants for CU692 and CU693 and by the presence of Val^r transductants for CU859 and CU860. Alleles *ilvG2095* and *-2096* confer Val^r, and alleles *ilvG2111* and *-2112* reverse Val^r phenotypes. See text for discussion.

[c] No transductants are formed by phage λ d*ilv73* and λ d*ilv62* with strains CU693, CU859, and CU860 as recipients (Table 2).

Thus, the data in Table 2, obtained by this deletion mapping technique, and the data of Smith et al. (27), obtained by three-factor crosses with phage P1, are mutually reinforcing and provide convincing genetic data that the activating mutations in *ilvG* (previously designated *ilvO* mutations) lie within the coding part of the *ilvG* structural gene. This conclusion has subsequently been confirmed by direct DNA sequence analysis (16, 16a). From these genetic data (Tables 2 and 3), confirmed by the physical analysis (see below), it can be determined that the λ d*ilv* phages carry the following combinations: *GEDAC* (λ d*ilv42*, *-58*), *'GEDAC* (λ d*ilv37*, *-43*, *-26*) *'EDAC* (λ d*ilv62*, *-73*), *'DAC* (λ d*ilv22*), and *C* (λ d*ilv29*).

Evidence interpreted to indicate the presence of several *ilv* promoters (P1, P2, and P3) has been reported (order, $_1G_2E_3DA$) from the analysis of strains with polar mutations or insertions in *ilvGEDA* (3–5, 29). A consensus promoter DNA sequence is located between *ilvG* and *ilvE* (16, 18). Since these λ d*ilv* prophages express the *ilv* genes only from *ilv* promoters (phage λ repressor inhibits transcription from phage promoters), the physical location and potential regulation from these promoters in the λ d*ilv* phage can be directly assessed in Δ*ilv* hosts (see below). In this regard, it should be noted that similar analyses from *ilv* segments cloned into many plasmids (e.g., pBR322) are often expressed by read-through from plasmid promoters. Also, interpretation of results from insertion elements (3–5, 29) can be complicated by (i) promoters present in the insertion elements, (ii) undetected transposition to other nearby sites, and (iii) ambiguous localization of these elements within structural or regulatory sites, in contrast to the physically defined termini in the λ d*ilv* phage.

Physical mapping of the λ d*ilv* phages. The λ d*ilv* phages were examined by restriction enzyme cleavage, using *Eco*RI, *Hin*dIII, *Sal*I, *Sma*I, *Pst*I, and *Kpn*I, and by electron microscopic analysis of heteroduplexes to give the results depicted in Fig. 2. The restriction enzyme analysis was simplified, since some restriction enzyme data have been published for the *ilvGEDAYC* genes present in λ h80 d*ilv* and the Clark and Carbon plasmids, including about 2.5 kilobases upstream or on the counterclockwise side (as the chromosomal map is usually represented) of *ilvG* and less than 0.10 kilobases downstream of *ilvC* (4, 9, 13, 16, 25). In addition, a phage (λ d*ilv5*) that carries *rrnC* (or *rrnX*) (21) and *ilv* has previously been studied, but with a primary focus on the rRNA and tRNA genes. The DNA downstream of *ilvC* has not previously been studied by restriction enzyme analysis. The *rho-115*$^+$ allele is carried by phages λ d*ilv22*, λ d*ilv62*, and λd*ilv73* (J. E. Gray, S. K. Guterman, and D. H. Calhoun, unpublished data). The rifampin hypersusceptibility of a chromosomal *rho-115* mutation is suppressed by a pBR322 derivative that carries the 6.6-kilobase *Hin*dIII fragment downstream of *ilvC* (Gray and Cal-

