Biosynthesis of the Branched-Chain Amino Acids

H. E. UMBARGER

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INTRODUCTION

This chapter will focus primarily on the current status of our knowledge of branched-chain amino biosynthesis. Less attention will be given to the studies that led to this current view.

The notable features of the pathways include (i) the parallel steps leading to isoleucine and valine, each of which is catalyzed by an enzyme catalyzing both parallel reactions; (ii) the intramolecular rearrangements in these two pathways which result in a substitution at the β-carbon of an aliphatic amino acid yielding the branched chain; and (iii) the leucine biosynthetic branch from the pathway to valine in which the α-keto precursor of valine is in effect lengthened by one -CH_2- unit to yield the α-keto precursor of leucine and conserving the isopropyl group formed by the intramolecular rearrangement in the valine biosynthetic pathway.

Although only one of the three branched-chain amino acids (isoleucine) has an asymmetric carbon atom in addition to the α-carbon, the stereochemistry of all of the reactions is an important topic that has been studied intensively in several laboratories. The stereochemical subtleties as they affect the enzymatic mechanisms of these pathways have been concisely reviewed with remarkable clarity by Crout (33).

Because of the structural relatedness of the three branched-chain amino acids, proteins with catalytic or binding sites for one of the branched-chain amino acids usually exhibit only a limited discrimination with respect to binding one or the other of the other two. Examples to be discussed below are transaminases B and C, the transport systems, the aminoacyl-tRNA synthetases, and the regulatory sites on threonine deaminase. As a result, metabolic antagonism among the three branched-chain amino acids is often encountered in bacteria as well as in plants and animals.

THE ENZYMATIC STEPS IN BRANCHED-CHAIN AMINO ACID BIOSYNTHESIS

Isoleucine and Valine

Because four of the five steps in isoleucine formation are catalyzed by enzymes catalyzing the corresponding steps in valine formation, the two pathways will be considered together. The pathways are shown in Fig. 1. The first common feature in the two pathways is a reaction in which an acetal group generated by the decarboxylation of pyruvate is condensed with a second pyruvate or with α-ketobutyrate at the α-carbonyl carbon. Which acceptor molecule is selected determines whether valine or isoleucine will be the final product. The resulting acetohydroxy acid is thus a branched-chain acid, but the branch is at the α-carbon rather than at the β-carbon as found in valine and isoleucine. However, an intramolecular rearrangement in which a methyl or ethyl group is transferred from the α-carbon to the β-carbon yields the carbon skeleton of valine or isoleucine. The enzyme catalyzing the rearrangement also catalyzes an NADPH-dependent reduction to yield the α,β-dihydroxy acid intermediates. Removal of a water molecule from these intermediates yields the α-keto acids which undergo transamination to yield valine and isoleucine. The valine precursor, α-ketoisovalerate, is a branch-point compound, serving as the starting point for leucine and pantothenate formation.

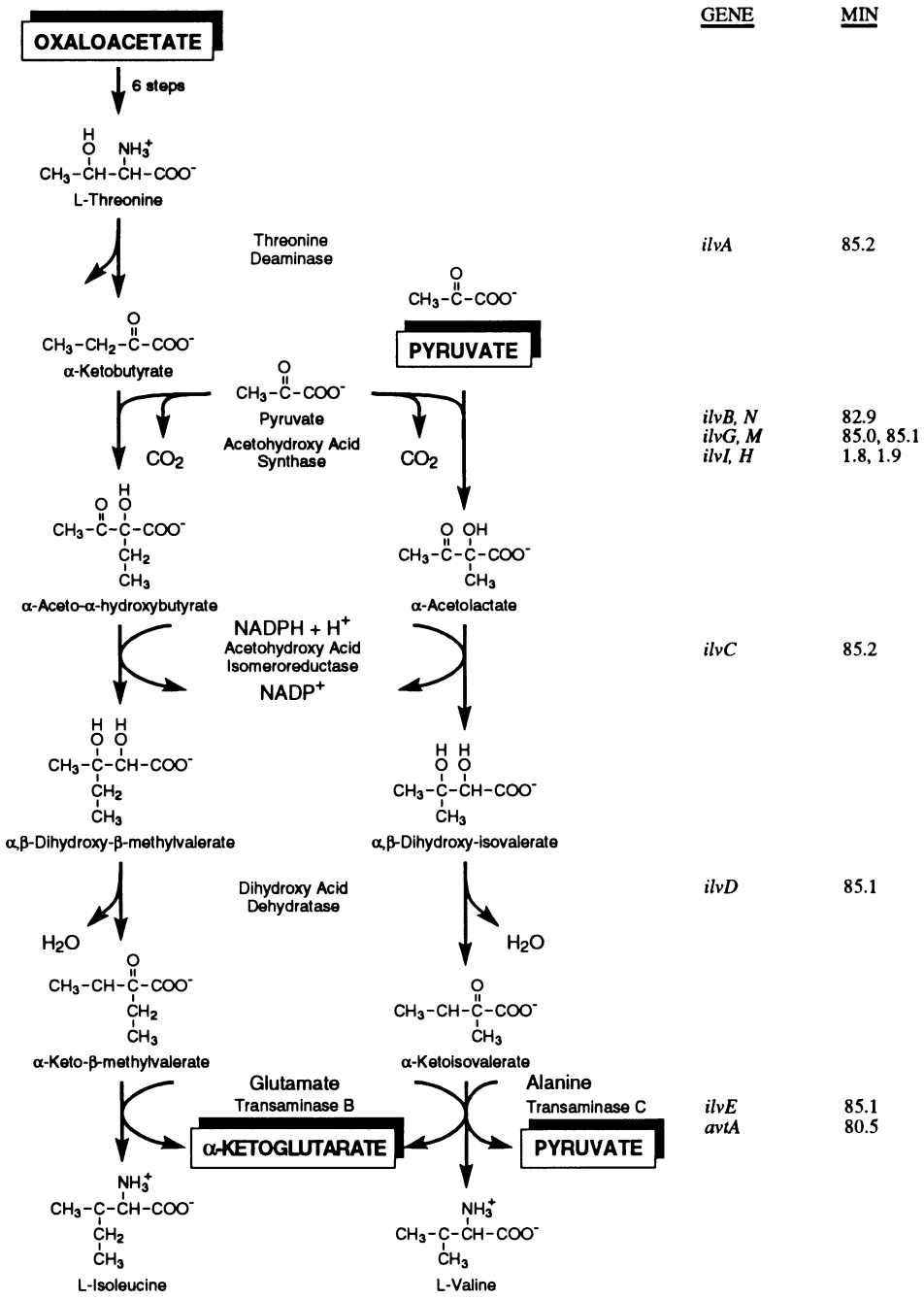

FIGURE 1 Biosynthesis of isoleucine and valine. The gene positions have been rounded off to the nearest 0.1 min from the values given in chapter 109.

Although the pyruvate needed for the synthesis of both amino acids is drawn from the pool of central metabolites, α-ketobutyrate is not a central metabolite and must be derived from some other source. The most common (but not the exclusive) source of α-ketobutyrate is the deamination of threonine.

Threonine Deaminase. L-Threonine deaminases are of two kinds, catabolic and biosynthetic (127). Both kinds also exhibit L-serine deaminase activity, although many have been found to be susceptible to a "serine toxicity" which causes a progressive inactivation of the enzyme. In general, the two types of enzyme are readily distinguished by the fact that the biosynthetic enzyme is sensitive to inhibition by isoleucine. This property underlies the feedback control of isoleucine biosynthesis by isoleucine itself and provides the mechanism that prevents normal cells from overproducing isoleucine.

The enzyme contains pyridoxal-5′-phosphate as a prosthetic group which is centrally involved in catalysis. The mechanism of the reaction has been well studied and is similar to that underlying a number of α-β elimination, addition, and substitution reactions, including the β-cleavage of cystathionine, the β-substitution reaction in tryptophan biosynthesis, and the desulfhydration of cysteine. All such reactions involve the loss of the α-proton (transimination) to yield the same kind of resonance-stabilized intermediate. Although each of the enzymes is highly specific, some have shown limited catalytic activity toward a substrate that may be the preferred substrate for another enzyme of this family. Furthermore, examination of the amino acid sequences of these enzymes reveals regions of sequence similarities.

In *Escherichia coli* and *Salmonella typhimurium* (official designation, *Salmonella enterica* serovar Typhimurium), the biosynthetic threonine deaminase is specified by the *ilvA* gene. The enzyme is a tetramer of 56-kDa subunits. Assuming removal of the formyl methionine on each subunit and a pyridoxal phosphate covalently bound in a Schiff's base linkage to the ε-amino group of a lysine residue, the mass of the active enzyme would be 225 kDa.

Comparison of the 514-amino-acid sequence of threonine deaminase of *E. coli* with that of the *S. typhimurium* enzyme reveals only 30 amino acid differences (94% identity) (32, 118). Both show strong similarity to other threonine deaminases of bacteria and plants, including the biodegradative enzyme specified by the *tdcB* gene of *E. coli* (35). As will be recalled, the latter enzyme differs from that specified by the *ilvA* gene in that it is not inhibited by isoleucine. This sensitivity of the biosynthetic enzyme to isoleucine was correlated with the presence of a C-terminal domain in the isoleucine-sensitive enzymes of *E. coli, S. typhimurium,* and *Saccharomyces cerevisiae* that was absent from the degradative enzyme (118). It was therefore concluded that the C-terminal portion of the biosynthetic enzyme was a regulatory domain. In support of this concept was the demonstration that two mutations giving rise to resistance to isoleucine inhibition were located in the C-terminal portion of the *ilvA* gene, whereas one giving rise to loss of catalytic activity was located in the N-terminal portion. Furthermore, there had been a report several years earlier of a mutant threonine deaminase in which much (perhaps 60%) of the C-terminal region, identified by Taillon et al. (118) as the regulatory region, had been truncated by a nonsense mutation (44). This enzyme was also insensitive to isoleucine, but it had also lost all but 0.3% of the activity. Thus, it appeared that the C-terminal portion of the enzyme bore the binding site for isoleucine, but the integrity of the protein for optimal catalysis was dependent on the presence of that domain.

