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Relationship Between Messenger Ribonucleic Acid and Enzyme Levels Specified by the Leucine Operon of *Escherichia coli* K-12

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The levels of leucine-forming enzymes in *Escherichia coli* K-12 varied over a several thousand-fold range, depending upon conditions of growth. The highest levels were achieved by growing auxotrophs in a chemostat under conditions of leucine limitation. Under such conditions, enzyme levels were increased 45- to 90-fold relative to cells grown in minimal medium containing leucine (the latter values arbitrarily called 1). Leucine operon-specific messenger ribonucleic acid levels were elevated to about the same extent as enzyme levels in cells grown in a chemostat. Growth in media of greater complexity resulted in progressively lower levels of leucine-forming enzymes, reaching a value of less than 0.02 for growth in a medium containing tryptone broth and yeast extract. The levels of leucine operon-specified enzymes and messenger ribonucleic acid were also measured in strains containing about 25 copies of plasmid pCV1(ColE1-*leu*) per chromosome. For such strains grown in minimal medium, enzyme levels were proportional to the number of plasmids per cell. Furthermore, they followed the same trends as those described above upon derepression in a chemostat or upon repression following growth in rich media. Leucine messenger ribonucleic acid, measured both by pulse-labeling and hybridization-competition experiments, was roughly proportional to enzyme levels over this entire range. For a plasmid-containing strain grown in a chemostat under conditions of leucine limitation (about 100 plasmids per chromosome), about 27% of pulse-labeled ribonucleic acid was coded for by genes in or adjacent to the leucine operon, and 10% of the total protein was β-isopropylmalate dehydrogenase.

The leucine operon of *Escherichia coli* B (36) and *Salmonella typhimurium* LT2 (9) consists of four contiguous structural genes (*leuDCBA*) that are transcribed as a unit and controlled by an adjacent operator and promoter region (*leuDCBAOP*). Less information is available for *E. coli* K-12, but what information there is suggests that the organization and control of the leucine operon in this organism is similar to that in other enteric bacteria (34, 5). The availability of phage λ-carrying portions of the leucine operon (15) has made it possible to study the relationship between messenger ribonucleic acid (mRNA) levels and enzyme levels specified by the leucine operon of *E. coli* K-12. We find that the levels of two of the enzymes specified by the operon vary more than 1,000-fold, depending upon the growth conditions, and that *leu* mRNA levels are approximately proportional to *leu* enzyme levels over this range.

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MATERIALS AND METHODS

The genotypes of the bacterial and phage strains used in this study are shown in Table 1. The compositions of tryptone broth, tryptone-yeast extract medium, and minimal medium are given in reference 15. The conditions for cell and phage growth, extract preparation, enzyme assays, deoxyribonucleic acid (DNA) isolation from phage, DNA strand separation, chemostat operation, digestion with endonuclease *Eco*RI, and agarose gel electrophoresis are also given in reference 15.

Isolation of RNA. RNA was isolated by a modification of the method of Ikemura and Dahlberg (20). Cultures (25 ml) grown in a chemostat or in Erlenmeyer flasks in minimal medium to about 5×10^8 cells/ml were poured over 35 ml of frozen, crushed medium containing 10 mM sodium azide and 400 μl of chloramphenicol per ml. The cells were centrifuged and suspended in 2 ml of a cold buffer containing 20 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.5), 40 mM ethylenediaminetetraacetic acid (EDTA), and 200 mM NaCl. The samples were mixed with an equal volume of boiling buffer containing 1% sodium dodecyl sulfate, swirled in a boiling water bath for 1 min, and trans-

TABLE 1. *Strains used in this study*

Strain	Relevant genotype and properties		Source
E. coli			
CV437	*leuA371 Δpro-lac thi*		This laboratory
CV438	*leuB061 Δpro-lac thi*		This laboratory
CV528	*leuD141* (λ)		R. Middleton
CSH26	*Δpro-lac thi*		Cold Spring Harbor strain kit
CSH62	*thi*		Cold Spring Harbor strain kit
CSH73	*Δara-leu Δlac thi*		Cold Spring Harbor strain kit
CGSC 4205	*leuB6*[a]		B. Bachmann
CV511	CGSC 4205/pCV1	Leu^{+b}; pCV1 is ColE1 attached to an intact leucine operon.	This study
CV510	CSH73/pCV2	Leu$^-$Tet^{r}λ^{imm}; pCV2 is pMB9 containing all of the *leu* operon except part of *leuD*.	This study
Bacteriophage λ			
λ*c*I857			M. Gottesman
λ*c*I*b*2			Cold Spring Harbor strain kit
λ*c*I*h*80			Cold Spring Harbor strain kit
λG4(p*leuABCD*)			This laboratory
λG5(p*leuABCD*)			This laboratory
λG7(d*leuABC*)			This laboratory

[a] Strain CGSC 4205 has α-IPM synthase activity but lacks β-IPM dehydrogenase activity. Therefore, the *leu-6* allele should be designated *leuB6*. Other markers in this strain are *argG6, metB1, his-1, recA1, mtl-2, xyl-7, malA1, gal-6, lacY1, str-104, tonA2, tsx-1, supE44*, and λ^{r}λ$^-$.