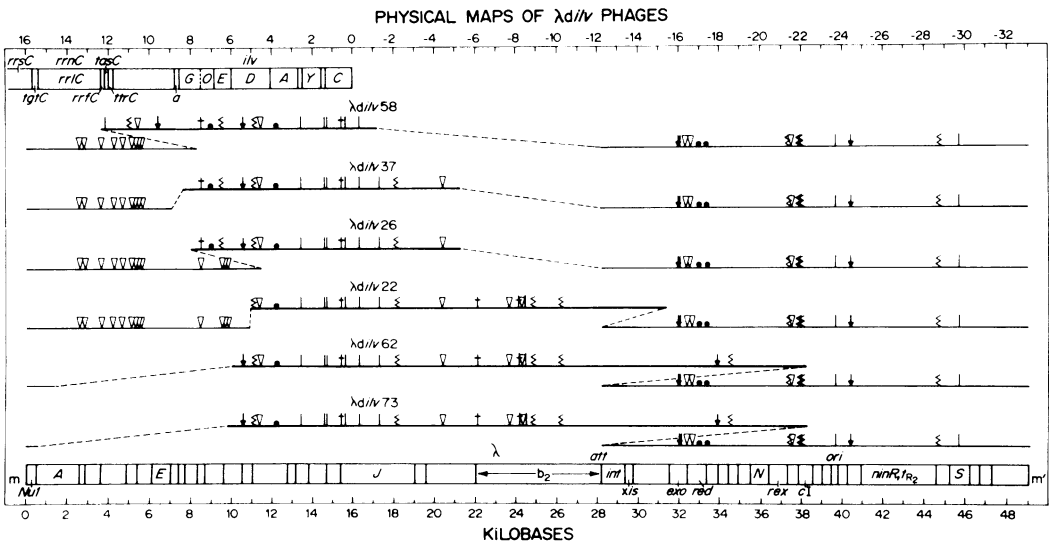

FIG. 2. Physical map of the DNA in the region of the *ilv* gene cluster carried on the *E. coli* chromosome and on several λ d*ilv* phages. The arbitrary kilobase coordinates for the chromosome are based on a zero point at the approximate beginning of the *ilvC* gene. The λ kilobase coordinates are based on the λ vegetative map. The "O" site indicates the presence of a naturally occurring frameshift site in the wild-type *ilvG* gene.
Symbols: |, *Eco*RI; ≷, *Hin*dIII; +, *Kpn*I; ▽, *Pst*I; ., *Sal*I; ↓, *Sma*I.

houn, unpublished data). Since none of the phages carries *cya*, these results support the order *ilv-rho-cya* reported by Das et al. (8), rather than *ilv-cya-rho* (1).

The structures of the λ d*ilv* phages as revealed by restriction enzyme analysis were supplemented and refined by examination of several heteroduplexes between the complementary strands of the parental phages and λ d*ilv58*, λ d*ilv37*, λ d*ilv26*, λ d*ilv62*, and λ d*ilv73*.

In addition, heteroduplexes between λ d*ilv58* and both λ d*ilv37* and λ d*ilv26*, between λ d*ilv37* and λ d*ilv26*, and between λ d*ilv62* and λ d*ilv73* were examined. These combinations were sufficient to verify that they resulted from excision of *ilv* DNA by prophages inserted at three different secondary λ attachment sites and that each had unique left-arm λ *ilv* DNA junctions. The precise junctions of λ d*ilv37* and λ d*ilv26* were important, since the genetic data of Table 2 indicated that λ d*ilv37* carried more *ilv* DNA than did λ d*ilv26*. This conclusion was verified by the heteroduplex analysis between the two (Fig. 3a), which revealed a bubble structure in which the larger bubble arm was formed by the extra left-arm λ DNA carried by λ d*ilv26* and the much smaller bubble arm was formed by the extra *ilv* DNA (about 300 bases) carried by λ d*ilv37*. On the other hand, neither the genetic tests nor the heteroduplexes permitted an unequivocal comparison of the *ilv* termini in λ d*ilv62* and λ d*ilv73*, since only a single-stranded loop was formed (Fig. 3b). This loop was made up primarily of left-arm λ DNA carried by λ d*ilv62* but missing from λ d*ilv73*. It might also have included some *ilv* DNA not present in λ d*ilv73*. It can thus be concluded only that, if λ d*ilv73* contains any *ilv* DNA not present in λ d*ilv62*, it is a length below the limits of resolution of the electron microscopic technique used.

A heteroduplex between λ d*ilv22* and the parental phage was not examined, since the restriction enzyme analysis allowed a clear demonstration that it was derived from a prophage inserted at a fourth secondary λ attachment site. Furthermore, the cleavage fragments were such that the junction between the left arm of λ and the *ilv* DNA could be as precisely defined as they could have been by heteroduplex mapping.

