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In Vitro Formation of β-Galactosidase with a Template Containing the *lac* Genes Fused to Gene *ilvD*

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An in vitro coupled transcription-translation system was used to synthesize transaminase B and β-galactosidase in the presence of a deoxyribonucleic acid template containing *lac* deoxyribonucleic acid under normal *lac*-specific control and in the presence of several deoxyribonucleic acid templates containing *lac* deoxyribonucleic acid fused to the *ilvD* gene. Time course experiments revealed that transcription of the *lacZ* gene from the fusion template required a longer time than did that initiated at the *lac* promoter. With a phage template containing an intact *ilvE* gene but lacking the normal *ilv*-specific promoter, synthesis of *ilvE* message was completed before synthesis of *lacZ* message. A phage template that contained the normal *ilv*-specific promoter but from which part of *ilvE* had been deleted also allowed formation of β-galactosidase. Three plasmids containing the *ilv-lac* fusion were also used as templates. Two plasmids that contained both an intact *ilvE* gene and the normal *ilv*-specific promoter required longer times for *lacZ* transcription but were more efficient templates than was a plasmid in which the *ilv-lac* fusion, the *ilvE* gene, and the contiguous non-specific *ilvE* promoter were inverted with respect to the normal *ilv*-specific promoter. β-Galactosidase synthesis was stimulated by guanosine 3'-pyrophosphate-5'-pyrophosphate with all templates tested except that in which the *ilv-lac* fusion had been inverted. Presumptive evidence was obtained for the generation of a limiting isoleucine signal by incorporating inhibitors of isoleucyl transfer ribonucleic acid synthetase into the coupled transcription-translation system.

Studies with *Escherichia coli* strains with the *lac* genes controlled by the *ilvEDA* promoter have indicated that the site of *ilv* operon control lies outside the *ilvO* region long thought to be the *cis*-acting control element for the *ilv* operon (5, 27). The strains carrying these fusions were prepared by the Casadaban (2) technique, so that it is possible to isolate λ derivatives carrying the fusion. With the DNA from such phages as template, it is possible to perform in vitro synthesis of β-galactosidase using the standard system of Zubay et al. (36). Such experiments are reported in this paper not only with the phages as templates but also with plasmid DNAs derived from them. The experiments provided presumptive evidence that a limiting isoleucine signal could be generated in vitro.

MATERIALS AND METHODS

Bacterial and bacteriophage strains and plasmids. The *E. coli* strains, bacteriophages, and plasmids used in this study are listed in Table 1.

Media and growth of cells and phage. The media used and the procedures for preparing S-30 extracts from strain CU903 were as described previously (33). The procedures for producing λ *h80 dlacZp* from a heat-inducible lysogen and for the lytic growth of the plaque-forming phages, λ p*ilv-lac*3 and λ p*ilv-lac*4 were also as described previously (33).

DNA template preparation. λ *h80 dlacZp*, λ p*ilv-lac*3, and λ p*ilv-lac*4 DNAs were prepared by extracting purified phage with phenol (30). The DNA solutions were dialyzed against two changes of 0.01 M sodium EDTA (pH 8.0) followed by dialysis against two changes of 0.02 M Tris-acetate (pH 8.0). The DNA concentration was estimated spectrophotometrically. Plasmid DNA was purified from transformed cells by the method of Sidikaro and Nomura (24).

In vitro protein synthesis. In vitro protein synthesis was performed by the procedure of Zubay et al. (36) as modified by Wild et al. (33). The S-30 extracts were prepared from a λ lysogen, strain CU903, and therefore contained λ repressor protein. The Mg^{2+} concentration optimal for the system was determined for each S-30 preparation. Cyclic AMP was employed only with the λ *h80 dlacZp* template. Except where otherwise indicated, 0.2 mM guanosine 3'-pyrophosphate-5'-pyrophosphate (ppGpp) was also present. Folinic acid (Lederle) solutions were prepared fresh every 2 weeks and stored at −20°C.

In vitro protein synthesis was followed by measuring the incorporation of [^{3}H]valine into the trichloroacetic acid-insoluble fraction. The 0.1-ml protein-syn-

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TABLE 1. *Bacterial strains, phages, and plasmids used*