Subsequent studies have largely supported the prediction of Taillon et al. (118). For example, Fisher and Eisenstein (45), using a selection procedure based on the toxicity of α-ketobutyrate (see below), selected 10 *E. coli* mutants with reduced threonine deaminase activity. Sequence analysis revealed that the mutations were located in the 5′ two-thirds of the *ilvA* gene. In my laboratory, Cindy Wolfe (Ph.D. thesis, Purdue University, West Lafayette, Ind., 1991) selected mutants resistant to the isoleucine analog isoleucine tetrazole (139). The resistant mutants all exhibited threonine deaminases with reduced sensitivity to inhibition by isoleucine. In each mutant, the resistance was due to an amino acid substitution; most of the mutations were in the presumed regulatory domain. Some of the resistant enzymes exhibited reduced catalytic activity, as did two mutant proteins in which portions (9 and 11%) of the C-terminal domain of the protein had been deleted. The two truncated proteins exhibited only 10 and 1% of the activity shown by the wild-type protein. It was also found that some of the isoleucine-resistant mutants carried substitutions only in the presumed catalytic region of the molecule.

That disruption of the regulatory domain leads to marked effects on catalytic activity indicates that the catalytic domain owes much of its integrity to the presence of the regulatory domain. The occurrence of lesions in the catalytic domain in which the primary effect is feedback resistance may not be surprising when it is considered that the two domains must interact and that perturbation in the catalytic domain might also affect its interaction with the regulatory domain.

A common but not universal feature of regulatory proteins subject to feedback control is the cooperative binding of the substrates and often of the inhibitor, a feature reminiscent of the binding of oxygen to hemoglobin, which had been so well known long before the significance of feedback inhibition was appreciated. This was first shown to be a feature of threonine deaminase (124).

Another feature of the enzyme is reversal of isoleucine inhibition by valine, first recognized as an activator by Changeux (27). Whereas isoleucine inhibited threonine deaminase by enhancing the

cooperativity of substrate binding, valine and other activators abolished the cooperativity and allowed the enzyme to bind threonine with normal Michaelis-Menten kinetics.

Until recently, the analysis of the *S. typhimurium* enzyme conducted by Burns and his associates (36, 66) was the most extensive investigation of a biosynthetic threonine deaminase. Briefly, the enzyme preparation studied by these workers was indeed a tetramer of identical subunits, but it was reported to have only two pyridoxal-5′-phosphate (active) sites, two inhibitor sites, and only a single activator (valine) site. More recent work in Eisenstein's laboratory and mine showed that this kind of half-the-sites reactivity did not occur with the *E. coli* enzyme. In both laboratories, four pyridoxal-5′-phosphates were found on the tetrameric enzyme, and ligand binding studies showed four isoleucine (inhibitor) sites. In retrospect, it may be that the peculiar behavior of the *S. typhimurium* enzyme was due to the presence of single disulfide bonds covalently linking the monomers into dimers. Since it is unlikely that disulfide bonds would occur in the cell, it may be that the enzyme was oxidized during its purification. That this problem has not arisen with the enzyme from the K-12 strain of *E. coli* may be because 1 of the 30 amino acid differences between the two enzymes is the presence of an extra cysteine residue in the *S. typhimurium* enzyme (118).

Since the three interacting ligands threonine, isoleucine, and valine are all β-substituted derivatives of α-aminobutyrate, it may be difficult to interpret the interactions that these ligands or their analogs exhibit with threonine deaminase. Thus, Burns studied the interaction between the activator, valine, and the inhibitor but assumed the inhibitory effect of valine, which occurred only at high concentrations, to be due to valine binding at the substrate site. Experiments in my laboratory by Cindy Wolfe indicated that valine inhibits by mimicking isoleucine, i.e., by binding at the inhibitor site and causing marked cooperativity in substrate binding.

Because it is unlikely that the results of the Wolfe thesis will be published, a summary of some of the kinetic parameters obtained with the enzyme purified from *E. coli* is given in Table 1. At the concentration range in which valine served as an activator, the Hill coefficient indicated noncooperative binding at the activator site. This observation was compatible with the direct binding data for valine, which indicated a single binding site. Binding studies at the higher (inhibitory) concentrations of valine were technically infeasible at the protein concentrations used in the binding assays. Also to be noted in Table 1 is the difference between the allosteric and isosteric inhibitors. The isosteric inhibitors, D-allothreonine and D-threonine, competing presumably for the substrate site, exhibit noncooperative binding (the Hill coefficient is 1). In contrast, the allosteric inhibitors, isoleucine, valine at a high concentration, and leucine, exhibited cooperative binding with n_H values approaching 3 or even 4. It was of interest that the enzyme 50% inhibited by D-threonine (an isosteric inhibitor presumably binding at the substrate site) still exhibited cooperative binding of but reduced affinity for isoleucine. This observation indicated that the substrate and substrate antagonist could act synergistically in excluding the inhibitor even though they competed with each other in binding at the substrate site. Surprisingly, the presence of D-threonine in the reaction mixture did not increase the cooperativity of isoleucine binding as elevated threonine concentrations did. In contrast, the enzyme 50% inhibited by valine (acting as an allosteric inhibitor) exhibited noncooperative binding of but also reduced affinity for isoleucine. This observation indicated that the two allosteric inhibitors (valine and isoleucine) could act synergistically in overcoming the antagonism by the substrate even though they competed with each other in binding at the inhibitor site. From these studies, it appeared that valine at moderate concentrations was an activator, binding presumably at a single site (in accord with Burns' results for the *S. typhimurium* enzyme), but at higher concentrations bound to presumably four inhibitor sites, although with only about 1/100 the affinity of isoleucine It is of interest that years ago Changeux (26) had noted that isoleucine at very low concentrations was also an activator of threonine deaminase. Thus, it may be that the difference between activation and inhibition by allosteric effectors is one of degree and that for either isoleucine or valine, the first molecule bound activates but as additional sites are bound, inhibition occurs.

The results in Table 1 are not completely in accord with the results of Eisenstein et al. (41), who, although not reporting valine binding data, did provide kinetic data indicating that valine cooperatively bound at four sites as an activator. Their studies did not examine the behavior of the enzyme in the presence of an inhibitory concentration of valine.

Much of the inhibitory effect of isoleucine can be explained by a reduced affinity (and increased cooperativity) for threonine by the isoleucine-bound enzyme. However, Eisenstein (40) has examined the

fluorescence increase accompanying the initial step in catalysis that occurs upon binding of D-threonine, which is not a substrate but does undergo the transimination step. It was found that isoleucine reduced the rate of this step. Thus, although isoleucine exerts its primary effect on K_m for substrate, it can also affect V_{max}. As judged from the fluorescence change, D-threonine bound to the four catalytic sites cooperatively. Valine activated the binding and abolished the cooperativity as it does for the substrate, L-threonine. Paradoxically, isoleucine, although impeding D-threonine binding as expected, acted like valine to abolish the cooperativity of D-threonine binding.

TABLE 1 Some kinetic parameters of *E. coli* threonine deaminase

Substrate[a]	Activator	Inhibitor[a]	S_{50}	n_H[b]	A_{50}	n_H	I_{50}	n_H
Thr			4.5	1.4				
Thr		Ile (0.02)	8.3	2.2				
Thr		Ile (0.04)	14.9	2.7				
Thr		Ile (0.06)	25.9	3.1				
Thr (4.5)		Ile					0.014	2.8
Thr (5.0)	Val	Ile (0.015)[c]			0.06	1.0		
Thr (5.0)		Val					14.3	3.6
Thr (6.0)		Leu					3.3	3.9
Thr (4.5)		D-Allo Thr					0.43	1.0
Thr (4.5)		D-Thr					21.2	1.0
Thr (4.5)		Ile + Val (14)[c]						
Thr (4.5)		Ile + D-Thr (21)[c]						
Thr (5.0)		Ile					0.017	2.8
Thr (80)		Ile					0.134	4.4

[a]The millimolar concentrations of the fixed ligands are given in parentheses. When the concentration not specified, the concentration of that ligand was varied.
[b]Apparent Hill coefficient of the variable ligand.
[c]This concentration of the ligand alone caused 50% inhibition.

Acetohydroxy Acid Synthase. Three different enzymes catalyzing the formation of the acetohydroxy acids have been found in wild-type strains of *E. coli* and *S. typhimurium* (15, 37, 92, 115). All three isozymes are similar to each other in that they catalyze a thiamine pyrophosphate-dependent decarboxylation of pyruvate and a transfer of the "active acetaldehyde" group to either pyruvate or α-ketobutyrate. All three also require flavin adenine dinucleotide (FAD) as a prosthetic group, although the reaction catalyzed does not involve FAD. FAD readily dissociates from isozymes I and II and must be added as a cofactor in the assay, whereas it is tightly bound to isozyme III and is removed only with difficulty (130). All three isozymes are composed of two large subunits and two small subunits. The large subunits have masses of approximately 60 kDa, whereas masses are around 10 kDa for the smaller subunits for two of the isozymes and around 17 kDa for that of the third isozyme. The similarities between the large subunits are considerably greater than those between the small.

An explanation for the noncatalytic FAD-binding site emerged when Chang and Cronan (24) provided evidence for a common ancestry of the large subunits of the three acetohydroxy acid synthases and the pyruvate oxidase of *E. coli.* This latter enzyme is a thiamine pyrophosphate-linked pyruvate decarboxylase with an FAD-containing oxidase domain that allows oxidation of the active acetaldehyde (presumably hydroxyethylthiamine pyrophosphate) to acetate. Studies with the oxidase itself have demonstrated that this enzyme can form acetolactate (or acetoin, if acetaldehyde is present as an acceptor) as a side reaction; either FAD or FAD analogs incapable of undergoing oxidation-reduction were active in the acetolactate (or acetoin)-forming reactions, but only FAD itself allowed the oxidase reaction (13). Thus, it appears that although FAD is not involved catalytically in the carboligase reaction, it must be present for the enzyme to be active, perhaps playing a structural role.