The genetic analysis (Tables 2 and 3) and the physical characterization (Fig. 2) permit a correlation of genes and fragments of genes with restriction enzyme sites. This correlation confirms and extends the analyses based on cloning fragments from λ h80 d*ilv* (7, 20).

Expression of *ilv* genes in the λ d*ilv* phages. The λ d*ilv* phages carry the *ilvGEDA* genes in overlapping segments, including *GEDA*, *'GEDA*,

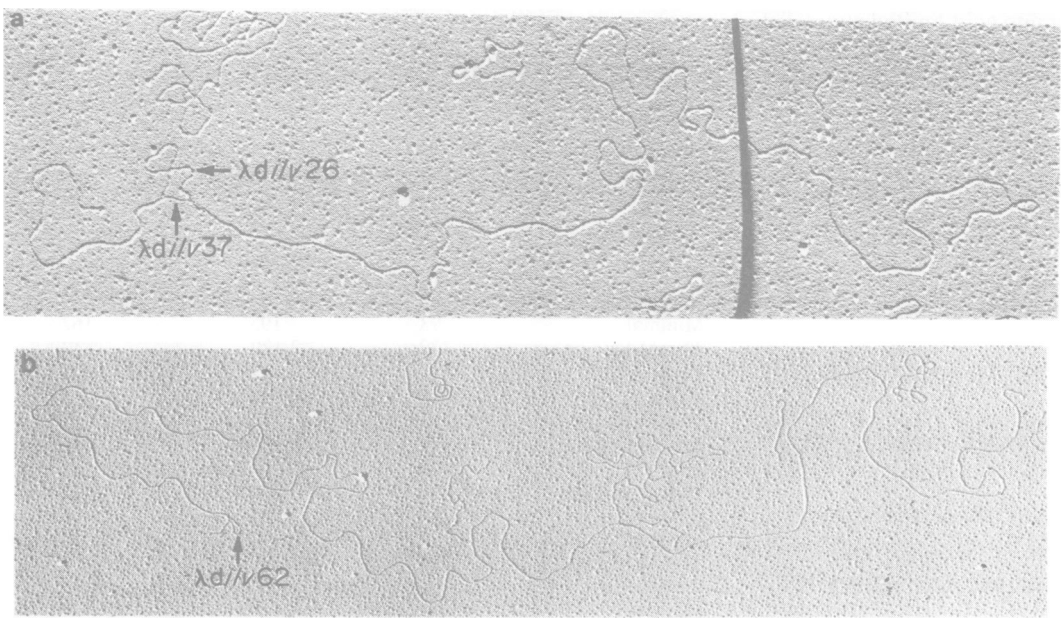

FIG. 3. (a) Heteroduplex prepared between DNA strands of λ d*ilv26* and λ d*ilv37*. The long single-stranded arm of the bubble structure is derived from left-arm λ DNA carried by λ d*ilv26*; the short single-stranded arm is derived from chromosomal DNA in the vicinity of the *ilvG* gene. (b) Heteroduplex prepared between DNA strands of λ d*ilv62* and λ d*ilv73*. The single-stranded DNA loop is derived from the left-arm λ DNA carried by λ d*ilv62* (but not by λ d*ilv73*). It could also be derived from any DNA in the vicinity of the *ilvE* gene carried by λ d*ilv62* but not by λ d*ilv73*. No *ilv* DNA in λ d*ilv73* can be detected that is not also carried by λ d*ilv62*.

'EDA, *'DA*, and *A*. Lysogens derived from strain AB3590[Δ(*ilvDAC*)*115*] were prepared to test for the presence of the *ilv*-specific regulation of *ilvD* and *ilvA* expression. The chromosomal *ilvGEDA* gene products are subject to repression in the presence of excess leucine, isoleucine, and valine. The synthesis of the *ilvD* gene product, dihydroxy acid dehydrase, and the *ilvA* gene product, threonine deaminase, were repressible (Table 4; compare growth with and without the branched-chain amino acid supplement) in lysogens containing λ d*ilv58* but not in lysogens containing λ d*ilv37*, λ d*ilv26*, or λ d*ilv73*. Thus, the site essential for multivalent repression control is present upstream of the *ilv* DNA carried by phages λ d*ilv26* and λ d*ilv37*; this site is presumably the attenuator identified by direct DNA sequence analysis (17, 22).