[b] Leu$^+$, able to grow in the absence of leucine; Tet^r, resistant to 20 μg of tetracycline per ml; λ^{imm}, immune to lysis by λ*c*I*b*2.

ferred to a 60°C water bath. An equal volume (4 ml) of water-saturated, redistilled phenol was added, and the samples were shaken for 3 min at 60°C with occasional vortexing. The samples were cooled to −10°C in an ice-ethanol bath and centrifuged for 5 min at 8,000 × *g* at −10°C. The upper layer, about 3.8 ml, was transferred to a freshly chilled tube, care being taken to exclude the precipitate at the interface. The sample was reextracted with phenol, and the RNA was precipitated by adding sodium acetate (pH 5.2) to 0.2 M and 2.5 volumes of cold 95% ethanol. After overnight incubation at −15°C, the precipitate was centrifuged, dissolved in 1 ml of 2× SSC (1× SSC is 150 mM NaCl plus 15 mM trisodium citrate), and dialyzed against 300 ml of 2× SSC.

Measurement of the rate of RNA synthesis. Cultures grown in a chemostat or in Erlenmeyer flasks to about 5 × 10^8 cells/ml were pulse-labeled for 3 min with [5-^{3}H]uridine (27 Ci/mmol; 10 μCi/ml). The cells were killed with azide and chloramphenicol, and RNA was isolated as described above. A 25-ml culture yielded 450 to 850 μg of RNA with a specific activity of 10,000 to 14,000 cpm/μg. The proportion of total label hybridizing to the separated strands of phage λ*c*I857 and λG4(p*leuABCD*) was determined by the liquid hybridization procedure of Bøvre and Szybalski (4). Occasionally, the filter hybridization method described by the same authors was employed (4).

Measurement of RNA levels by hybridization-competition. A limiting amount of λG5(p*leuABCD*) *r*-strand DNA, usually 0.2 μg, was added to a small siliconized tube. An amount of [^{3}H]RNA sufficient to saturate this DNA was added, together with 0 to 200 μg of unlabeled RNA and sufficient phenol-saturated 2× SSC to bring the total volume to 0.1 ml. The labeled RNA was prepared from strain CV437(*leuA371*) grown in a chemostat under conditions of leucine limitation and pulse-labeled for 3 min with [^{3}H]uridine (about 4% of the total label is in *leu* mRNA). After incubation at 67°C for 18 h, samples were treated with ribonuclease (RNase), diluted, filtered, dried, and counted as described by Bøvre and Szybalski for the liquid hybridization method (4). Data were analyzed by the method of Lavalle and DeHauwer (25) by the equation $Hy_{max}/Hy = \beta\hat{Z} + 1$, where Hy_{max} and Hy are counts per minute hybridized in the absence and presence of competing RNA, respectively, and $\hat{Z}$ is the ratio of the weights of unlabeled RNA and labeled RNA. The slope β is proportional to the weight percentage of mRNA in the sample of competing RNA.

Formation of plasmid pCV2. Fragment *Eco*RI-B (2 μg) was isolated from phage λG7(d*leuABC*) after

digestion with endonuclease *Eco*RI and gel electrophoresis as described (15). Ligation to plasmid pMB9 (3 μg), similarly treated with endonuclease *Eco*RI, was carried out in a total volume of 0.1 ml containing 12 mM $MgCl_2$, 10 mM dithioerythritol, 0.05 mM adenosine 5′-triphosphate, 50 mM Tris-hydrochloride (pH 7.5), 25 mM NaCl, and 0.1 Miles U of T4 ligase (Miles Laboratories, Inc., Elkhart, Ind.). After 4 h at 11°C, the mixture was used to transform strain CSH73(Δ*leu*), following procedures described by Lederberg and Cohen (27). After the chilling and heat-pulse steps and a 2-h recovery period in L-broth, samples were plated on nutrient agar plates containing 20 μg of tetracycline per ml and separately on similar plates previously spread with 10^9 particles each of λ*cIb*2 and λ*cIh*80. Transformants were purified, grown in M9 medium containing glucose and Casamino Acids (10), and treated for 10 h with 170 μg of chloramphenicol per ml to amplify plasmid DNA (10). After lysis by lysozyme-detergent treatment (23), cleared lysates were prepared (23), and samples of the latter were analyzed by agarose gel electrophoresis (15). In some cases, plasmid DNA was purified from cleared lysates by centrifugation in cesium chloride-ethidium bromide gradients (23). Strains carrying plasmid pCV2 were always grown in the presence of 20 μg of tetracycline per ml, because otherwise the plasmid was lost at a high frequency.

Estimation of the number of plasmids per chromosome. For the isolation of total cellular DNA, cells were centrifuged, washed in 1 volume of buffer containing 18.9 mM KH_2PO_4 and 10.4 mM Na_2HPO_4 (pH 7.5), and suspended in 1 ml of buffer containing 10 mM Tris-hydrochloride (pH 8.0), 10 mM EDTA, and 50 mM NaCl. To each sample was added 50 μl of 10% sodium dodecyl sulfate and 50 μl of a solution containing 1 mg of proteinase K (EM Laboratories, Elmsford, N.Y.) per ml (18). [^{14}C]thymine-labeled DNA (16,000 cpm, isolated from strain CSH73 by the same procedure) was added to allow estimation of DNA recovery, and the samples were incubated for 12 h at 37°C. An equal volume of phenol was added, each sample was shaken gently and centrifuged, and the water layers were removed and dialyzed against the second buffer described above. After dialysis, samples were treated with 50 μg of RNase A per ml for 4 h and then with 0.5% sodium dodecyl sulfate and 50 μg of proteinase K per ml for 12 h at 37°C. Phenol extraction was repeated, and the water layers were dialyzed against a solution containing 10 mM Tris-hydrochloride (pH 8.0) and 10 mM EDTA. This DNA isolation procedure is similar to one known to yield a plasmid preparation that is almost entirely nicked (13). Portions were added to 2 ml of 1 mM EDTA, denatured by heating in a boiling water bath for 3 min, and then cooled quickly on ice. The denatured DNA was adhered to nitrocellulose filters as described by Bøvre and Szybalski for the filter hybridization method (4).