It is of interest that glyoxylate carboligase, another enzyme in *E. coli* catalyzing a carboligase reaction with thiamine pyrophosphate as a cofactor, 2 glyoxylate → tartronic semialdehyde + CO_2, is a

flavoprotein with FAD as a noncatalytic group (59). Its structural gene, *gcl,* has been cloned, and its nucleotide sequence has been compared with those of the genes for the large subunits of the acetohydroxy acid synthase isozymes and with *pox,* the structural gene for pyruvate oxidase (25). The proteins show a clear relationship to each other, including a seven-residue segment that is present in the proteins binding FAD tightly (glyoxylate carboligase, pyruvate oxidase, and the large subunit of acetohydroxy acid synthase III) but is missing in those that bind FAD weakly (acetohydroxy acid synthases I and II).

Why are there multiple enzymes for acetohydroxy acid synthesis? Clearly, any of the three isozymes can catalyze the formation of both acetolactate and acetohydroxybutyrate. Speculation on the answer to this question gives rise to another: How do the three proteins differ from each other in their properties? The most striking property is that of sensitivity to end product inhibition. As the reaction is the first committed step in valine biosynthesis, inhibition of the reaction by valine itself would be typical of the pattern found in most biosynthetic pathways. That the same enzyme catalyzes the second step in the isoleucine pathway means that effective inhibition by valine would prevent isoleucine biosynthesis. For most organisms, the solution is the formation of an acetohydroxy acid synthase that is not inhibited by valine, and indeed one of the three enteric isozymes (acetohydroxy acid synthase II) is valine insensitive. In the much-studied K-12 strain of *E. coli,* the gene for the large subunit for this isozyme contains a frameshift, so that the valine-insensitive isozyme is missing (83). It was the analysis of acquisition of this activity and the sequential loss, regain, and loss of valine resistance in a series of mutants by DeFelice et al. (37) that revealed the existence of acetohydroxy acid synthase III in *E. coli* K-12. Interestingly, the most studied strain of *S. typhimurium* (LT2) lacks isozyme III owing to a nonsense mutation near the beginning of the gene for the large subunit (111). On the basis of the sensitivity to end product control, it would appear that isozyme II is more suitable for isoleucine biosynthesis than isozymes I and III, which are inhibited by valine. However, the relative affinities of isozymes I and III for the keto acid receptors, pyruvate and α-ketobutyrate (see below), would indicate that isozyme III is more suited to isoleucine biosynthesis than is isozyme I (7).

Differences in the regulation of formation of the three isozymes also point toward specialized roles for each. Although regulation of gene expression will be considered in more detail below, it is appropriate to consider here that only isozyme II is multivalently repressed by all three branched-chain amino acids. Isozyme I is repressed bivalently by valine and leucine, and isozyme III is repressed by leucine alone. A very important difference in gene expression is the catabolite repressibility of isozyme I, an observation suggesting that isozyme I might be more important when carbon sources other than glucose are encountered. Indeed, Daily and Cronan (34) observed that although *E. coli* K-12 mutants lacking isozyme I as well as isozyme II can grow prototrophically with glucose as the carbon source, they require both isoleucine and valine when acetate is the carbon source.

A more definitive analysis of the three isozymes was done by Chipman and Barak and their colleagues (7, 57), who had developed analytical procedures for specifically determining acetolactate (the valine precursor) and acetohydroxybutyrate (the isoleucine precursor). They defined a parameter *R,*

$$R = \frac{V_{AHB}/V_{AL}}{[\alpha - \text{ketobutyrate}]/[\text{pyruvate}]}$$

(where AHB is acetohydroxybutyrate and AL is acetolactate) and compared the *R* values for the three isozymes. As the equation above indicates, the rates of formation of the two products will be equal (i.e., $V_{AHB}/V_{AL} = 1.0$) when [pyruvate]/[α-ketobutyrate] equals *R.* The larger the value of *R,* the more effective is the enzyme in using α-ketobutyrate as the acceptor. For *E. coli* isozyme I, *R* was 1; for isozyme III, *R* was 60; and for isozyme II, *R* was 185. Thus, isozymes II and III are much more suited for acetohydroxybutyrate formation than is isozyme I. Barak et al. (7) have estimated that at their assumed values for pyruvate and α-ketobutyrate (assuming uniform distribution over the entire cell), only 1 to 2% of the products produced by isozyme I consist of acetohydroxybutyrate, whereas the other two isozymes produce the two products in close to equal amounts. That α-ketobutyrate is so effective in suppressing acetolactate formation by either isozyme II or isozyme III suggests that the real deficiency in *E. coli* strains forming only isozyme III during growth on acetate is for valine and that the double requirement stems from the need for isoleucine to reverse valine inhibition.

The LT2 strain of *S. typhimurium* and the K-12 strain of *E. coli* represent examples of chance isolation of *ilvI* and *ilvG* mutants, respectively (83, 111). As such, the genes are sometimes referred to as "cryptic." In these two specific examples, there is evidence, based on examination of closely related strains, for their possible origin. There are two other examples of apparently cryptic acetohydroxy acid synthase genes, but for which the relationship to genes in other strains or the basis of their crypticity has yet to be established. One is the *ilvF* gene, which following mutation gives rise to an acetohydroxy acid synthase that is completely valine resistant (2, 100). When transferred to a K-12 strain bearing deletions for the genes specifying isozymes I and II, the *ilvF* mutation allowed prototrophic growth on acetate as well as glucose as the sole carbon source. The enzyme appears unique in that following extensive dialysis, there was little or no resolution of the enzyme for thiamine pyrophosphate or FAD (2). The nucleotide sequence has not been reported, and thus its structural similarity to the other isozymes is unknown.

Another mutation (*ilvJ662*) giving rise to valine resistance has been described by Jackson et al. (69). The locus is close to that of the gene for isozyme III. The resulting enzyme, designated acetohydroxy acid synthase IV, exhibits surprisingly about the same level of valine sensitivity as does isozyme III. Both isozymes III and IV exhibit about 20% residual activity at very high valine concentrations. Isozyme IV, however, has a higher affinity for α-ketobutyrate, and the argument is made that during valine inhibition, isozyme IV can keep α-ketobutyrate at a lower level than can isozyme III. In view of the toxicity of α-ketobutyrate (see below), this property is thought to underlie the effectiveness of isozyme IV in overcoming valine inhibition. Interestingly, isozyme IV also resembles isozyme III in not requiring added FAD for activity. It is, however, only about one-half the molecular weight of isozyme III. For either *ilvF* or *ilvJ,* it is not known whether the Val^r mutations compensate for inactivating mutations within the structural gene(s) for an enzyme or allow the expression of a gene not normally expressed in the K-12 strain.

α-Ketobutyrate toxicity. One of the consequences of the inhibition of acetohydroxy acid synthase by valine is the accumulation of α-ketobutyrate unless isoleucine is added. The addition of isoleucine does two things: it fulfills the requirement for isoleucine that was caused by the valine inhibition, and it inhibits threonine deaminase, thereby blocking α-ketobutyrate accumulation. It has been suggested that the toxicity of α-ketobutyrate contributes to the growth inhibition resulting from adding valine to cultures of *E. coli* K-12. α-Ketobutyrate toxicity has also been thought to be a factor in the inhibition of plants treated with those herbicides that interfere with acetolactate and acetohydroxybutyrate (e.g., sulfometuron methyl).

There are several potential target sites at which α-ketobutyrate might interfere with metabolism. An obvious class consists of those enzymes for which pyruvate is a substrate. Another example is acetohydroxy acid synthase III, which requires a high pyruvate/ketobutyrate ratio to form acetolactate (7).

A target recognized by Primerano and Burns (101) in *S. typhimurium* is in the conversion of α-ketoisovalerate to ketopantoate, the first step in pantothenate biosynthesis. This site of toxicity was found upon examination of an auxotroph of *S. typhimurium* that exhibited a requirement for isoleucine, pantothenate, or methionine. The basis of accumulation of α-ketobutyrate in this strain was the presence of an *ilvG* mutation leaving only acetohydroxy acid synthase I for acetohydroxybutyrate formation. As discussed above, this enzyme forms acetohydroxybutyrate only when α-ketobutyrate is at a very high concentration, which, in turn, competed with α-ketoisovalerate in pantothenate synthesis. The addition of isoleucine bypassed the blocked reaction and thus quenched α-ketobutyrate formation and eliminated pantothenate formation. That methionine alone replaced the pantothenate requirement was taken to indicate that the reaction involving succinyl coenzyme A, needed for methionine synthesis, was the most sensitive of the coenzyme A-linked reactions. When the mutant also had an *ilvA* lesion that rendered threonine deaminase resistant to inhibition by isoleucine, only pantothenate or methionine allowed growth.

Other potential targets of α-ketobutyrate toxicity have been considered by LaRossa et al. (82).

Acetohydroxy Acid Isomeroreductase. Both acetolactate and acetohydroxybutyrate are converted to the dihydroxy acids by a single enzyme, acetohydroxy acid isomeroreductase. The enzyme is the product of the *ilvC* gene and is a tetramer of 53-kDa subunits. Mg^{2+} is required, but no prosthetic group is required.