Strain	Genotype[a]	Source or reference
Bacteria		
CU903	F⁻ Δ*ilvE2050 thi arg trp* Δ*lac* λ⁺	Watson et al. (31)
CU1152	pPU34/F⁻ Δ(*ilvGEDAC*)*2049 ara* Δ(*pro-lac*) *thi* λ⁻	Subrahmanyam et al. (27)
CU1153	pPU35/F⁻ Δ(*ilvGEDAC*)*2049 ara* Δ(*pro-lac*) *thi* λ⁻	Subrahmanyam et al. (27)
CU1154	pPU36/F⁻ Δ(*ilvGEDAC*)*2049 ara* Δ(*pro-lac*) *thi* λ⁻	Subrahmanyam et al. (27)
PP70	Δ*lac* λ *h80 c*I857 *St*68 λ *h80 c*I857 *St*68 d*lacZp*	H. Weissbach (3, 13)
Phages		
λ p*ilv-lac*3	Δ(*b-xis*)[*ilv'GOED' trp'BA' lac'ZoZY*]	Leathers et al. (15)
λ p*ilv-lac*4	Δ(*b-xis*)[(+'Mu')::(*ilvG'*-Δ2130-*'ED' trp'BA' lac'ZoZY*)]	Gayda et al. (5)
Plasmids		
pPU34	pBR313Ω5[0.375kb:λ *h80* d*ilv ilvGOEDAYC* −1.5–11.6kb(−)]Δ1 [*ilv'GOEDYAC'* 0.55–7.32kb]Ω7[2.42kb: λ p*ilv-lac*3 *ilv'GOED' trp'AB' lac'ZoZY* λ' 7.34(K-12)–17.28(λ)kb(−)]	Subrahmanyam et al. (27)
pPU35	pBR322Ω7[0.375kb:λ *h80* d*ilvO2200 ilvE2201 ilvGOEDAYC* −1.5–11.6kb(+)]Δ9[*ilv'GOEDAYC'* 0.55–7.34kb]Ω15[4.7kb:λ p*ilv-lac*3 *ilv'GOED' trp'AB' lac'ZoZY* λ' 7.34(K-12)–17.28(λ)kb(+)]	Subrahmanyam et al. (27)
pPU36	pBR322Ω7[0.37kb:λ *h80* d*ilvO2200 ilvE2201 ilvGOEDAYC* −1.5–11.6kb(+)]Δ10[*ilv'GOEDAYC'* 0.55–7.34kb]Ω16[4.7kg:λ p*ilv-lac3 ilv'GOED' trp'AB' lac'ZoZY* λ' 7.34(K-12)–18.96(λ)kb(−)]	Subrahmanyam et al. (27)

[a] The conventions described in the preceding paper (27) have been used in designating bacterial, phage, and plasmid genotypes. Thus, pPU34 contained a *Bam*HI insert of *ilv* genes from λ *h80* d*ilv* (18) and a replacement of the *ilv* DNA between two *Kpn*I fragment containing the *ilv-lac* fusion from λ p*ilv-lac*3. For more details, see footnote of Table 2 and Fig. 2 of reference 27.

thesizing system contained 5 μCi of [³H]valine. The synthesis was terminated by the addition of 3 ml of cold 5% trichloroacetic acid; after 15 min at 0°C, the precipitate was heated to 95 to 100°C for 15 min and removed by filtration through a membrane filter (0.45-μm pore size; Millipore Corp.). The filters were washed with 30 ml of 5% trichloroacetic acid and dried, and the radioactivity was determined.

Enzyme assays. Transaminase B was assayed by the method of Duggan and Wechsler (4). β-Galactosidase was assayed by the method of Zubay et al. (36).

Chemicals. Pseudomonic acid was kindly supplied by N. H. Rogers, Research Division, Beecham Pharmaceuticals, Betchworth, Surrey, United Kingdom. *Kpn*I restriction enzyme and T4 DNA ligase were obtained from New England BioLabs. All other chemicals were of the purest grades obtainable from commercial sources.

RESULTS

Kinetics of β-galactosidase formation with several templates. A series of templates that contained the *lacZ* gene was compared with respect to the time required for formation of the transcription initiation complex, elongation, and completion of the message and the additional time required for translation of the completed message. Similar kinetic measurements were made on the formation of transaminase B with one of the templates that carried both the *ilvE* and *lacZ* messages as part of the same transcript.

As a source of DNA with a *lac*-controlled *lac* operon, λ *h80* d*lacZp* was used. The times required for the initiation, elongation, and translation of β-galactosidase message were determined by the addition of rifampin, actinomycin D, or chloramphenicol at various times to a complete protein-synthesizing system incubated at 37°C. An additional 30-min incubation period allowed complete translation of initiated or completed transcripts. As shown in Fig. 1, 1.8 min was required for the initiation of *lacZ* message. The time between the initiation and completion of the *lacZ* message (difference between the time of the appearance of enzyme in tubes receiving rifampin and the time of its first appearance in tubes receiving actinomycin D) was 4.0 min.