There have been several reports showing that prototrophic strains of *E. coli* growing in minimal medium supplemented with leucine exhibit an increased expression of the *ilv* genes due to an isoleucine limitation (13, 15, 23, 31). In preliminary experiments with strain CU4, it was observed that the maximal increase in *ilv* gene expression was obtained when the medium was supplemented with 20 mM leucine. This concentration also increased the generation time 1.5-fold (Table 4). The very high level of threonine deaminase activity exceeded the typical derepressed levels seen in regulatory mutants or in amino acid-limited auxotrophs by approximately 10-fold; it was approximately the same as that observed when an auxotrophic *ilvG468* (activator) mutant was limited for valine or isoleucine (28).

It is striking that the leucine-induced elevation in activity of the dehydrase was less than that of threonine deaminase. In this respect, the leucine effect was reminiscent of the downstream amplification observed when isoleucine is limited (29). It was of interest to determine whether this effect of *ilv* gene expression was dependent on the normal *ilv*-specific regulatory locus carried by strain CU4 and by λ d*ilv58* but missing from the other λ d*ilv* derivatives listed in Table 4. As the table shows, the effect of 20 mM leucine was exhibited not only by the *ilv* genes carried on the phage with the normal *ilvGEDA* control region but also by two of the three that had not retained that site. Only in λ d*ilv73*, in which the *ilv* DNA extended from a point downstream of *ilvC* only into, but not through, *ilvE*, did the addition of 20 mM leucine fail to elevate the level of threonine deaminase activity but, instead, was inhibitory to growth. It should be noted, however, in this strain that in minimal medium the expression of the *ilvD* and *ilvA* genes was very low, and growth of the strain was extremely slow. The question remains of whether the failure of the strain to grow was due to loss of some element between *ilvG* and *ilvE* that responds to excess leucine or to the inability of the low level of threonine deaminase to overcome the inhibitory effect of leucine on the enzyme. It is clear, however, that the response to 20 mM leucine is not dependent upon the presence of the *ilvGEDA* promoter-attenuator region.

TABLE 4. Expression of threonine deaminase (*ilvA*) and dihydroxy acid dehydrase (*ilvD*) specified by the genes carried by several λ d*ilv* prophages

Strain[a] [Δ(*ilvDAC*)*115*]	Growth medium	Generation time (min)	Sp act	
			Threonine deaminase	Dihydroxy acid dehydrase[b]
CU4$^+$(*ilv*$^+$ λ$^-$)	Repressing	90	123	99
	Minimal	93	195	162
	20 mM leucine	132	2,120	530
MSR170(λ d*ilv73*) [*'EDAC*][c]	Repressing	102	11	5
	Minimal	780	14	6
	20 mM leucine	NG[d]	NG	NG
MSR169(λ d*ilv26*) [*'GEDAC*]	Repressing	72	84	36
	Minimal	78	67	37
	20 mM leucine	138	260	98
MSR114(λ d*ilv37*) [*'GEDAC*]	Repressing	84	46	14
	Minimal	90	45	20
	20 mM leucine	156	221	78
MSR168(λ d*ilv58*) [*GEDAC*]	Repressing	90	61	18
	Minimal	84	126	99
	20 mM leucine	120	700	296

[a] Strains MSR170, MSR169, MSR168, and MSR114 contain the Δ(*ilvDAC*)*115* deletion and are lysogens of strain AB3590 containing the indicated prophage. Strain CU4 is an *ilv*$^+$ nonlysogenic control strain.

[b] The substrate used was α,β-dihydroxyisovalerate.

[c] *ilv* gene carried on the phage genome.

[d] NG, No growth.