The reaction features an alkyl group migration which results in the carbon skeleton configuration found in isoleucine and valine and a reduction with NADPH as the hydrogen donor. The overall reaction had long been assumed to occur as two separate steps in which isomerization occurred before reduction, because the enzyme from *S. typhimurium* would act on the unreduced rearranged compound, α-keto-β-methylbutyrate (HKIV), but not on the reduced, unrearranged compound, α-hydroxy-α-methyl-β-hydroxybutyrate (5, 107). More recent kinetic analysis with the *E. coli* enzyme has confirmed this concept and has shown that the substrate binds to the enzyme only after both Mg^{2+} and NADPH, which had randomly been bound. Mn^{2+} will not substitute for Mg^{2+} in the overall reaction, but it will support the reduction of HKIV (30). The enzyme will also reduce ketopantoate to pantoate (see chapter 44) and α-ketoisovalerate to α-hydroxyisovalerate (90, 102). The former reaction allows the synthesis of pantothenate by *panE* mutants.

Analyses of the pH profiles of the forward and reverse reactions have led to a model in which a base-catalyzed reaction shuttles a proton from the C-2 hydroxyl of the acetohydroxy acid to the C-3 carbonyl as the alkyl group migrates from C-2 to C-3, after which an acid-catalyzed protonation at C-2 occurs (90). The finding that the metal ion requirement was more stringent in the isomerization step than in the reduction step was interpreted to imply that the former reaction required a more precise orientation of substrate and metal than did reduction and that the metal interacts directly with the substrate (30).

Dihydroxy Acid Dehydrase. The enzyme converting the dihydroxy acid precursors to the α-keto acids is the product of the *ilvD* gene and is a dimer of 66 kDa. The enzyme has attracted interest because of its rapid inactivation in the cell by exposure to hyperbaric oxygen (19, 46, 78). This inactivation can be explained by the fact that the enzyme contains a $[4Fe\text{-}4S]^{2+}$ cluster that is converted to a $[3Fe\text{-}4S]^{3+}$ cluster, which is then lost from the protein. The protein is one of a family of enzymes that includes fumarase and aconitase, which are also inactivated in vivo by hyperbaric oxygen and in vitro by superoxide ($O_2^{\cdot}$). Unlike the case for aconitase inactivated in vitro by $O_2^{\cdot}$, which retains a $[3Fe\text{-}4S]^{3+}$ and can be reactivated by treatment with Fe^{2+} and a thiol, it has not been possible to repair the dehydrase inactivated in vitro, since the dehydrase does not retain the $[3Fe\text{-}4S]^{3+}$ cluster (46). Because the enzyme can be reactivated in the cell after oxygen inactivation (i.e., enzyme activity is restored in the presence of chloramphenicol after reduction of the oxygen tension), it is apparent that the protein can exist in the cell in two forms, one with and one without the $[4Fe\text{-}4S]^{2+}$ cluster (47).

One of the irons in the cluster is not ligated to a cysteine residue and is presumed to take part in the catalysis, as has been proposed for aconitase. It is this iron that is presumably oxidized and lost from the cluster.

Transaminase B. The final step in the biosynthesis of both isoleucine and valine is a transamination reaction with glutamate as an amino donor (113). The enzyme was shown by Lee-Peng et al. (87) to be a hexamer of identical subunits with a molecular weight of about 31,500. Cross-linking experiments with dimethylsuberimidate yielded a mixture of dimers and trimers but, curiously, no hexamers. The enzyme has a greater affinity for the branched-chain keto acids than for the branched-chain amino acids, i.e., the biosynthetic direction of the reversible reaction. The enzyme had the greatest affinity for α-keto-β-methylvalerate. The latter point is of interest, since α-ketoisovalerate and α-ketoisocaproate can be converted to valine by transaminase C and to leucine by the aromatic transaminase, respectively.

The *ilvE* gene, specifying transaminase B, has been cloned and shown to specify a protein with a molecular weight of 33,960 (including the N-terminal methionine, which is absent from the native protein) (79).

More recently, the enzyme has been crystallized, and a preliminary report of the molecular structure has been published (72). Two crystal forms were obtained, both of which contained three subunits per asymmetric unit rather than the six found to make up the active enzyme.

Transaminase C. The second route for valine formation from α-ketoisovalerate is via transaminase C, which can use either alanine or α-aminobutyrate as the amino donor (113). Transaminase C is the product of the *avt* gene (135). It accounts for the fact that *ilvE* mutants, lacking transaminase B, do not exhibit an absolute auxotrophy for valine, in contrast to the absolute requirement for isoleucine.

Although *ilvE* avt^+ strains have an absolute requirement for isoleucine and are nearly prototrophic for valine, when the avt^+ gene is present in high copy number, *ilvE* strains are also prototrophic for isoleucine. This phenomenon has been termed "multicopy suppression" by Berg and colleagues (12).

They also noted that in high copy number, the *avt* product could also form a limited amount of leucine, so that there was partial suppression of the absolute leucine requirement that normally results from loss of *ilvE* and *tyrB* (specifying the aromatic transaminase) function. They extended this study to demonstrate that in multicopy (but not single copy), *aspC* allowed amination of α-ketoisocaproate and *ilvE* allowed amination of *p*-hydroxyphenyl pyruvate (12).

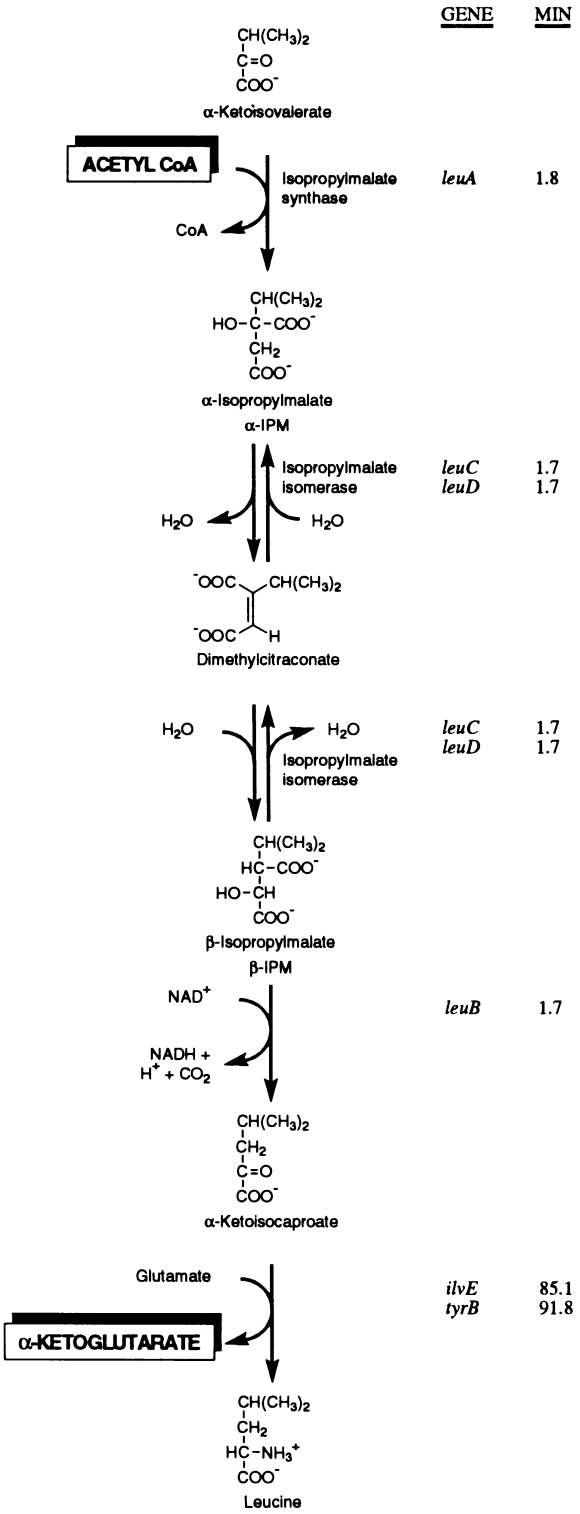

FIGURE 2 The biosynthesis of leucine from α-ketoisovalerate. The gene positions have been rounded off to the nearest 0.1 min from the values given in chapter 109. CoA, coenzyme A.

Leucine

α-Isopropylmalate Synthase. The first step in the lengthening of α-ketoisovalerate to α-ketocaproate (Fig. 2) is the condensation of the acetyl group of acetyl coenyzme A with α-ketoisovalerate to yield α-isopropylmalate, in a reaction analogous to the formation of citrate from oxaloacetate. The same general reaction occurs in the first step in lysine biosynthesis in fungi, in which α-ketoglutarate is transformed to α-ketoadipate. The enzyme has been purified from *S. typhimurium* and was observed to be a multimer of approximately 50-kDa subunits (86). Although the average molecular mass of the enzyme approached 200 kDa, indicating a tetramer, the enzyme underwent a rapid association-dissociation that was influenced by the protein concentration and the concentration of the feedback inhibitor, leucine. Inhibition and dilution favored dissociation to a monomer-dimer equilibrium, with the dimeric form predominating. That there was less tendency for leucine to dissociate the multimers to the monomeric form may reflect the fact that leucine binding reached saturation at 0.5 leucine per subunit, an observation indicating half-the-sites reactivity or strong negative cooperativity.

The nucleotide sequences of the *leuA* genes of both *S. typhimurium* and *E. coli* have been reported and shown to specify proteins of 57.6 and 57.2 kDa, respectively (110, 143). The NH_2-terminal amino acid of the *S. typhimurium* enzyme was shown to be serine, a finding indicating removal of only the formyl methionine of the *leuA* gene product (8).

Although the enzyme has a much greater affinity for a-ketoisovalerate than for any other α-keto acid, it can transfer the acetyl group to a variety of other α-keto acids, including pyruvate, α-ketobutyrate, and α-ketovalerate. Significantly, the transfer of the acetyl group to these straight-chain keto acids initiates the lengthening of the chain by the action of the other two enzymes in the leucine pathway. These "abnormal" conversions can lead to pyruvate being sequentially converted to α-ketobutyrate, α-ketovalerate, and α-ketocaproate. Under normal conditions of wild-type cells growing in a minimal medium, the formation of these keto acids (and their subsequent amination) is not a significant occurrence. However, under conditions of derepression of the *leu* operon by limiting leucine, these side products can become significant, because not only has derepression occurred but also feedback control over the pathway is absent (see below).