It was then of interest to compare two templates in which the *lacZ* gene had been fused to *ilv* DNA. One of these, λ p*ilv-lac*3, had exhibited a coordinate formation of transaminase B and β-galactosidase in vivo, but the formation of neither enzyme was under *ilv*-specific control. The other, λ p*ilv-lac*4, containing a deletion of *ilv* DNA that allowed it to have picked up the *ilv* control region, exhibited an *ilv*-specific control over β-galactosidase formation in vivo, but

owing to the deletion did not exhibit transaminase B formation.

The in vitro formation of β-galactosidase with λ *pilv-lac*3 as template is shown in Fig. 2. The time required for the formation of the rifampin-resistant complex required about the same length of time as did that with λ *h80 dlacZp*, but the time required for completion of the *lacZ* message was much longer, 7.8 min. The longer time required for complete transcription of *lacZ* from this template is probably due to the fact that there are a small amount of *trp* DNA and about 3.6 kilobases (kb) of *ilv* DNA including the *ilvE* gene carried by this phage (15). Since the *ilvE* gene is upstream of the *lacZ* gene, the completion of the transaminase B message (Fig. 3) required only a little more than 1 min. Although the resolutions of the transcription times measured in these experiments do not allow one to estimate the site of transcription initiation of the various templates, it would appear that initiation of the transcript from λ *pilv-lac*3 DNA was quite close to the beginning of the *ilvE* gene itself.

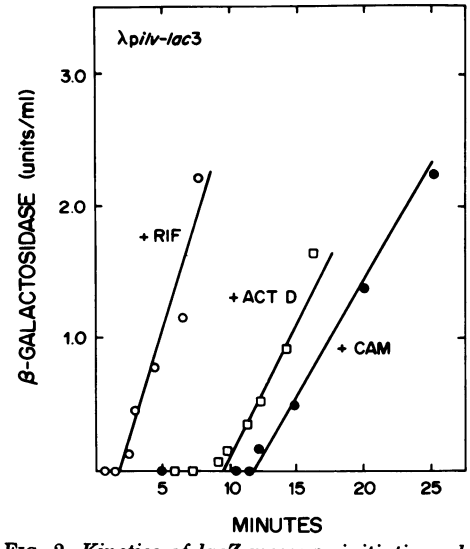

FIG. 2. *Kinetics of lacZ message initiation, elongation, and translation with λ pilv-lac3 DNA (70 μg/ml) as template. Other conditions as described in the legend to Fig. 1.*

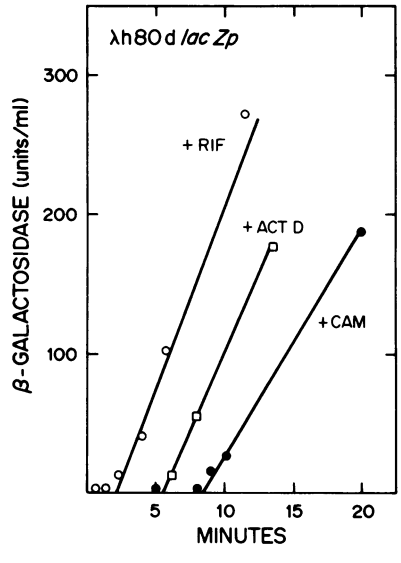

FIG. 1. *Kinetics of lacZ message initiation, elongation, and translation with λ h80 dlacZp DNA as template. Rifampin (○; 5 μg/ml), actinomycin D (□; 5 μg/ml), or chloramphenicol (●; 100 μg/ml) were added at various times after the initiation of protein synthesis with a coupled transcription-translation system containing λ h80 dlacZp (52 μg/ml) as template. The times indicated are the times after the beginning of the reaction at which the indicated additions were made, each to a separate reaction mixture. Incubation was continued for 30 min after each addition, after which the reaction mixtures were chilled at 0°C.*

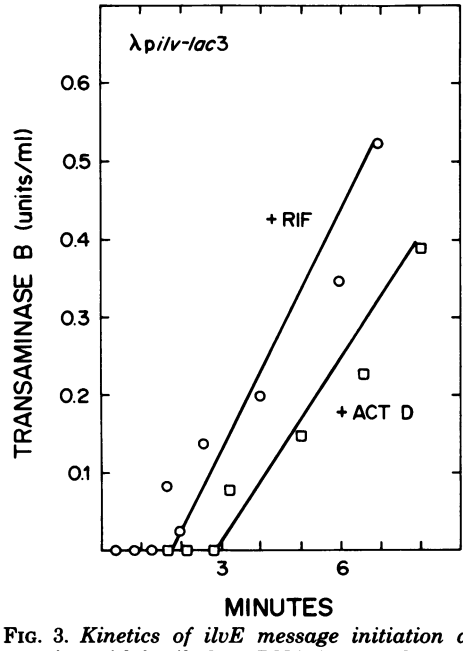

FIG. 3. *Kinetics of ilvE message initiation and elongation with λ pilv-lac3 DNA (70 μg/ml) as template. Addition of rifampin and actinomycin D as described in the legend to Fig. 1.*