Derepression of the *ilv* genes in the λd*ilv* phages by limiting isoleucine and by limiting leucine. The capacity of the *ilv* genes carried on the λ d*ilv* phages to respond to a limiting amino acid signal was examined in strains carrying the *leu-455* marker. In that way it was possible to examine the effects of limiting leucine and, since the strains were valine susceptible, of limiting isoleucine. The phage recipients carried deletions so that the enzyme activities measured were those specified by the *ilvE*, *-D*, and *-A* genes of the phages (or the *ilvD* and *-A* genes of λ d*ilv73*). Table 5 shows the activities observed in cells grown under the various conditions.

Strain CU505 lysogenized with λ d*ilv58* exhibited the same quantitative pattern of derepression as did the control strain, CU504, which carried the *ilv* gene cluster on the chromosome. The apparent derepression of threonine deaminase was much greater than that of transaminase B when isoleucine was limiting. This noncoordinate behavior of the *ilvGEDA* operon has been seen before and is thought to be unrelated to the *ilv*-specific transcriptional control exhibited over the operon (28). The same effect on the activity of threonine deaminase was observed in the strains lysogenized with λ d*ilv37* and λ d*ilv26*, neither of which exhibited derepression of threonine deaminase with leucine limiting or of transaminase B with either amino acid limiting. These phages lack the attenuator-promoter region of the *ilv* gene cluster. That an effect of limiting isoleucine is also exhibited by the *ilvA* gene carried on λ d*ilv73* indicates that the effect is mediated by the *ilvA* gene itself or by DNA that lies well after the weak promoter between *ilvG* and *ilvE* and which is probably functional in λ d*ilv26* and λ d*ilv37*.

DISCUSSION

The results reported here establish the genetic and physical structure of a set of λ d*ilv* phages that are being used to probe the nature of the controlling elements regulating isoleucine and valine biosynthesis. The restriction enzyme analysis of the bacterial DNA in the λ d*ilv* phages and the examination of several heteroduplexes has permitted a correlation of genes with DNA segments that is in accord with previously published data (7, 20, 21). The λ d*ilv* phages arose by insertion at four distinct sites downstream or to the right of *ilvC* (as the chromosome is represented in Fig. 2) and to the right of the fusion between chromosomal DNA and the left arm φ80 DNA of λ h80 d*ilv*. The existence of such a nonrandom cluster of secondary prophage insertion sites was also observed by Shimada et al. (26). Since the DNA of bacterial origin is inserted immediately to the left (as drawn in Fig. 2) of the phage *att* site, it is clear that they arose by the λ *gal* type of aberrant excision (26, 27). These phages have also made it possible to extend the physical analysis to DNA upstream of *ilvG* into the *rrnC* operon and downstream of *ilvC* beyond *rho*.

Examination of *ilvA* and *ilvD* gene expression in λ d*ilv* phages containing all or part of the *ilvG* and *ilvE* genes has confirmed that the site essential for multivalent repression by the branched-chain amino acids is upstream of *ilvG*. This site presumably includes, at least, *ilvG* proximal promoter and leader sequence with an attenua-

TABLE 5. Derepression of the *ilv* genes carried by several λ d*ilv* prophages

Strain and relevant genotype	Phage and *ilv* DNA carried	Medium	Sp act		
			Threonine deaminase	Dihydroxy acid dehydrase[a]	Transaminase B
CU504	None	Repressing	46	9	35[b]
leu-455 ilv$^+$		Limiting isoleucine	247	32	77
		Limiting leucine	97	32	88
CU505	λ d*ilv58*, *GEDAYC*	Repressing	101	12	44
Δ(*ilvGEDAYC*)*2049 leu-455*		Limiting isoleucine	429	35	73
		Limiting leucine	174	38	92
CU505	λ d*ilv37*, *'GEDAYC*	Repressing	105	8	44
Δ(*ilvGEAYC*)*2049 leu-455*		Limiting isoleucine	155	12	49
		Limiting leucine	116	14	58
CU505	λ d*ilv26*, *'GEDAYC*	Repressing	29	10	31
Δ(*ilvGEAYC*)*2049 leu-455*		Limiting isoleucine	51	15	40
		Limiting leucine	39	14	41
CU519	λ d*ilv73*, *'EDAYC*	Repressing	32	5	43
Δ(*ilvDAC*)*115 leu-455*		Limiting isoleucine	52	6	73
		Limiting leucine	25	8	89

[a] α,β-Dihydroxy-β-methylvalerate was the substrate.
[b] Values of enzyme activities derived from a chromosomal gene are underlined.

tion site that has been identified by nucleotide sequence analysis and by in vitro transcription experiments (17, 22). The control at this site is demonstrated by (i) repression by growth of prototrophic lysogens in the presence of the three branched-chain amino acids (Table 4) or (ii) derepression by growth of valine-susceptible leucine-auxotrophic lysogens in the presence of limiting isoleucine or limiting leucine (Table 5).