α-Isopropylmalate Isomerase. The second step in the chain- lengthening sequence in leucine biosynthesis is the *trans* elimination of water to yield dimethylcitraconate which, like *cis*-aconitate, diffuses freely from the enzyme. To complete the reaction, it is necessary for dimethylcitraconate to be bound to the enzyme in an orientation rotated 180° from the orientation that it had at its formation or, if not released from the enzyme, to undergo a "flip" on the enzyme surface (33). The *trans* addition of the water molecule to a flipped dimethylcitraconate yields β-isopropylmalate.

The enzyme from *S. typhimurium* has been partially characterized and reported to consist of a heterodimer of 51- and 23.5-kDa subunits specified by the *leuC* and *leuD* genes, respectively (55). The sequences of the genes from *S. typhimurium* and *E. coli* are shown to specify proteins of 49.3 and 22.5 kDa in *S. typhimurium* and 49.6 and 22.5 kDa in *E. coli.* The enzymes from the yeast *S. cerevisiae,* which has been much more thoroughly studied, and from *Neurospora crassa* are both monomeric species of about 90 kDa (14, 108).

The reaction is very much like that catalyzed by aconitase, and studies by Emptage (42) with the enzyme from *S. cerevisiae* have indicated that the mechanism is very similar to that of the aconitase reaction. As for aconitase, fumarases A and B, and dihydroxy acid dehydrase, the reaction center is a 4Fe-4S center with three of the irons liganded to cysteines. In the yeast enzyme, the unliganded iron is considered to be the one that forms a five-member ring with the α-carboxyl, the α-carbon, and the hydroxy group of α-isopropylmalate, and it forms another one with the intermediate dimethylcitraconate to yield β-isopropylmalate. It is also this Fe that is removed upon oxidation. Unlike aconitase, but like the bacterial dihydroxy acid dehydrase, the yeast isomerase cannot be reactivated after oxidation of the unliganded iron.

An interesting example of a cryptic gene was discovered by Kemper and Margolin (75) when a *leuD* mutant was selected for suppressing mutations allowing prototrophic growth. One mutation at the *supQ* locus was necessary for the suppressing activity of a closely linked, previously unknown gene designated

newD. Because the suppression was due to a gene substitution, it was effective in suppressing both nonsense and deletion mutations in *leuD* (73).

Some of the *supQ* mutations are also deletion mutations, and in general these suppressing mutations are more effective in complementing *leuD* mutations than are missense mutations. The model proposed by Kemper (74) was that *newD* and *supQ* specify subunits of an enzyme complex (of unknown function) and the SupQ subunit has a greater affinity for NewD than does the *leuC* product. Mutations destroying SupQ function thus make NewD available for combination with LeuC and restoration of isomerase activity.

β-Isopropylmalate Dehydrogenase. The final step in the chain-lengthening process is the oxidative decarboxylation of β-isopropylmalate to yield α-ketoisocaproate. The enzyme had been purified by Parsons and Burns (96) from *S. typhimurium* and shown to be specific for NAD^+ as the hydrogen acceptor. The enzyme was shown to be a dimer of identical subunits estimated to be around 35 kDa. The sequence of the structural gene, *leuB,* reveals a protein of 38.6 kDa.

Amino Group Transfer to α-Ketoisocaproate. Although transaminase B is the predominant transaminase for the amination of α-ketoisocaproate, *ilvE* mutants exhibit a significant growth rate in the absence of added leucine owing to the activity of the aromatic transaminase, the *tyrB* (tyrosine-repressible) product.

The conversion of α-ketoisocaproate to leucine via the aromatic transaminase requires some qualification in view of the report of Vartak et al. (128) that lesions in the *leu* operon prevented *ilvE* mutants from growing on α-ketoisocaproate even in the presence of a intact *tyrB* gene. This observation remains unexplained. It has been suggested that the accumulation of α-ketoisovalerate can compete with α-ketoisocaproate amination by the aromatic transaminase and that the presence of an intact pathway to α-ketoisocaproate will reduce the level of α-ketoisovalerate accumulation. However, it would be anticipated that any block except one in the *leuA* gene would serve equally well to divert α-ketoisovalerate into the leucine pathway and reduce the amount of α-ketoisovalerate.

REGULATION OF GENE EXPRESSION

Isoleucine and Valine

In very general terms, the regulation of the isoleucine and valine biosynthetic enzymes is multivalent, with derepression occurring whenever one of the three branched-chain amino acids is limiting, and repression requiring all three to be in excess. That generalization must be tempered because of the fact that the five enzyme activities involve the products of one quinquecistronic, two bicistronic, and two unicistronic transcriptional units (operons). It would be true for *S. typhimurium* LT2 in that the activities of the five reactions in the dual pathway are all elevated when any one of the three amino acids is in limiting supply (125). That statement would not be true for the K-12 strain of *E. coli,* because that strain does not form the isozyme of acetohydroxy acid synthase that is insensitive to valine inhibition (acetohydroxy acid synthase II), the only one of the three isozymes that is derepressed when isoleucine is limiting. As will be discussed below, a secondary consequence of E. coli K-12 lacking an active acetohydroxy acid synthase II is that the isomeroreductase is derepressed in that strain by limiting valine only.

To understand the pattern of control of expression of the *ilv* genes, it is necessary to consider each transcriptional unit separately.

The *ilvGMEDA* Operon. The *ilv* gene cluster contains seven genes, of which five, *ilvG, ilvM, ilvE, ilvD,* and *ilvA,* are in a single operon. In considering the regulation of any biosynthetic pathway, it is necessary to distinguish between factors stimulating or impeding gene expression in response to the amount of the end product available to the cell and factors that affect gene expression quite independently of end product availability.

"Availability" is not necessarily restricted to presence or absence of the end product in the medium. For amino acids, availability might be a function of ease of entry to the cell for an auxotroph, the extent of the block for a bradytroph, or ease of transferring the amino acid to its cognate tRNA for organisms with intact pathways.

In the isoleucine and valine pathway, controls responding to branched-chain amino acid availability are considered to provide "*ilv*-specific" controls, whereas factors that are independent of the branched-chain amino acids are considered "non-*ilv*-specific" factors. Indeed, whether the latter are to be considered "controls" depends on whether the effects of the factor are modulated or are constant.

To date, the only ilv-specific control over expression of the ilvGMEDA operon that has been established in E. coli is by attenuation of transcription that can occur at a Rho-independent termination site to yield a 183-base leader (84, 91). Whether attenuation or readthrough occurs at the Rho-independent terminator depends on whether the stem-and-loop structure can form at the potential termination site, because the upstream arm of the potential stem-and-loop of this terminator can also be base paired with a region further upstream to form an alternative stem-and-loop structure, the preemptor or antiterminator. If the preemptor were formed, transcription would not be terminated but would instead be continued into the ilvG gene and the rest of the operon. Preemptor formation itself can be prevented because the bases of the upstream arm of that stem and loop can be base paired with bases still further upstream to yield a protector. The formation of the protector stem instead of a preemptor stem would thus allow formation of the intact terminator and presumably ensure termination at that site (attenuation of transcription or repression of operon expression).

Modulation and selection of these mutually exclusive potential structures by the supply of the branched-chain amino acids is possible because the leader specifies a peptide of 32 amino acids, of which 15 are branched-chain amino acids. Stalling of the lead ribosome at a codon for one of the branched-chain amino acids can be readily pictured as a means to preclude protector formation, ensure preemptor formation, and allow transcription into the operon (derepression). If these amino acids are in excess, ribosome stalling would not occur, and translation would proceed to the amber codon of the leader. In the absence of stalling, those bases in the leader that provide the upstream arm of the protector would be covered by the ribosome and could not interfere with terminator formation (repression).

Because of the protector stem and loop, transcription will be terminated at the end of the leader when no ribosomes are able to initiate translation, as might occur, for example, in the presence of chloramphenicol.

As emphasized in the general chapter on attenuation control (chapter 81), an important feature in attenuation is thought to be the presence of polymerase pause sites, which presumably allow the more slowly moving but otherwise unimpeded lead ribosomes to approach more closely the transcribing polymerases. Evidence for polymerase pausing at one specific site has been demonstrated for the *ilvGMEDA* operon (64). Such pausing would presumably prevent preemptor formation, except for a transcript being translated by a ribosome severely retarded by a limitation for leucine, valine, or isoleucine.

The outline described above is a very simplified description of the attenuation process. A variety of stem-and-loop structures, some mutually exclusive, in addition to those described above can be postulated for the *ilvGMEDA* leader. The importance of these additional structures cannot presently be assessed. Such factors as codon selection, relative rate of transcription and translation, and pause sites could all be involved in whether attenuation or continued transcription into the rest of the operon occurs. What appears to have been selected in the nucleotide sequence of the *ilv* leader regions of *E. coli* and *S. typhimurium* (which are identical in those parts of the sequences that are thought to be responsible for modulation of transcription [61, 119]) is a subtle balance of these factors that results in an efficient, multivalent control of gene expression in the operon.

The *ilv* leader is a remarkably efficient regulatory structure when one considers how few codons for each of the three branched-chain amino acids are contained in the *ilv* leader, in contrast to the leader for the histidine operon (seven His codons), the leader for the *pheST* (five Phe codons), and the leader for *pheA* (seven Phe codons) (38, 116, 144). The significance of attenuation in control of the *ilvGMEDA* operon has been studied by the examination of mutations involving deletions of various parts of the leader and substitution of the three leucine codons that are thought to be pertinent to control of the operon by leucine (29).

There is little doubt that the *ilvGMEDA* operon is regulated by attenuation, just as is the *trp* operon. The question arises as to whether there is any additional control over gene expression in that operon. Is there a repressor activated by or an activator antagonized by one or more of the branched-chain amino acids? In the absence of attenuation, is there any regulation of gene expression in the cluster?