Similar experiments were performed with λ *pilv-lac*4 DNA as template; this DNA differs from that of λ *pilv-lac*3 in that it contains the region of DNA shown in the previous paper (27) to contain the probable *ilv*-specific transcription

initiation site but has a deletion extending from early in the *ilvE* structural gene to beyond the point where the *ilv* DNA in λ p*ilv-lac*3 terminates. Again, about 2 min was sufficient for formation of the rifampin-resistant complex; about 6.9 min later the *lacZ* message was completed (Fig. 4). Earlier studies (5) had revealed that the 1.75-kb deletion carried in λ p*ilv-lac*4 included about 1.4 kb of *ilv* DNA contained in λ p*ilv-lac*3. Since it carried an additional 1-kb length of chromosomal DNA from beyond the deletion, the terminus of chromosomal DNA in λ p*ilv-lac*4 is about 1.35 kb beyond that in λ p*ilv-lac*3. As the normal *ilv*-specific transcription initiation point is within this 1.35-kb length, it is reasonable that about the same transcription time was required for β-galactosidase message from λ p*ilv-lac*4 as was required from λ p*ilv-lac*3.

It was of interest to examine three additional *ilv-lac* templates, plasmids pPU34, pPU35, and pPU36, since they each contained both the presumed *ilv*-specific promoter carried by λ p*ilv-lac*4 and the presumed *ilv*-nonspecific *ilvE* promoter carried by λ p*ilv-lac*3 (27). All three were prepared by substituting a *Kpn*I fragment of λ p*ilv-lac*3 containing the *ilv-lac* fusion for the region between two *Kpn*I sites in the *ilv* gene cluster. pPU34 and pPU35 were similar in that the weak *ilvE* promoter and the *ilv*-specific promoter had the same relationship to each other as they do on *Escherichia coli* chromosomal DNA. They differed from each other in that pPU34 contained the *ilv*-specific promoter in the same orientation as the *tet* gene into which it had been inserted, and the vector was pBR313, whereas pPU35 contained the *ilv*-specific promoter in the orientation opposite to the *tet* genes, and the vector was pBR322. pPU36 was like pPU35, except that the *Kpn*I fragment containing the *ilvE* promoter and the *ilv-lac* fusion had been inserted in the opposite orientation to that in pPU35. Thus, in pPU36 the *ilv*-noncontrolled promoter and the *ilv*-controlled one were not contiguous. In the preceding paper, it was shown that pPU35 exhibited *ilv*-specific control in vivo, whereas pPU36 did not (27). Figure 5 shows the kinetics of β-galactosidase formation after the addition of the three inhibitors at various times. With pPU34 and pPU35, the templates with the *ilv-lac* fusion in the correct orientation with respect to the region before *ilvG*, the time for initiation of transcription was 1.5 min, and the time for completion of the tran-

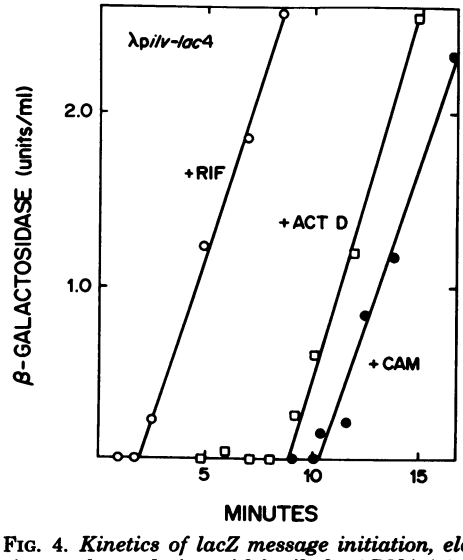

FIG. 4. *Kinetics of lacZ message initiation, elongation, and translation with λ pilv-lac4 DNA (16 μg/ml) as template. Rifampin (○), actinomycin D (□), or chloramphenicol (●) was added at indicated times. Other conditions as described in the legend to Fig. 1.*