pCV1 DNA was isolated from strain CSH73 by cesium chloride-ethidium bromide gradient centrifugation (23) as described above for pCV2 DNA. A reaction mixture for in vitro transcription of this DNA contained the following in a total volume of 0.1 ml: 20 mM Tris-hydrochloride (pH 7.9), 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.1 mM EDTA, 50 mM KCl, 0.3 mM cytidine 5′-triphosphate, 0.3 mM guanosine 5′-triphosphate, 0.3 mM adenosine 5′-triphosphate, 50 μCi of [^{3}H]uridine 5′-triphosphate (1 Ci/mmol), 5 μg of pCV1 DNA, and 3 U of RNA polymerase (Miles Laboratories). After 30 min at 37°C, 200 μg of *E. coli* B transfer RNA (tRNA) was added, and RNA was isolated as described.

Total cellular DNA bound to filters was hybridized to a constant amount of [^{3}H]RNA transcribed from pCV1 DNA in vitro, using the filter hybridization technique of Bøvre and Szybalski (4). Experiments were run in triplicate, and for each plasmid number determination, at least two different amounts of DNA were analyzed. The amount of plasmid DNA in a sample was determined from a standard curve prepared from samples containing known amounts of pCV1 DNA mixed with a 25- to 35-fold excess of chromosomal DNA. The number of plasmids per chromosome, n, was calculated from the equation $n = PM/(T - P)$, where P is the amount of plasmid DNA in the sample, M is the ratio of molecular weights of the *E. coli* chromosome and the plasmid chromosome ($2.5 \times 10^9/1.1 \times 10^7$), and T is the total amount of DNA in the sample. The recovery of DNA was about 80% for most samples.

RESULTS

Comparison of *leu* enzyme levels with the rate of synthesis of *leu*-specific mRNA in *E. coli* strains grown in a chemostat. Initial experiments were performed with a chemostat to achieve uniformity of growth conditions and the ability to repress or derepress the leucine operon. Strains having lesions in *leuA* (CV437), *leuB* (CV438), *leuD* (CV528), or *leuABCD*-(CSH73) were grown in a chemostat under conditions of leucine limitation (Table 2). The specific activities of α-isopropylmalate (IPM) synthase (*leuA* gene product; α-IPM α-ketoisovalerate lyase [coenzyme A acetylating], EC 4.13) and β-IPM dehydrogenase (*leuB* gene product; 2-hydroxy-4-methyl-3-carboxyvalerate:NAD^+ oxidoreductase, EC 1.1.1.85) were determined in crude extracts prepared from these strains. For comparison, data are also presented for prototrophic strain CSH62 grown in an Erlenmeyer flask with an excess of leucine and for strain CSH26 grown in a chemostat with excess leucine present but with a proline limitation. Clearly, limitation of leucine causes significant derepression of the leucine operon of *E. coli*, in the range of 45- to 90-fold above repressed levels. The specific activities of α-IPM synthase and β-IPM dehydrogenase were usually observed to vary coordinately, as they do in *S. typhimurium* (7, 8).

To determine the rate of synthesis of *leu*-specific mRNA, a portion of each of the cultures described in Table 2 was pulse-labeled with

[^{3}H]uridine. [^{3}H]RNA isolated from these cultures was hybridized to the separated strands of phages λ and λG4(p*leuABCD*) (Table 3). We showed previously that the *r* strand of λG4 corresponds to the *r* strand of λ(14). Some 4 to 5% of pulse-labeled RNA isolated from cultures grown under leucine limitation hybridized specifically with the *r* strand of λG4(p*leuABCD*). An estimate of the rate of synthesis of *leu*-specific mRNA in repressed cultures was made as follows. Strain CV437, a proline auxotroph, was grown in a chemostat and pulse-labeled under conditions of excess leucine and limiting proline. A small (0.23%) but significant fraction of [^{3}H]RNA isolated from this strain hybridized to the *r* strand of phage λG4 (Table 2, line 2). Another estimate, made using the filter hybridization method (4), was 0.27 ± 0.1% (average of five determinations). A similar value was obtained for strain CSH26 grown under the same conditions (Table 2). If it is assumed that all of this RNA is *leu* mRNA, then limiting an auxotroph for leucine causes about a 20-fold increase in the rate of *leu* mRNA synthesis (Table 2, compare lines 2 and 3). However, some of the hybridization is probably not due to

TABLE 2. *Comparison of leu enzyme levels and the rate of synthesis of leu mRNA in E. coli strains grown in a chemostat*

Strain	Limiting amino acid[a]	Sp act[b]		Rate of synthesis of *leu*-specific mRNA[c]
		α-IPM synthase	β-IPM dehydrogenase	
CSH26 (Δ*pro*)	Proline	0.72 (1)	1.46 (1)	0.25 (1)
CV437(*leuA371* Δ*pro*)	Proline	<0.001 (1)	0.78 ± 0.22 (6)	0.23 ± 0.06 (5)
	Leucine	<0.001 (1)	70 ± 41 (5)	4.8 ± 0.76 (13)
CV438(*leuB061* Δ*pro*)	Leucine	12.5 (1)	<0.006 (1)	3.8 (1)
CV528(*leuD141*)	Leucine	14.8 (1)	31.1 (1)	4.2 (1)
CSH73(Δ*leu*)	Leucine	<0.001 (1)	<0.006 (1)	0.18 ± 0.16 (12)
CSH62	None; excess leucine present[d]	0.24 (1)	1.5 (1)	

[a] Strains except CSH62 were grown in a chemostat at a generation time of 134 min in minimal medium. During leucine limitation, fresh medium was introduced that contained 10 μg of L-leucine per ml and 50 μg of L-proline per ml; during proline limitation, the concentrations were 100 μg of L-leucine per ml and 5 μg of L-proline per ml.