Two additional regulatory features of *ilv* gene expression have been exhibited by these phages that cannot yet be fully explained. One is the elevated activity of threonine deaminase that is observed in cells grown on limiting isoleucine even in the absence of the *cis*-acting *ilv*-specific attenuation control that occurs upstream of *ilvG*. The increased activity did not occur when the same lysogen as grown on limiting leucine and thus was not simply a consequence of the *ilv*-specific multivalent regulation. In the lysogen containing λ d*ilv58*, which contains the *ilv*-specific regulatory region, the unique effect of limiting isoleucine on threonine deaminase activity is superimposed on the derepression that occurs when either leucine or isoleucine is limiting and which is exhibited by all three of the enzymes examined. This isoleucine-specific effect has been reported before (29) and could be due either to a specific site of attenuation preceding the *ilvA* gene or to paucity of isoleucine residues in threonine deaminase relative to the occurrence of isoleucine in *E. coli* bulk protein. These possibilities are currently being tested by examining regulation from specifically constructed plasmids with *lacZ* substituted for *ilv* structural genes (Gray and Calhoun, unpublished data).

The second unexplained regulatory feature is also independent of the regulatory region upstream of *ilvG*. This effect was observed when the prototrophic strains were grown in the presence of excess leucine (Table 4). The effect may be related to the effect of limiting isoleucine described above and is similar to the downstream amplification described by Smith et al. (29) in that the increase of threonine deaminase activity is greater than that of the dehydrase. One possible explanation of the effect of these high levels of leucine is that the isoleucine pool has been reduced because of the previously reported inhibition of threonine deaminase by leucine (6, 32). If so, the apparent derepression would thus be the result of an indirect limitation of isoleucine. Compatible with this possibility is the fact that the high concentration of leucine reduced the growth rate of all the prototrophic strains examined, and the growth inhibition was completely reversed by isoleucine (2, 6, 25; Calhoun, unpublished data). Furthermore, the growth of the prototrophic lysogen carrying λ d*ilv73*, which grew only slowly in minimal medium and exhibited a low level of *ilvD* and *ilvA* expression, was completely inhibited by that amount of leucine. However, until the phenomenon is examined further, its basis must remain unexplained.

Finally, the combination of genetic and physical characterization reported here for λ d*ilv* phages provides the essential foundation for the interpretation of our nucleic acid sequence determinations (16, 16a), indicating specific base changes associated with *ilvG*-activating mutations (previously *ilvO*). Without such a specific and unambiguous foundation, we could not have realistically attempted the DNA sequence determination of *ilvG*-activating mutations, and their interpretation would be highly speculative. We view DNA sequencing as an adjunct to conventional genetic and biochemical analyses. DNA sequence immeasurably sharpens our interpretation of phenotype. At present, however, DNA sequence information alone does not adequately predict, and obviously cannot confirm, phenotype.

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ADDENDUM IN PROOF

After this manuscript was submitted for publication, the paper by M. Uzan, R. Favre, E. Gallay, and L. Caro (Mol. Gen. Genet. **182**:462–470, 1982, "1981") was published. Their structural analyses of the DNA present in these phage are in substantial agreement with ours except for phage λ d*ilv73*. Their preparation of this phage, but not ours, has undergone a gene rearrangement relative to the other λ d*ilv* phages.

The intercistronic region between *ilvG* and *ilvE* present on a *Kpn*I to *Hin*dIII segment contains an active and regulatable promoter as judged by its activity in plasmid pMC81 (Gray and Calhoun, Mid-Atlantic Extrachromosomal Elements Conference, 1981).

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