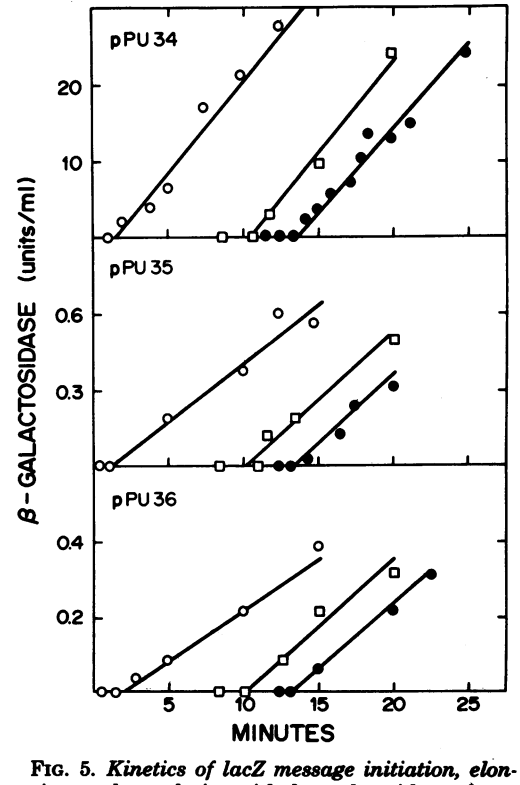

FIG. 5. *Kinetics of lacZ message initiation, elongation, and translation with three plasmid templates, pJN101 (29 μg/ml), pJN102 (4 μg/ml), and pJN103 (16 μg/ml). Other conditions as described in the legend to Fig. 1.*

script was 9.2 min. With pPU36, in which the *ilv-lac* fusion was in the wrong orientation with respect to the region before *ilvG*, the time for initiation was a little longer, 2.2 min, but the time for completion of the transcript was reduced to 8 min, as might be expected if the promoter were closer to the *lacZ* gene. Although the time required for transcription was reduced when the DNA upstream of *ilvG* was not contiguous with the *ilvE* gene, the efficiency of the template was also reduced.

Effect of isoleucine restriction on in vitro β-galactosidase synthesis. The *ilv* genes are normally repressed in the presence of excess isoelucine, valine, and leucine. Limitation of one of the amino acids leads simultaneously to a reduced rate of protein synthesis and a relative preferential expression of the *ilv* genes. A variety of treatments were employed to remove free amino acids so that an isoleucine limitation might be generated in vitro. Among these was the addition of 19 amino acids with only isoleucine omitted after extensive dialysis of the S-30 preparation. In the few cases in which extensive dialysis caused a dependence of protein synthesis upon added isoleucine, the synthetic activity of the extracts was so reduced that only the best template (that from λ *h80* d*lacZp*) permitted significant synthesis of β-galactosidase. For this reason, two antimetabolites thought to interfere with the charging of isoleucine acceptor tRNA were examined.

Effect of pseudomonic acid. The antibiotic pseudomonic acid has been shown to be an inhibitor of *E. coli* B isoleucyl tRNA synthetase that prevents the formation of the enzyme-aminoacyl adenylate complex (8). Therefore, this antibiotic was employed in vitro to alter the amount of charged isoleucyl tRNA. For this purpose pPU34 was used as a template, since of the two plasmids containing *lacZ* under *ilv*-specific control, it was more active in in vitro expression than was pPU35 (Table 2). Figure 6 shows the effect of increasing the concentration of pseudomonic acid on β-galactosidase synthesis and total protein synthesis with λ *h80* d*lacZp* and pPU34 as templates. The results are expressed as the percentage of enzyme activity remaining (or [^{3}H]valine incorporation) relative to the control containing no pseudomonic acid. β-Galactosidase activity was relatively unaffected at concentrations of pseudomonic acid up to 0.04 μg/ml when either template was employed (Fig. 6). Thereafter, a sharp decline in β-galactosidase activity occurred when the concentration was increased to 0.06 μg/ml in systems containing either template. However, when the concentration was increased to 0.08 μg/ml, β-galactosidase activity declined to zero in systems containing the λ *h80* d*lacZp* template, but virtually no further loss in activity occurred in systems containing the pPU34 template. The decrease in [^{3}H]valine incorporation was the same with either template and closely paralleled the decrease in β-galactosidase with pPU34 as template. As expected, [^{3}H]valine incorporation did not decrease to zero with either template, since some incomplete peptide synthesis might still occur. The low rate of β-galactosidase synthesis that persisted with pPU34 as template at concentrations of pseudomonic acid that prevented synthesis with λ *h80* d*lacZp* DNA as template may be an in vitro demonstration of a derepression of the *ilv* operon. It is difficult to explain the biphasic kinetics seen in the enzyme activity and incorporation experiments, since isoleucyl tRNA synthetase exhibits Michaelis-Menten kinetics in the binding of isoleucine, ATP, and tRNA (7, 23). It is possible that pseudomonic acid at low concentrations is tightly bound to other receptors not concerned with protein synthesis. If so, no effect on protein synthesis would occur until these were saturated.