[b] Micromoles of product formed per hour per milligram of protein. For each determination, duplicate samples were run. The number of determinations is given in parentheses.

[c] Percent of input [^{3}H]RNA that hybridized to the *r* strand of λG4 phage (see Table 3). The value for CSH26 was obtained by the filter hybridization technique described by Bøvre and Szybalski (4) and is the average of three determinations.

[d] CSH62 was grown in an Erlenmeyer flask in minimal medium containing 50 μg of L-leucine per ml.

TABLE 3. *Hybridization of pulse-labeled [^{3}H]RNA isolated from leucine auxotrophs grown in a chemostat with the separated strands of phage λpleu*

Strain	Limiting amino acid[a]	RNA input[b] (cpm)	cpm bound to[b]:					Input cpm bound to λG4*r*[c] (%)
			No DNA	1 μg of DNA from:				
				λ*l*	λ*r*	λG4*l*	λG4*r*	
CV437(*leuA371* Δ*pro*)	Proline	256,000	128	202	186	249	726	0.23 ± 0.06 (5)
	Leucine	32,000	41	140	152	124	1,692	4.8 ± 0.76 (13)
CV438(*leuB061* Δ*pro*)	Leucine	32,700	62	60	81	140	1,330	3.8 (1)
CSH73 (Δ*leu*)	Leucine	91,400	130	164	146	244	400	0.18 ± 0.16 (12)

[a] RNA was isolated from the same cells that were used in the experiment described in Table 2. See footnote *a*, Table 2, for the conditions of growth. The length of the pulse was 3 min.

[b] Data are for a single experiment. The relatively high background in some experiments is probably due to a small amount of single-stranded chromosomal DNA contaminating the RNA preparation.

[c] Because values for no DNA, λ*l*, λ*r*, and λG4*l* were so similar, an average of the four was taken as a measure of the nonspecific bindings of label to the filters. This average was subtracted from the counts per minute hybridizing to λG4*r* DNA, and the latter number was divided by the input counts per minute to give percent input counts per minute bound to λG4*r*. The average from several experiments is shown, together with the standard deviation and the number of determinations (in parentheses). Each determination employed duplicate filters.

leu mRNA, because about 40% of the bacterial DNA carried by λG4 is not part of the leucine operon (15). An estimate of non-*leu* mRNA that hybridizes to the *r* strand of λG4 DNA was obtained by carrying out the hybridization with pulse-labeled RNA isolated from strain CSH73(Δ*ara-leu*) grown under conditions of leucine limitation (Table 3). There is sufficient variation in these data that an accurate estimate cannot be made. However, it seems likely that at least half of the hybridization observed for cells grown in excess leucine is due to non-*leu* mRNA. This would place the increase in mRNA synthesis (about 40-fold) in the same range as the increase in enzyme levels (45- to 90-fold). We conclude that under these conditions of growth, leucine-forming enzyme levels are roughly proportional to *leu*-specific mRNA levels (compare columns 4 and 5 in Table 2).

Levels of leucine-forming enzymes in crude extracts of *E. coli* K-12 grown in different media. The study described above employed leucine auxotrophs grown in a chemostat. To investigate more stringent conditions of repression, enzyme levels were measured in crude extracts of leucine prototrophs grown in minimal, supplemented minimal, and rich media (Table 4). The addition of L-leucine at 50 μg/ml to a minimal medium repressed β-IPM dehydrogenase formation by a factor of 3 to 4. Addition of more leucine had no further effect upon dehydrogenase levels (data not shown). However, enriching the minimal medium by adding Casamino Acids or a mixture of amino acids, purines, pyrimidines, vitamins, and trace minerals caused a significant reduction in the specific activity of β-IPM dehydrogenase. A further large reduction in specific activity was observed when cells were grown in a very rich medium such as tryptone broth or tryptone broth containing yeast extract. A mixing experiment was performed to determine whether extracts prepared from cells grown in rich medium contain substances that inhibit these enzymes. Extracts prepared from strain CSH73 (Δ*ara-leu*) grown in tryptone broth or tryptone broth and yeast extract were mixed with an extract of strain CSH26 grown in minimal medium. No inhibition of either enzyme was observed.

The data in Tables 2 and 4 taken together demonstrate that leucine-forming enzyme levels in *E. coli* K-12 vary more than 1,000-fold, depending upon conditions of growth. The hybridization techniques described in the previous section are not sensitive enough to determine whether the proportionality between mRNA and enzyme levels is maintained under conditions of severe repression. However, the isolation of strains possessing multiple copies of the leucine operon, described below, allowed us to investigate this question.

Isolation of plasmids carrying portions of the leucine operon of *E. coli* K-12. λG7(d*leuABC*) is a defective phage carrying all of the leucine operon except the operator-distal portion of *leuD* (15) (Fig. 1). A portion of the λ genome extending from the *b2* region through gene *N* is missing in phage λG7, but as indicated below, gene *c*I857 is present. Endonuclease *Eco*RI cleaves λG7 DNA into four fragments, the second largest of which (fragment B, molecular weight 6.7 × 10^6) contains the leucine operon (Fig. 2).