Some evidence that there is no *ilv*-specific control other than attenuation came from studies with deletions that removed the terminator at the end of the end of the leader region and extended either

beyond the site of the frameshift in *ilvG* that destroys *ilvG* function in *E. coli* K-12 or beyond the Rho-dependent polarity sites (10, 133). Strains bearing these deletions exhibited no derepression when any of the branched-chain amino acids was limiting or no repression upon addition of the three amino acids in excess (28). As a corollary, it was shown by Lawther et al. (85) that attenuation of transcription was dependent only on the intact leader region and not on the promoter itself or on sequences upstream of the promoter that are known to enhance promoter strength (93). The experiments with the deletion strains might be criticized, since the deletions also removed the region between the leader termination site and *ilvG* that could be necessary for an *ilv*-specific blocking of transcription or be a downstream repressor binding site acting in concert with a site upstream of the promoter that resulted in DNA looping. As yet, there is no evidence for any such role for the deleted region.

Possible evidence contradicting the notion that attenuation may not be the sole mechanism of *ilvGMEDA* operon regulation came from isolation of mutants in which expression from the *ilvG* promoter was elevated owing to lesions in a locus designated *ileR* (71). The same lesions elevated expression from the *thrA* promoter. In each case, expression was examined in strains containing *lacZ* under control of the *ilvG* or *thrA* promoter in the absence of the cognate attenuator regions. Although these experiments showed an elevation of expression as a result of the *ileR* lesion and a reversal by a plasmid bearing $ileR^+$, there was no evidence provided indicating *ilv*-specific control or indeed that the repressing effect of the *ileR* region was subject to any modulation.

Nevertheless, it is interesting that there are regions in the vicinity of the *ilvG* promoter with some similarity to the *trpR* operator (17). Indeed, isoleucine and valine along with leucine, threonine, phenylalanine, tyrosine, and serine are required for growth in the presence of tryptophan when the *trpR* gene is strongly overexpressed.

Adjacent to the *ileR* locus is a region that appears to exhibit both activating and repressing functions on the *ilv* promoter (71). Sites in this region at which F factor-derived γδ insertions inactivated the repressing functions of this region were interspersed with those that destroyed the activating function. The results raised the possibility that the two activities are in the same gene (designated *ilvR*). The experiments were not addressed to the question of whether these *trans*-acting elements were involved in any *ilv*-specific control or whether their effects on the *ilvGMEDA* operon were at all modulated.

Another locus, *leuJ,* was identified as the site of lesions conferring trifluoroleucine resistance and leading to elevated *ilvA* and *leuA* expression (70). Again, no evidence was obtained indicating that the repressing element was involved in any *ilv*-specific or *leu*-specific regulation or that the repressing effect was subject to any modulation.

Although the products of *ilvR* and *ileR* loci appear to interact with the *ilvGMEDA* promoter and thereby affect transcription, there is no evidence that they play a role in *ilv* regulation. They may be proteins that do bind to DNA and bind sufficiently well to *ilv* promoter DNA to exert their effects. Thus, although their binding may be incidental to *ilv* function, these proteins may be factors for which the *ilvGMEDA* operon has evolved to compensate.

The *ilvGMEDA* operon is subject to at least one kind of global regulation, i.e., the rich-medium depression that has been observed with many biosynthetic pathways. For the isoleucine and valine biosynthetic pathway, the low enzyme activities in cells grown in a rich medium, such as L broth, cannot be explained merely by the presence of the branched-chain amino acids in the rich medium. Indeed, *ilv*-specific regulation of the *ilvGMEDA* operon can still be derepressed by limiting one of the branched-chain amino acids in an otherwise rich medium, although not to the extent exhibited in a minimal medium-based culture medium. Part of this phenomenon might be merely competition for polymerases by the genes for the protein-synthesizing machinery; part may be accounted for by the low levels of ppGpp. ppGpp stimulates in vitro transcription from the $ilvGp_2$ promoter (D. K. Agrawal, Ph.D. thesis, Purdue University, 1991). The Pribnow box and the discriminator region fit a pattern of ppGpp-activated promoters recognized by Riggs et al. (112). The stimulation by ppGpp would serve to amplify derepression that results from branched-chain amino acid limitation, since ppGpp levels would rise as the growth rate was reduced owing to the amino acid limitation.

Another factor that affects the *ilvGMEDA* operon in a non-*ilv*-specific way is integration host factor (IHF), the product of the *himA* and *himD* genes, which has been shown to affect expression of a broad spectrum of genes (53). The protein binds to two sites upstream of *ilvG* (97, 122). One is at a region that had been shown to be needed for maximal expression of the *ilvGMEDA* operon (93). This binding site overlaps with one of the two polymerase-binding sites that are detected in vitro, that designated $ilvGp_1$ (94, 122, 140). Binding at this site interferes with transcription from $ilvGp_1$ but activates transcription at

the downstream promoter, *ilvGp*$_2$ (94, 95). These interactions may account for the fact that *ilvGp*$_2$ is the predominant promoter detected in vivo.

The second IHF binding site is within the leader region just downstream of the polymerase pause site (122). Binding at this site increases the polymerase pause period in in vitro transcription experiments (94). The in vivo consequence of this in vitro effect is difficult to assess. A polymerase pause that is too brief might not allow a ribosome to begin leader transcription in time for an arrested ribosome to allow preemptor formation (antitermination) to occur. For the *ilvGMEDA* leader, it has been assumed that the assembly of the ribosome at the first (AUG) codon will disrupt the stem-and-loop structure required for polymerase pausing (29). It may be that IHF ensures that polymerase will remain at the pause until the ribosome has initiated translation.

The activation of polymerase function at *ilvGp*$_2$ is probably accounted for by the enhanced bending of the DNA in the region of its binding (95). As found with many promoters, the region upstream of *ilvGp*$_2$ exhibits intrinsic bending, which alone is responsible for a sevenfold activation of transcription for *ilvGp*$_2$ (95). Interestingly, the stimulation of expression by the presence of IHF occurs even when this intrinsic DNA bending is abolished by changing the helical phasing in the region. Again, the *cis* effect of the intrinsic DNA bending and the *trans* effect of IHF seem to be factors affecting *ilvGMEDA* operon expression independently of regulation related to branched-chain amino acid biosynthesis. Any factors regulating the amount of IHF might thereby be recognized as global regulators of *ilvGMEDA* expression as well as that of other operons that are affected by IHF. The question might be raised as to how many more DNA-binding proteins in the cell in addition to IHF and the *ileR* protein have positive or negative effects on transcription initiated from the *ilvGp*$_2$ promoter. Might the histone-like proteins that bind to the bacterial chromosome have either negative or positive effects on *ilv* operon expression, depending on the site of binding? Because of the seemingly complete loss of any *ilv*-specific control when attenuation control is abolished, it is unlikely that any will be responsive to a branched-chain amino acid signal.

The *ilvBN* Operon. The *ilvB* and *ilvN* genes, specifying the large and small subunits, respectively, of acetohydroxy acid synthase I, constitute an operon that is near the *ilv* cluster but unlinked by P1 transduction in *E. coli.* It, like the *ilvGMEDA* operon, is regulated by an attenuation mechanism, although its leader contains three leucine codons (one solitary and a tandem pair) and runs of four and five valine codons but no isoleucine codons (50, 62). Therefore, the *ilvBN* operon is responsive only to valyl- and leucyl-tRNA availability. Conflicting reports have appeared concerning whether transcription of the operon is also deattenuated when growth is limited by the supply of either alanine (or alanyl-tRNA) or threonine (or threonyl-tRNA), for which tandem codons are found in the *ilvBN* leader (63, 121).

In addition to the *ilv*-specific regulation by valine and leucine, the *ilvBN* operon is subject to the global catabolite repression signal inversely related to cyclic AMP (cAMP) levels (117). It is interesting that this effect had been observed by Coukell and Polglase (31) on acetolactate formation long before the isozymic nature of the reaction had been discovered. Catabolite repression of a biosynthetic enzyme does seem unusual, but for the formation of the acetohydroxy acid synthase I isozyme, there is a clear selective advantage as mentioned earlier in citing the fact that isozyme I is much more effective than either isozyme II or isozyme III in forming acetolactate (rather than acetohydroxybutyrate) at low pyruvate concentrations such as would obtain when acetate is the carbon source (34).

The stimulation of *ilvBN* expression by cAMP is independent of the control of the operon by transcript attenuation but, rather, appears to improve transcript initiation. The cAMP-cAMP receptor protein-binding region appears to be at base pairs –44 to –82 upstream of the transcription start site (51). Binding in this region may prevent binding of RNA polymerase at a second (nonproductive) polymerase-binding site and thereby increase binding at the one at which transcription of the operon is initiated.

ilvBN expression is also stimulated by another global regulatory metabolite, ppGpp (49). The role of ppGpp in *ilvBN* expression is reflected in the low level of derepression that is observed in *relA* strains. It may also contribute to the rich-medium depression of enzyme formation that is observed with the *ilvBN* operon as well as with other isoleucine- and valine-forming enzymes and indeed amino acid biosynthetic enzymes in general. A direct effect in vitro of ppGpp on transcription initiated from the *ilvBN* promoter appears not be have been reported. Although the early report implied that the ppGpp stimulation could be replaced by cAMP, the two effectors undoubtedly are completely independent of and therefore additive to each other.

The effect of IHF on the isoleucine and valine biosynthetic enzymes was first noted by Friedman et al. (54) for the *ilvBN* operon. The effect was examined in more detail by Friden et al. (52), who verified the

effect on *ilvBN* expression and provided evidence for the effect of IHF on the *ilvGMEDA* operon described earlier. IHF was reported also to stimulate *ilvBN* transcription in vitro. Later, Tsui and Freundlich (123) showed that IHF bound upstream of the *ilvBN* promoter in the region of an intrinsic bend in the DNA and upon binding enhanced the degree of bending.