TABLE 2. *Effect of ppGpp on in vitro β-galactosidase and protein synthesis with several templates*

DNA template	DNA concn (pmol/ml)	β-Galactosidase formed[a]		[^{3}H]valine incorporated[b]	
		No ppGpp	0.16 mM ppGpp	No ppGpp	0.2 mM ppGpp
λ *h80* d*lacZp*	1.67	92	247	53,733	53,677
pPU34	1.74	97	164	75,297	54,173
pPU35	0.29	8.7	12.5	8,140	3,963
pPU36	1.21	23	10	18,668	10,292
λ p*ilv-lac*3	2.22	0.5	1.5	47,928	46,240
λ p*ilv-lac*4	0.50	2.7	4.2	22,791	13,180
pBR322	8.96			26,582	13,594
λ *c*I857*S*7	4.35			17,143	7,971

[a] Nanomoles per minute per milliliter of transcription-translation incubation mixture.
[b] Trichloroacetic acid-precipitable radioactivity incorporated by the 0.1-ml incubation mixture in 15 min.

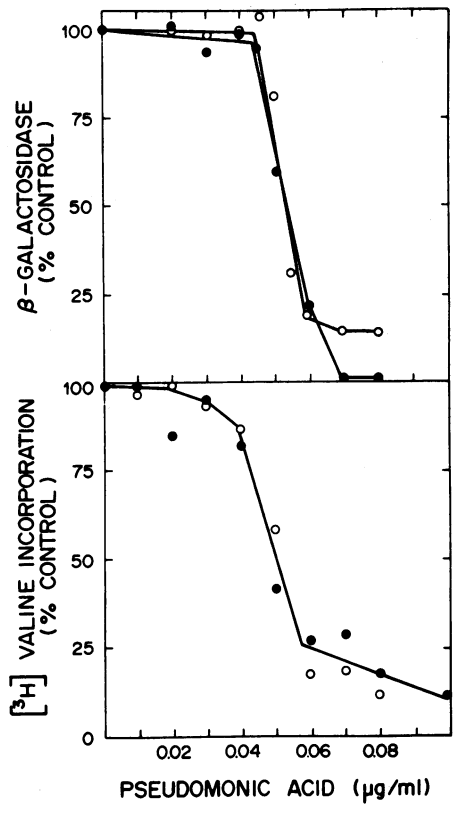

FIG. 6. *Effect of pseudomonic acid on the in vitro coupled transcription-translation of lacZ with λ h80 dlacZp (●; 52 μg/ml) DNA as template or with pJN101 (○; 25 μg/ml) as template. In vitro protein synthesis proceeded for 60 min in the presence of the indicated concentrations of pseudomonic acid. At the end of the incubation period, 25-μl samples were removed from each 0.1-ml reaction mixture for the determination of β-galactosidase, and 50-μl samples were removed for measurement of [^{3}H]valine incorporation. Results are expressed as percentage of the values in the control tube containing no pseudomonic acid.*

Effect of isoleucine hydroxamate. Several amino acid hydroxamates have been shown to inhibit amino acyl tRNA synthetases (28). Although isoleucine hydroxamate has not been thoroughly investigated in *E. coli*, a *Serratia marcescens* culture grown in its presence exhibits a derepression of the *ilvEDA* operon (12). Indirect evidence indicated that the analog inhibited not only threonine deaminase but also isoleucyl tRNA synthetase. Increasing the concentration of isoleucine hydroxamate resulted in a decrease in β-galactosidase activity when λ *h80 dlacZp* was the template employed, but significantly less inhibition was seen when pPU34 was employed (Fig. 7). Particularly, the higher concentrations resulted in greater differences between the two templates. Total protein synthesis from the two templates was decreased to the same extent, although not as much as the inhibition of β-galactosidase activity from the λ *h80 dlacZp* template.

Effect of ppGpp on protein and β-galactosidase syntheses with several templates. The nucleotide ppGpp is known to stimulate transcription from some promoters and to impede transcription initiated from others (22, 29). In vitro transcripts of several biosynthetic operons, as well as those of several catabolic operons such as the *lac* operon, have been shown to be stimulated by ppGpp (6, 10, 21, 22, 25, 26, 33, 34). The effect of ppGpp on in vitro β-galactosidase synthesis with several templates used here is shown in Table 2. The formation of β-galactosidase was stimulated by ppGpp with the template containing *lacZ* under *lac*-specific con-