Plasmid pMB9, a derivative of plasmid ColE1, exists in about 20 copies per cell, is

TABLE 4. *Specific activity of β-IPM dehydrogenase in crude extracts of E. coli K-12 grown in different media*

Strain	Medium[a]	Generation time (min)	Sp act[b]	
			α-IPM synthase	β-IPM dehydrogenase
CSH62(*thi*)	Minimal	60	0.74	4.4
	Minimal + leucine	60	0.35	1.5
	Minimal + Casamino Acids	25	0.08	0.48
	Tryptone broth	25	0.01	0.02
	Tryptone broth + yeast extract	25	0.013	0.01
CSH26(*thi* Δ*lac-pro*)	Minimal	60	0.24	2.7
	Minimal + leucine	60	0.09	0.70
	Minimal + Casamino Acids	25	0.04	0.41
	Synthetic rich medium	25	0.02	0.10
	Tryptone broth	25	0.008	0.04
	Tryptone broth + yeast extract	25	0.002	0.009

[a] Leucine and Casamino Acids were added at concentrations of 50 μg/ml and 0.12%, respectively. Thiamine (1 μg/ml) and proline (50 μg/ml) were added where required. Synthetic rich medium is minimal medium containing 20 amino acids, vitamins, minerals, and nucleotide bases (30).

[b] Micromoles of product formed per hour per milligram of protein. Values are for a single determination, each done in duplicate.

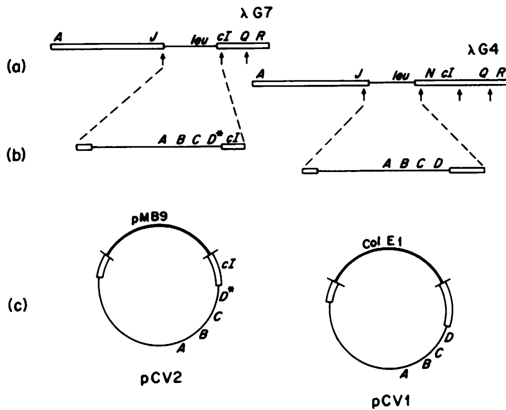

FIG. 1. *Diagrammatic representation of the formation of plasmids pCV1 and pCV2. (a) Phages λG7(dleuABCD*) and λG4(pleuABCD). Arrows indicate positions of cleavage by endonuclease EcoRI. Double line, Phage DNA; single line, bacterial DNA. (b) Expanded view of fragments carrying leu genes. An asterisk indicates that only a portion of the D gene is present. (c) Plasmids pCV1 and pCV2. Regions derived from ColE1 and pMB9 are represented by a thickened line.*

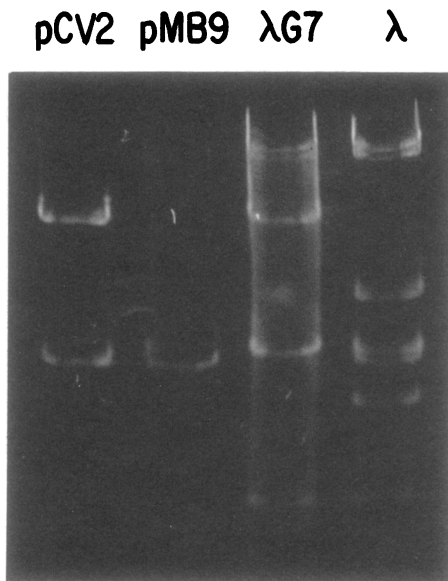

FIG. 2. *Agarose gel electrophoresis of EcoRI digests of λ, λG7(dleuABC), pMB9, and pCV2(pMB9-leuABC) DNAs.*

cleaved at a single site by *Eco*RI, and carries a gene specifying resistance to tetracycline (32). Fragment λG7 *Eco*RI-B, isolated by gel electrophoresis, was ligated to *Eco*RI-treated pMB9 DNA, and the mixture was used to transform CSH73(*Δleu*). Samples of the mixture were spread on plates containing tetracyline and separately on plates containing tetracycline and particles of λ*cIb*2 and λ*cIh*80. The frequency of Tet^r and $Tet^r\lambda^{imm}$ cells was 2×10^{-5} and 2×10^{-6}, respectively. Six of 32 clones selected for Tet^r were λ^{imm}.

Cultures of 11 $Tet^r\lambda^{imm}$ clones and 1 Tet^r clone were treated with chloramphenicol to amplify the plasmid, and samples of cleared lysates prepared from them were electrophoresed on agarose gels after treatment with *Eco*RI. Plasmid DNA from 9 of 11 $Tet^r\lambda^{imm}$ clones showed two bands corresponding in mobility to fragments λG7 *Eco*RI-B and pMB9 *Eco*RI-A (see track 1, Fig. 2 for an example). The plasmid from the Tet^r clone was pMB9 as expected. The recombinant plasmid is designated pCV2.

Phage λG4(p*leuABCD*) carries the entire leucine operon of *E. coli* K-12 (15). The latter is intact on an *Eco*RI-generated fragment (fragment B, molecular weight 7.3×10^6) of the phage DNA (Fig. 1). With methods similar to those described above, a recombinant plasmid was cloned after ligation of λG4 *Eco*RI-B with *Eco*RI-treated ColE1 (M. J. Finley and J. M. Calvo, unpublished data). This recombinant plasmid was designated pCV1.