The *ilvC* Gene. The *ilvC* gene specifying the isomeroreductase appeared to be derepressed in *E. coli* K-12 only when valine was limiting, whereas in *S. typhimurium,* derepression was observed when any of the branched-chain amino acids was in limited supply (126). This difference was readily accounted for by the fact that expression of the *ilvC* gene is induced by the substrates acetolactate and acetohydroxybutyrate, which are more readily formed at low valine levels and would not be formed in the presence of excess valine. Thus, end product regulation of expression of the *ilvC* gene is indirect.

The induction is dependent on the presence of upsilon, the product of the divergently transcribed *ilvY* gene (4, 131). Upsilon is a protein of 33.2 kDa and is a member of the *lysR* family of regulatory proteins (65).

Wek and Hatfield (131) have studied in detail the nucleotide sequences of the *ilvY* and *ilvC* genes and the interactions of upsilon with two operators. The binding of upsilon to O_1, which includes the early part of the *ilvY* coding region, and the binding to O_2, which overlaps the *ilvC* –35 region, are cooperative and strongly stimulated by the inducing intermediates. Binding at O_1 serves two functions: (i) it serves to repress *ilvY* expression, and, (ii) it enhances binding at O_2, which if inducer is present allows RNA polymerase to initiate transcription. It is thought that upsilon binds in the major grooves flanking the –35 region, allowing RNA to bind in the major groove at the –35 promoter on the opposite side of the DNA.

The *ilvIH* Operon. The *ilvIH* operon was known very early to be repressible by leucine, but there was no indication of a leucine-dependent attenuation mechanism as was known to regulate the leucine-repressible *leu* operon. Pursuing the lead that an unlinked locus was involved in *ilvIH* control, Ricca et al. (109) identified a 43-kDa dimeric protein that bound to the region upstream of the *ilvIH* promoter. In the presence of leucine, the amount of protein bound was reduced. The protein, initially called IHB, was eventually shown to be identical with a protein with many other functions and was renamed Lrp, the leucine- responsive protein (99; chapter 94). Lrp is a basic protein structurally related to AsnC, the protein that stimulates expression of the *asnA* promoter and responds negatively to asparagine (76, 138).

Lrp binds to six sites distributed over a 250-bp sequence upstream of the *ilvIH* promoter of *E. coli* (129). Binding at sites 1 and 2 is cooperative, as is binding to sites 3, 4, and 5. Binding of Lrp enhances the intrinsic bend that occurs in regions upstream of *ilvIH.*

In vitro, transcription in the absence of Lrp is initiated from a second promoter which overlaps one of the Lrp binding sites and is upstream of the promoter that is active in vivo. Lrp causes transcription to be initiated exclusively from the in vivo promoter. The addition of leucine could be shown to reduce the effect of Lrp and even to restore some of the transcription initiated from the second promoter.

Regulation of *avtA* Gene Expression. Regulation of the *avt* gene has been most thoroughly studied in the laboratory of C. M. Berg (11). The effects of amino acid supplementation and limitation on transaminase C activity have been examined in *S. typhimurium* and *E. coli.* In addition, the effects of the same conditions on β-galactosidase activity under control of the *avtA* promoter have been examined in *E. coli.* Some repression was observed in all three test systems when alanine or leucine was added to the medium. In addition, the nonprotein amino acids α-aminobutyrate, norvaline, and norleucine also caused repression. The common element here is the absence of a methyl group on the β-carbon, in contrast to valine and isoleucine, which do not repress *avtA* when added to a minimal medium.

Attempts to demonstrate a derepression of the *avtA* gene appear to have been unsuccessful. Indeed, limitation of growth even by limiting leucine (which represses when added in excess) also results in a repression, as do limitations of eight other amino acids. It is now concluded that limiting an amino acid results, in effect, in alanine excess, resulting, in turn, in repression. It was concluded that alanine and α-aminobutyrate were the true repressing metabolites and that the repression resulting from adding amino acids lacking β-methyl substitutions was caused by these amino acids mimicking alanine (or α-aminobutyrate) and acting as "gratuitous" corepressors. The repressibility by alanine may be indicative of a role in alanine biosynthesis (discussed in chapter 24), whereas repressibility by α-aminobutyrate may indicate a role for transaminase C in removal of the toxic metabolite, α-ketobutyrate, by converting it to

α-aminobutyrate (indeed, during valine inhibition of *E. coli* K-12, α-aminobutyrate accumulates in the medium).

Leucine

Regulation of the *leuABCD* Operon. The primary mechanism for regulation of *leu* operon expression is an attenuation mechanism mediated by the rate at which a ribosome can traverse a run of four leucine codons (56, 134). In *E. coli,* all four of these codons are the rarely used CUA; in *S. typhimurium,* three of them are CUA. The significance of the presence of rare codons was examined by replacing them with the commonly used CUG (22). Such a replacement led to lower basal levels of operon expression and less derepression upon limiting the leucyl-tRNA level. In another study, the four leucine codons were changed by two flanking frameshift mutations to four threonine (ACU) codons (23). Although leucine control was greatly reduced but not completely abolished, the derepression resulting from limited threonyl-tRNA levels was not nearly as high as that resulting from leucine limitation with a wild-type leader region. The presence of only four consecutive codons in the leader may not be sufficient with the readily used ACU threonine codons. (The *thr* leader region contains eight threonine codons.)

The finding that there was indeed a low level of apparent derepression of the *leu* operon in the mutant containing no leucine control codons raises the question of whether there is some control (albeit very weak) by leucine in addition to attenuation. With the existence of the Lrp protein, which has a leucine-binding site, a candidate for a leucine-mediated repressor or a leucine-antagonized activator, another kind of control is readily envisioned. Some indication for a role of Lrp as a positive regulatory element for the *leu* operon comes from the isolation of mutants containing λp*lac* Mu insertions in the *leu* operon that exhibited higher levels of β-galactosidase in an Lrp^+ background than in an Lrp^- background (88). However, since such strains were dependent on exogenous leucine for growth, the Lrp effect could have occurred via the LIV transport system, which is repressed by leucine via its interaction with Lrp (60).

The question of the effect of the number of leucine codons in the *leu* leader was addressed by Bartkus et al. (9). The four consecutive leucine codons in *S. typhimurium* are $(CUA)_3CUC$. These were replaced in a series of constructs to yield leader transcripts containing from one to seven consecutive CUA codons (CUA is a rarely used codon for leucine). The strains forming *leu* leaders with six or seven CUA codons did respond a little more sensitively to leucine limitation than did the wild type. On the other hand, the strains with only two CUA codons responded almost as well as the wild type. That there was so little difference between attenuation with $(CUA)_2$ in the leader and that with $(CUA)_7$ in the leader is reflected in the fact that the predicted secondary structures thought to be important in regulating the *leu* operon are essentially the same in each case.

Another feature of the *leu* operon and its control should be mentioned. Among the early experiments by Calvo and colleagues (20, 21) were some that led to the isolation of mutants of *S. typhimurium* derepressed for the *leu* operon. These were distinguished from the wild-type parent by their excretion of leucine. It thus appeared that although carbon flow through the pathway to leucine could be effectively controlled by end product inhibition in strains containing normal enzyme levels, when the enzyme levels were elevated, end product inhibition was ineffective. If this limitation of the effectiveness of end product inhibition is also true of *E. coli,* it may be possible to account for the formation of norleucine and its incorporation in place of methionine when high-level expression systems are stressed to form leucine-rich proteins. There have been two such examples. In one, a high-expression vector used to make methionyl bovine somatostatin, which contains 191 amino acids residues, of which 27 are leucine (18). In the other, the protein made was interleukin-2, in which 26 of the 152 amino acid residues are leucine (120). In each case, the drain on the leucine pool appears to have resulted in (i) a derepression of the *leu* operon and (ii) insufficient leucine to exert feedback control over the pathway. Therefore, the largely uninhibited α-isopropylmalate synthase was free to initiate the chain-lengthening sequence with pyruvate (or perhaps α-ketobutyrate): (pyruvate) → α-ketobutyrate → α-ketovalerate → α-ketocaproate → norleucine.

TRANSPORT OF THE BRANCHED-CHAIN AMINO ACIDS

The transport of the branched-chain amino acids is catalyzed by several systems with different specificities. Three classifications have emerged from three different research groups using different approaches in the genetic and biochemical analyses of the several transport systems (Table 2). Perhaps because of the different approaches or perhaps because of strain differences, different spectra of mutants

have been isolated and studied by the various groups. As a result, the three classifications are difficult to combine into a single system of classification. There is some overlap and consistency among the three, however.

The most thoroughly studied are those (in Oxender's classification) of the LIV-I and LS systems (106). Both are lost upon osmotic shock and dependent on the presence of binding proteins in the periplasmic space and energetically coupled to the hydrolysis of ATP (141). There are two different binding proteins associated with a common membrane-associated complex. The LIV binding protein, specified by the *livJ* gene, is formed as a 39-kDa precursor protein from which is cleaved a 23-residue leader peptide upon transfer to the periplasm. This protein binds all three branched-chain amino acids. The LS binding protein, specified by the *livK* gene, is formed as a 39.3-kDa precursor including its 23-residue leader peptide. This protein binds only leucine. The membrane-associated components are a pair of inner membrane proteins of 32.9 and 46.1 kDa specified by the *livH* and *livM* genes, respectively. The ATP-binding and -cleaving component is a heterodimer of 28.5- and 26.2-kDa proteins specified by the *livG* and *livF* genes (1). The LivG and LivF proteins are cytoplasmic but are tightly associated with the inner membrane. An additional point of interest is that the leucine-specific binding protein contains two regions separated by 14 amino acid residues that bear a strong similarity to two regions in leucyl-tRNA synthetase (136).