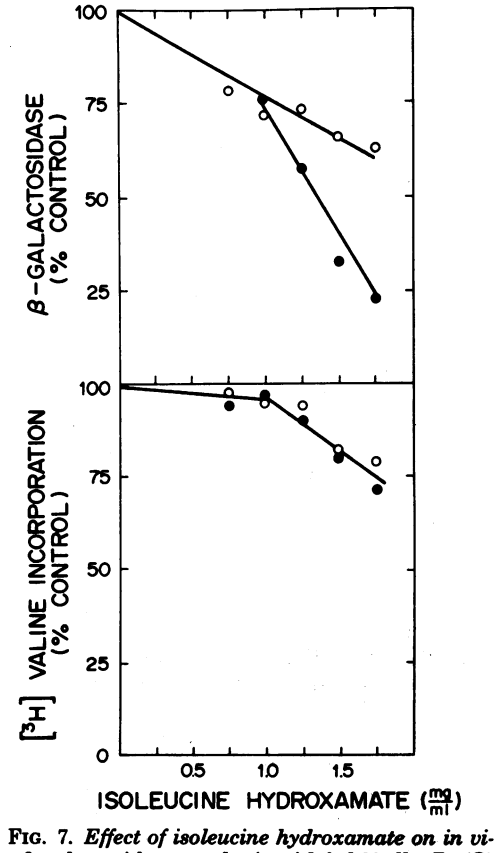

FIG. 7. *Effect of isoleucine hydroxamate on in vitro β-galactosidase synthesis with λ h80 dlacZp (●) or with pJN101 (○) DNA as template. The reaction mixtures contained isoleucine hydroxamate as indicated. Other conditions as described in the legend to Fig. 6.*

trol (λ *h80* d*lacZp*) as well as those under *ilv*-specific control (pPU34, pPU35, and λ p*ilv-lac*4). There was also stimulation of synthesis when λ p*ilv-lac*3 was used, even though *lac* expression is not under *ilv*-specific control and the in vitro template activity of the DNA is poor. Only with the plasmid in which the *ilv-lac* fusion had been inserted into *ilv* DNA so that an inversion occurred between the presumed *ilvGEDA* promoter and the beginning of the *ilvE* gene did ppGpp inhibit expression. In contrast, bulk protein synthesis was inhibited by ppGpp with all templates except λ *h80* d*lacZp*, in which incorporation was unaffected. It may be that with that template, the very active transcription that occurs with the "up-promoter" mutation in the *lac* operon compensates for the decrease in protein synthesis that ppGpp causes for other operons carried on the phage.

DISCUSSION

An obvious goal in using an in vitro coupled transcription-translation system with a defined DNA template was not only to demonstrate but also to regulate gene expression. Such a demonstration has been successful with several systems in which a protein-DNA interaction is an important feature in the regulation (20, 32, 37). In two amino acid-forming systems, those leading to tryptophan and to arginine, it has been possible to demonstrate repression upon adding preparations containing repressor protein to the transcription-translation system (11, 35, 38). The corepressors tryptophan and arginine, also part of the repression signal, are always present as part of the complete protein-synthesizing system. Similarly, in the inducible step in isoleucine and valine biosynthesis, the necessity for a positive control protein for coupled transcription-translation has been demonstrated (31).

For those biosynthetic pathways for which the only "repress-derepress" signal is generated independently of an added protein and is more likely inherently coupled to the translatability of the leader (attenuation control), a direct in vitro demonstration of regulation will be more difficult. For systems examined so far, in which the signal for transcription of the structural genes is dependent upon charging of the homologous acceptor tRNA, the very conditions that allow the optimal transcription of the operon are those that reduce translation (i.e., amino acid starvation). In studies of histidine biosynthesis in vitro it was possible to demonstrate one aspect of the specific regulation by using a tRNA preparation (from cells bearing a *hisT* lesion) that was itself probably defective in the efficient translation of the leader that is required for attenuation of transcription (1, 9).

The isoleucine and valine biosynthetic pathway is another for which evidence supporting the general pattern of attenuation control has been demonstrated. The multivalent repression signal for the *ilvGEDA* operon is one that is dependent upon efficient charging of the isoacceptor for the three branched-chain amino acids. There are as yet no lesions that result in branched-chain amino acyl tRNA's that are defective in signaling attenuation as occurs with *hisT* tRNA in regulation of the *his* operon. In the experiments reported here, a template was employed that was known from in vivo experiments to exhibit regulation by the *ilv*-specific mechanism (27). It was thus anticipated that if protein synthesis was retarded because of limited charging of one of the branched-chain amino acid acceptor tRNA's, there would be a preferential expression of the genes controlled by the *ilv* promoter. Such a condition might be obtained in vitro by a system completely dependent upon added amino acids and by providing isoleucine, for example, in a slowly released form. Such S-30 preparations could not be obtained even by prolonged dialysis. This failure may have been due to a significant amount of proteolysis occurring in the extracts.