Comparison of *leu* enzyme and mRNA levels in strain CV511(CGSC 4205/pCV1) grown in different media. Since strain CV511(CGSC 4205/pCV1) lacks a functional *leuB* gene on the chromosome, β-IPM dehydrogenase produced by this strain is specified entirely by the *leuB* gene carried on plasmid pCV1. The level of β-IPM dehydrogenase in this strain grown in minimal medium containing leucine was about 20 times higher than that for a wild-type strain grown under the same conditions (Table 5, line 2).

The elevated levels of β-IPM dehydrogenase in strain CV511 might be due to a gene dosage effect, since ColE1-related plasmids are known to be present in multiple copies per cell (10). The number of copies of plasmid per cell was determined by RNA-DNA hybridization. Total cellular DNA, isolated by phenol extraction after lysis by sodium dodecyl sulfate and digestion with proteinase K and RNase, was denatured, adsorbed to nitrocellulose filters, and hybridized to ^{3}H-labeled complementary RNA prepared by transcription of pCV1 DNA in vitro. A standard curve of counts per minute hybridized versus amount of pCV1 DNA was not linear (Fig. 3), and therefore a set of pCV1 DNA standards was included in each determination of an unknown. Using this assay, we estimated that strain CV511 grown in minimal medium plus leucine contained about 20 copies of plasmid pCV1 per chromosome (Table 5, line

TABLE 5. *Comparison of leu enzyme levels, leu mRNA levels, and the number of pCV1 plasmids for strain CV511(CGSC 4205/pCV1) grown in different media*

Medium[a]	Sp act[b] of β-IPM dehydrogenase in strain:			*leu*-specific mRNA in strain CV511 as determined by:		No. of plasmids/chromosome[e]
	CSH26	CSH62	CV511	Pulse-label expt[c]	Competition hybridization[d]	
Min	2.7 (3.9)	4.4 (2.9)	74 ± 7 (2.3)		0.38 (2.1)	
Min + leu	0.70 (1)	1.5 (1)	32 (1)	1.37 (1)	0.18 (1)	18
Min + CAA	0.41 (0.59)	0.48 (0.32)	8.2 ± 1.5 (0.26)	0.90 (0.65)	0.06 (0.3)	19
TB	0.04 (0.057)	0.2 (0.013)	2.7 ± 0.5 (0.08)	0.18 (0.13)	0.04 (0.22)	25
TYE	0.009 (0.013)	0.01 (0.007)	0.84 ± 0.18 (0.026)	0.07 (0.05)	0.0075 (0.04)	50

[a] Min, Minimal medium (15); leu, 50 μg of L-leucine per ml; CAA, 0.12% Casamino Acids; TB, tryptone broth (5); TYE, tryptone broth plus yeast extract (5).

[b] Micromoles of product formed per hour per milligram of protein. The values for CSH26 and CSH62, taken from Table 4, are shown for comparison.

[c] Determined as indicated in Table 3 and in Materials and Methods. The values represent the percentage of total pulse-labeled RNA that hybridize to the *r* strand of λG4 DNA.

[d] The values represent the slopes of curves of the type shown in Fig. 4. When RNA from strain CSH73(Δ*ara-leu*) served as competitor, the slope was 0.0015. The data above have not been corrected for any such background.

[e] Determined as shown in Fig. 3.

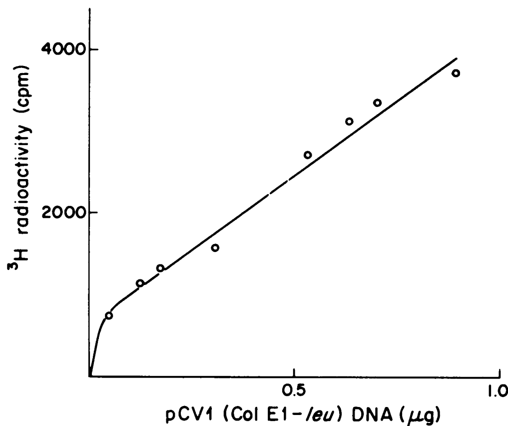

FIG. 3. *Standard curve for plasmid number determination. The ordinate represents counts per minute from [^{3}H]RNA transcribed in vitro from plasmid pCV1 DNA that hybridized to the indicated amounts of pCV1 DNA. Each hybridization took place in the presence of a 25-fold excess of E. coli DNA.*

2). Thus, under these conditions of growth, *leu* enzyme levels were directly proportional to the number of *leu* genes per cell.

The experiments described above were also carried out with strain CV511 grown in minimal medium, in supplemented minimal media, and in rich media (Table 5, lines 1, 3, 4, 5). For comparison, similar values for strains CSH26 and CSH62 are included in Table 5. Whereas the number of plasmids per chromosome did not vary appreciably for strain CV511 grown in different media, the level of β-IPM dehydrogenase decreased markedly as the medium became richer. Moreover, if the amount of dehydrogenase in cells grown in minimal medium containing leucine is arbitrarily set at 1, then the relative increase or decrease in level was roughly the same for the three strains. This suggests that the expression of one *leu* gene in strain CV511 is not being affected by expression of other *leu* genes in the same strain. This, in turn, makes mRNA measurements on this strain meaningful.

leu-specific mRNA in strain CV511(CGSC 4205/pCV1) was determined by two different methods. Data from the pulse-label method are shown in Table 5. The relative decrease in the rate of mRNA synthesis dependent upon growth conditions was similar to the relative decrease observed in enzyme levels. However, the results obtained by the pulse-label method are difficult to evaluate when different conditions of growth are employed because of possible differences in uridine pool sizes and in the rates of ribosomal RNA synthesis. For three growth conditions, at least, the generation times were the same (25 min; Table 4). Another potential source of error stems from the fact that the phage used to measure *leu* mRNA contains about 2 × 10^6 daltons of non-*leu* bacterial DNA (15). One could argue that most of the hybridization observed was from non-*leu* mRNA, and that growth of cells in rich medium repressed synthesis of this non-*leu* mRNA.