The LIV-II system is a low-affinity branched-chain amino acid transport system (103). This system is resistant to osmotic shock and is presumably dependent on the proton motive force. In the absence of both the proton motive force and the ATP-coupled system, leucine is excreted into the medium (3). This observation along with the repressibility of transport by leucine (see below) leads to the idea that an important role of the transport system is retention of endogenously formed leucine. In the presence of exogenous amino acids, the advantage of energy-coupled transport as well as endogenous synthesis is lessened or abolished, and the response of the cell is repression of both processes.

The approach of the Iaccarino laboratory in Naples (68) was to isolate mutants selected for resistance to valine (but sensitivity to valine peptides) and for reduced uptake of isoleucine. Their selection procedure resulted in recognition of a very high affinity system and two high-affinity systems for all three branched-chain amino acids, a high-affinity system specific for leucine, and a low-affinity transport for isoleucine (and perhaps valine and leucine as well). The system identified as the very high affinity system for leucine, isoleucine, and valine was assumed to be the same as the LIV-I system of Oxender's classification (106). The resolution of the LIV-II system into the high-affinity 1 and the high-affinity 2 systems was based on a differential sensitivity to inhibition by threonine (68).

The analysis by Anraku's group (142) helps to bridge some of the discrepancies between the Oxender and Iaccarino descriptions. In their clarification, the LIV-1 and LIV-I systems seem to be equivalent, and the "low-affinity" LIV-2 system dependent on the *hrbA* gene seems to be equivalent to the "high-affinity 1" system described by Guardiola et al. (58).

The repressibility of the LIV-I and LS transport systems by leucine has been studied intensively by Oxender and colleagues. Both the *ilvJ* gene (specifying the LIV binding protein) and the *ilvKHMGF* operon (specifying the LS binding protein and the four-component membrane complex functioning in concert with both binding proteins) are repressed by Lrp in the presence of leucine (60).

A second level of control also appears to effect gene expression of the *ilvJ* gene. The LIV binding protein is elevated in Rho mutants and under conditions of restricted leucyl-tRNA formation (104, 105). One model is that transcription termination at the end of the *ilvJ* leader occurs when there is ample supply of leucyl-tRNA and that this termination is Rho dependent (137).

The LIV-II (low-affinity) system is less repressible in the presence of exogenous amino acids, allowing it to transport the exogenous amino acids into the cell.

TRANSFER OF THE BRANCHED-CHAIN AMINO ACIDS TO COGNATE tRNAs

The structural similarity between isoleucine and valine underlies the impetus of much study of the incorporation of these two amino acids into protein via their respective aminoacyl-tRNAs. For example, isoleucyl-tRNA synthetase does indeed activate valine at a rate of 1 in 200. However, as discussed by Schmidt and Schimmel (114), there are several "sieves" through which the synthetase itself reduces the misincorporation to a level of one in many thousands. Any valyl AMP that passed through the initial discrimination step is subject to a hydrolysis by the synthetase itself. Should valyl AMP pass through this sieve and the valyl group be transferred to $tRNA^{Ile}$, the valyl-$tRNA^{Ile}$ is subject to hydrolysis, again by the synthetase itself. A mutant isoleucyl- tRNA synthetase that was unable to discriminate between isoleucine

and valine in the formation of the aminoacyl AMP was still able to hydrolyze AMP and deacylate valyl-tRNA^{Ile}. Another mutant studied by these workers was unable to discriminate in the transfer reaction so that valyl-tRNA^{Ile} was formed at a high rate. However, this mutant enzyme was able to deacylate valyl-tRNA^{Ile} as well as the wild-type enzyme could. Posttransfer cleavage of tRNA^{Val} misacylated with γ-hydroxy analogs of valine and isoleucine has also been postulated for the proofreading by the corresponding synthetase (98). With the hydroxy acid analogs, the hydrolysis is expedited by the formation of the corresponding lactones. The proofreading reactions lead to greater than stoichiometric consumption of ATP during aminoacylation.

TABLE 2 Transport systems for the branched-chain amino acids in *E. coli*

Classification scheme	System	Affinity	Identifying feature(s)	Gene(s)	Map position
Anraku	LIV-1	High	Osmotic shock sensitive	*hrbC*	ca. 77.4
	LIV-2	Low	Osmotic shock sensitive	*hrbB*	ca. 77.4
	LIV-3	Low	Osmotic shock sensitive	*hrbA*	ca. 9.0
	LIV-4	Low	Osmotic shock resistant	*hrbD*	ca. 78.5
Oxender	LIV-I	High	Osmotic shock sensitive	*livF, livG, livM, livH*	77.4
				livJ	77.5
	LIV-II	Low	Osmotic shock resistant	*livP* ?	?
	LS	High	Osmotic shock sensitive	*livK, livF, livG, livM, livH*	77.4
Iaccarino	Very high affinity		Met represses and Met and Thr inhibit		
	High affinity 1		Thr does not inhibit	*brnQ, brnR*	9.0, ca. 8.0
	High affinity 2		Thr inhibits	*brnS, brnR*	ca. 1.0, ca. 8.0
	Low affinity		Residual transport in absence of "very high" and "high" systems	*brnT* (affects Ile transport only)	ca. 64.1

Freist and Cramer (48) have analyzed the various discrimination or sieve reactions for the mischarging of tRNA^{Ile} by valine and recognize two discrimination steps in the initial activation in addition to the pre- and posttransfer proofreading reactions. Their data revealed that with equal concentrations of valine and isoleucine and under conditions that yielded initially one misactivation of valine (i.e., formation of valyl AMP by isoleucyl-tRNA synthetase) in 53 proper activations, there was a final discrimination against valyl-tRNA^{Ile} persistence as being 1 in 72,000 and, in the presence of EF-Tu·GTP, 1 in 144,000.

Because three of the operons specifying enzymes for branched-chain amino acid biosynthesis are regulated by attenuation, the efficiencies of the respective amino acyl-tRNA synthetases have regulatory consequences. Commonly encountered mutants are those in which the synthetase has a reduced affinity for the amino acid (16, 67) or is temperature sensitive (39, 80). At least one mutant leucyl-tRNA synthetase exhibited both an elevated K_m for leucine and sensitivity to elevated temperatures (89). The consequences of a branched-chain aminoacyl-tRNA synthetase with reduced affinity for its amino acid substrate might be a derepression in minimal medium and a requirement for a very high level of the amino acid for repression or even auxotrophy for the amino acid.

Whether derepression will result from the defects in aminoacyl-tRNA synthetase function can also depend on how much of a compensatory derepression of a poor synthetase can occur. Thus, one thiaisoleucine-resistant mutant derived from *E. coli* was found to carry three separate mutations, all of which contributed to the thiaisoleucine resistance (43).

One mutation in *ileS,* the structural gene for isoleucyl-tRNA synthetase, did result in a synthetase with a reduced affinity for isoleucine and, presumably, thiaisoleucine. However, by itself the *ileS* lesion had little effect on derepression of the *ilv* genes, because the synthetase became derepressed, thus compensating for the low activity. In the presence of the second, unlinked lesion, *ilvU,* the *ileS* gene was no longer derepressible, and derepression of the *ilvGMEDA* operon resulted, owing to the reduced isoleucyl-tRNA level. The third lesion, referred to as *ilvT* at the time, was of less importance but did add

somewhat to the derepression. The basis of the *ilvU* effect was not determined, but it appeared to retard the conversion of a species of each isoleucyl-tRNA and valyl-tRNA emerging as second components from reverse-phase columns, so that the second species of $tRNA^{Val}$ and $tRNA^{Ile}$ were not found in *ilvU* extracts. It thus may be thought that the second (undermodified) species of $tRNA^{Ile}$ charged with isoleucine repressed *ileS*.

Mutants selected for compensatory mutations allowing *E. coli* mutants with temperature-sensitive valyl- and leucyl-tRNA synthetases to grow at 42°C have been studied in the Söll laboratory (6, 80, 81). Of interest are those in which the synthetases were still temperature sensitive but were produced in larger amounts. Two types of mutants were found in which the leucyl-tRNA synthetases were overproduced. In one type, the lesions were in the operator-promoter region of *leuS,* but in the other, the lesions were unlinked and had affected the structural gene for a protein (80). Among the mutations affecting the level of valyl-tRNA synthetase, only those with lesions in the operator-promoter region of *valS* were found (6).

The genes for the branched-chain amino acid acceptors have all been located on the *E. coli* chromosome by Komine et al. (77). For $tRNA_1^{Ile}$, there are triplicate genes (*ileT, ileU,* and *ileV*), each in tandem with genes for $tRNA_{1B}^{Ala}$ as a spacer in the *rrnA, rrnD,* and *rrnH* operons, respectively. In addition, there is a single gene (*ileX*) specifying $tRNA_2^{Ile}$. For $tRNA_1^{Val}$, there are four genes; three of them, *valU, valX,* and *valY,* are consecutive in an operon in which the terminal gene is a gene for $tRNA^{Lys,}$ and a fourth gene (*valT*) is in an operon containing flanking genes for $tRNA^{Lys.}$ Another operon of two genes, *valN* and *valW,* specifies $tRNA_{2B}^{Val}$ and $tRNA_{2A}^{Val}$, respectively. For $tRNA^{Leu}$, there are four genes specifying the major species of $tRNA_1^{Leu}$, three of which, *leuV, leuP,* and *leuQ,* are in a single operon, and the fourth, *leuT,* is in the *argT* operon specifying acceptors for arginine, histidine, and proline. $tRNA_2^{Leu}$ is specified by the *leuU* gene in a single-gene expression unit. $tRNA_3^{Leu}$ is specified by *leuW,* which is in the *metT* operon in a cluster of six additional tRNA genes. $tRNA_4^{Leu}$ is specified by the *leuZ* gene in an operon with genes for glycine and cysteine acceptors. Finally, *leuX* specifies $tRNA_6^{Leu}$, which upon mutation of its anticodon becomes a suppressor of UAG codons (formerly called Su-6). It is also a single gene expression unit. (For the locations of these genes, see chapter 109.)

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