A reduced rate of protein synthesis was achieved by using two antimetabolites that are thought to interfere with the formation of isoleucyl tRNA, pseudomonic acid and isoleucine hydroxamate (8, 12). With each agent, overall protein synthesis was retarded, as was synthesis of β-galactosidase. However, there was some indication that β-galactosidase formation controlled by an *ilv*-specific promoter was more refractory to the effect of the antimetabolite than was that controlled by a *lac*-specific promoter. The latter was affected more nearly the way overall protein synthesis was affected.

At most, these experiments can only be taken as presumptive evidence for an in vitro demonstration of derepression of the *ilvGEDA* operon. However, it should be emphasized that in a system controlled only by the translatability of a leader, it is to be expected that derepression will only occur with normal templates, as it does in vivo, when protein synthesis is reduced owing to a restricted supply of the amino acid specifically involved in the regulatory process. Should a template be used in which the regulatory region in the vicinity of the attenuator has been altered, or an S-30 system containing an appropriately defective tRNA, derepression might result even with excess amino acids.

The nucleotide ppGpp was shown to stimulate

β-galactosidase synthesis in vitro when pPU34 and pPU35 were employed as templates. It appeared unlikely that this stimulation was the result of read-through from a plasmid promoter, as overall protein synthesis decreased when pBR322 was employed as template. Furthermore, if the effect of ppGpp on β-galactosidase formation from pPU34 had been due to stimulation of a plasmid promoter, that same promoter should have been stimulated in pPU36 (see below), in which the *lacZ* gene would be even closer to the promoter. Nevertheless, the DNA lying between any plasmid promoter and the *lacZ* gene in pPU34 and pPU36 is different, so that the possibility of read-through cannot be completely eliminated.

ppGpp was also shown to stimulate β-galactosidase synthesis when λ p*ilv-lac*3 DNA was employed as template, although only the weak *ilvE* promoter is present on the bacterial DNA carried by the phage. Because ppGpp decreases overall protein synthesis 54% with λ *c*I857 *S*7 DNA, it is unlikely that the stimulation was the result of increased transcriptions at the leftward p_L phage promoter. Indeed, Lindahl et al. (17) had shown that the synthesis of a protein probably originating from a gene in the left arm of λ was inhibited 42% by ppGpp.

The decrease in β-galactosidase synthesis by ppGpp with pPU36 DNA was unexpected, since β-galactosidase synthesis with λ p*ilv-lac*3 DNA with a nearly similar *ilv* region was stimulated. Like λ p*ilv-lac*3, it is a relatively inefficient template. It may be that some additional DNA which is upstream of the *Kpn*I site and is carried by λ p*ilv-lac*3 but is no longer contiguous with the weak *ilvE* promoter in pPU36 is essential for ppGpp to act. It is also possible that a necessary secondary structure has been lost by separating this fragment from the contiguous phage DNA. A similar phenomenon was observed by Kung et al. (14) in an experiment with wild-type *lac* operon fragments. It was shown that, although the entire *lac* regulatory region was present on a restriction fragment, ppGpp did not stimulate β-galactosidase synthesis when this fragment was transferred to the plasmid pMC3. If, as Travers (29) assumes, ppGpp binds to RNA polymerase and alters its specificity, it is to be expected that stimulation of transcription would occur at some polymerase binding sites and reduction of transcription would occur at others.

It is also possible that ppGpp could have increased transcription initiations at the *ilvG* promoter of pPU36 and thus could interfere with the completion of transcription through the *lacZ* gene, which is in the opposite orientation. A parallel finding was made in a *Salmonella typhimurium* mutant in which the *rfb* and *his* operons were fused in opposite orientations; inhibition of expression of the terminal portion of one operon occurred when expression of the other was elevated (16).

These experiments illustrate the difficulty of demonstrating the in vitro counterpart of the derepression of the *ilv* genes that is so readily observed in whole cells. The attenuation control model postulated for the *ilv* operon (19) predicts a repression signal under conditions of completely blocked protein synthesis or completely unimpeded protein synthesis and a derepression signal only when protein synthesis is retarded by a limitation of one of the three branched-chain amino acids. An analog can be used only if the sole effect of the analog is to inhibit charging and the analog is not itself incorporated into protein. More fruitful may be a search for mutants in which one of the branched-chain amino acid isoaccepting tRNA's exhibits a reduced efficiency in protein synthesis such as is postulated as the mechanism for the derepression of the *his* operon in *hisT* mutants (9). Another approach is to seek mutants in which transcription of the *ilvGEDA* operon itself is no longer dependent upon limited translation of the leader.

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