As a check on the results of the pulse-label method, we determined mRNA levels by hybridization competition, a method that is not beset by problems of labeling. Unlabeled RNA

from cultures described in Table 5 was used as a competitor in hybridizations involving pulse-labeled [^{3}H]RNA isolated from strain CV437(*leuA371*) grown in a chemostat under conditions of leucine limitation (Fig. 4). The pulse-label method measures the rate of mRNA synthesis, whereas the hybridization-competition method measures steady-state levels of mRNA. If the values for cells grown in minimal medium containing leucine are arbitrarily set at 1, then the relative increase or decrease in these values dependent upon growth conditions is very similar for the two methods (Table 5). The simplest interpretation of these results is that the rate of *leu* mRNA degradation is the same for all of these growth conditions, and that therefore the decrease in steady-state levels is due to a decrease in the rate of synthesis of *leu* mRNA. These results also demonstrate that the pulse-labeled RNA hybridizing to λG4(p*leu*) DNA is, in fact, *leu* mRNA.

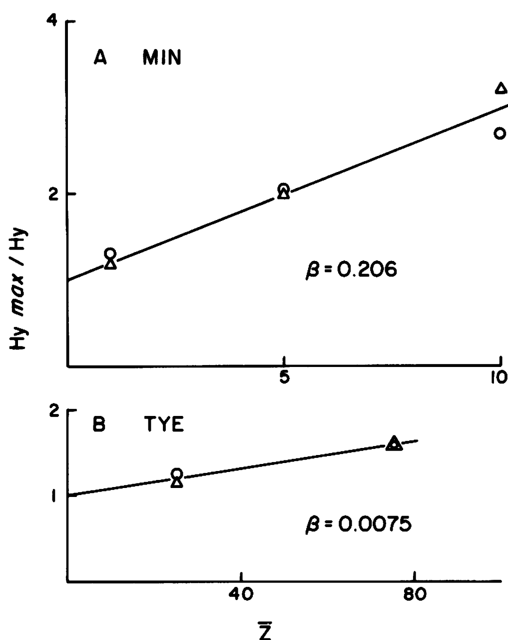

FIG. 4. *Hybridization-competition data. [^{3}H[RNA isolated from a leucine auxotroph grown under conditions of leucine limitation was mixed with unlabeled RNA and hybridized to the r strand of λG5-(leuABCD) DNA. Hy is the counts per minute hybridized. Hy_{max} is the counts per minute hybridized in the absence of competitor. $\bar{Z}$ is the ratio of the weights of unlabeled and labeled RNA. (A and B) Unlabeled RNA isolated from strain CV511 grown in minimal medium and in tryptone-yeast extract, respectively. β represents the slope of each curve. Circles and triangles represent results from two different experiments.*

The important point is that mRNA levels were considerably reduced under conditions of severe repression of the leucine operon (Table 5). Thus, the lowered enzyme levels observed for cells grown in rich medium is due, at least in part, to a reduced rate of transcription of *leu* mRNA. Since strain CV511 has about 20 copies of the *leu* operon per chromosome, and since the expression of one *leu* operon seems to be unaffected by other *leu* operons, the rate of *leu* mRNA synthesis in a wild-type strain can be estimated as 1/20 that in strain CV511. Therefore, the difference between the highest and lowest rate of *leu* mRNA synthesis was greater than 1,000-fold (4.8% for a culture grown in a chemostat versus 0.07/20 = 0.0035 for a culture grown in tryptone plus yeast extract).

Comparison of *leu* enzyme and mRNA levels in strain CV510(CSH73/pCV2) grown in a chemostat. As described earlier, increasing the number of leucine operons per cell by a factor of 20 resulted in a proportional increase in levels of leucine-forming enzymes. One possibility is that there was sufficient putative repressor so that in the presence of excess leucine, expression of each leucine operon remained relatively low. Another possibility is that expression of the leucine operon in strain CV511(CGSC 4205/pCV1) grown in minimal medium was near maximal due to the limitation of some factor necessary for expression of the operon. To eliminate one of these two possibilities, we grew strain CV510[CSH73(Δ*leu*)/pCV2 *leuABC*] in a chemostat under conditions of leucine limitation and measured *leu* enzyme levels and the rate of *leu* mRNA formation (Table 6). Clearly, the leucine operon of strain CV510 was dere-

TABLE 6. *Comparison of leu enzyme levels, leu mRNA levels, and the number of pCV2 plasmids for strain CV510(CSH73/pCV2) grown under conditions of repression and derepression*

Growth conditions[a]	β-IPM dehydrogenase[b]	Input cpm (%) bound to[c]: λl	λr	λG4l	λG4r	No. of plasmids/chromosome[d]
Chemostat, leucine limiting	500	1.6	1.0	3.2	28.3	114
Minimal + leucine	46					45

[a] Strain CV510 was grown in minimal medium containing 20 μg of tetracycline per ml in a chemostat (generation time, 134 min; L-leucine in fresh medium at 10 μg/ml) or in shake cultures supplemented with 50 μg of L-leucine per ml.

[b] Micromoles of product formed per minute per milligram of protein.

[c] Determined as indicated in Table 3 and in Materials and Methods. Input was 37,000 cpm. Hybridization to a filter lacking DNA gave 193 cpm.

[d] Determined as shown in Fig. 3.

pressed under conditions of leucine limitation. The specific activity of β-IPM dehydrogenase was 10-fold higher than that observed for the same cells grown in the presence of excess leucine (Table 6) and several hundred-fold higher than that for a wild-type strain grown in minimal medium containing excess leucine. A specific activity of 500 μmol/h per mg of protein corresponded to 10% of the total protein in the cell being β-IPM dehydrogenase.

Growth of plasmid-containing strains under conditions of partial starvation for an amino acid might be expected to cause amplification of the plasmid DNA. Certainly, plasmid DNA is amplified when protein synthesis is inhibited by chloramphenicol (10) and when cells are totally starved for an amino acid (11). An increase in the number of plasmids per cell was observed for chemostat-grown cells, but the increase (2.5 times) was not large enough to account for the degree of derepression observed (10 times; Table 6).

A high proportion of the pulse-labeled RNA isolated in this experiment hybridized to the *r* strand of λ4(p*leuABCD*) (28%; Table 6). Almost all of this RNA was complementary to bacterial DNA on λG4, since only 1% of the label hybridized to λ*r* DNA. It remains to be determined how much of the hybridizable label is in *leu* mRNA and how much is in other bacterial mRNA.

DISCUSSION

Expression of the leucine operon of *S. typhimurium* was shown to vary from a relative value of 1 (cells grown with excess leucine) to a relative value of about 40 to 60 (cells grown under conditions of leucine limitation) (8). Here we demonstrate that the same is true for *E. coli* K-12. Furthermore, the minimum expression of the leucine operon of *E. coli* is very low (relative value less than 0.02 for cells grown in a medium containing tryptone and yeast extract), so that the total range of expression in this organism is at least several thousand-fold. Expression of the operon over the range of 1 to 40 is clearly controlled by the availability of leucine. The intracellular concentration of leucine determines the level of leucyl-tRNA, and the latter probably plays a more direct role in repression than does leucine (1, 12, 31). Is expression of the leucine operon over the range of 1 to 0.02 also under the control of leucine? This could be the case, for example, if Casamino Acids facilitated entry of leucine or if leucine-containing peptides in tryptone broth, transported by an independent mechanism, resulted in a higher intracellular pool of leucine. Another possibility is that expression of the leucine operon is determined in part by the growth rate of the cells. This cannot be the predominant factor, because a considerable amount of repression occurs under conditions where the generation time is a constant 25 min (Table 4). A third possibility is that one or several components of complex media signal the presence of a rich medium and that this in turn leads to further repression of the leucine operon by an unknown mechanism.

It may be noted that $tRNA^{Leu}$ isolated from cells grown in minimal medium containing leucine is charged to the extent of about 98% (1). Thus, if leucyl-tRNA is a co-repressor regulating the entire range of expression of the leucine operon, then charging the remaining 2% of $tRNA^{Leu}$ results in more than 50-fold repression of the operon.

Regardless of the nature of the repression signal, it is pertinent to ask whether a single mechanism functions in controlling the operon over a several thousand-fold range of expression. An operator-repressor interaction controls the *lac* operon over a 1,000-fold range of expression (17). On the other hand, regulation of the *trp* operon occurs by two mechanisms, one involving an operator-repressor interaction (33) and a second involving a transcription attenuation mechanism (3). Also, there have been suggestions that some operons are controlled in part at the level of translation (24, 26). The fact that *leu*-specific mRNA levels are roughly proportional to enzyme levels over a wide range of values is consistent with the leucine operon being controlled predominantly at the level of transcription. We have no direct evidence for either an operator-repressor mechanism or a transcription-attenuation mechanism. The properties of operator-constitutive mutations described previously (9) can be easily reconciled with either mechanism. Indeed, some *hisO* mutations are now interpreted as being deletions within a putative-transcription attenuator (2, 20). An unlinked regulatory locus affecting leucine operon expression (16) could code for a repressor, but some other role for this locus (an involvement in tRNA synthesis or maturation, for example [6]) cannot be excluded. In some preliminary experiments designed with the transcription-attenuation model in mind, we measured leucine-forming enzyme levels in *suA* strain 2054 studied by Wasmuth and Umbarger (35) and in three *psu* strains described by Korn and Yanofsky (22). Leucine enzyme levels in these strains were identical with those in the parent strains.

The results in Table 6 indicate that in the absence of a signal for leucine limitation, the

leucine operon remains extensively repressed, even when the number of copies of the operon is elevated by a factor of 20 to 30. The same conclusion was reached when the number of copies of the operon was amplified by multiplication of phage λG4(p*leuABCD*) (15). Therefore, there is no indication of escape synthesis from the leucine operon, as is the case when the *lac* repressor is titrated out by multiple copies of the *lacO* gene (28). Hershfield et al. similarly observed that tryptophan-forming enzyme levels were repressed in a strain containing multiple copies of the *trp* operon as ColE-*trp* (19). We conclude that if a *leu* repressor exists, it must either be coded for by a gene carried by plasmids pCV1 and pCV2 or be present in higher concentration (>50 copies per cell) than is the *lac* repressor (about 20 copies per cell) (17). Furthermore, the fact that such a large proportion of the cells' transcriptional and translational machinery can be mobilized toward leucine biosynthesis (Table 6) indicates that, if a positive regulatory factor is required for expression of the operon, it must exist in at least hundreds of copies per cell.

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