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Translational Coupling in the Threonine Operon of *Escherichia coli* K-12

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In an attempt to express the two distal genes of the *Escherichia coli* threonine operon, the majority of the first gene in the operon, *thrA*, was removed and a series of transcriptional fusions were constructed placing the *thrB* and *thrC* genes downstream of either the *trp* or hybrid *tac* promoter. Analysis of the proteins produced by cells containing these fusions revealed that although the distal gene, *thrC*, was efficiently expressed, the proximal gene, *thrB*, was not expressed at a detectable level. A translational fusion was constructed which fused the *cat* gene in phase to the last 800 base pairs of *thrA* followed by *thrB* and *thrC*. Cells containing this fusion produced high levels of both the *thrB* and *thrC* gene products, showing that translation of *thrB* requires translation through *thrA*; thus, *thrA* and *thrB* are translationally coupled. In addition, it was found that a sequence between 220 and 57 base pairs before the start of *thrB* was necessary to allow translational coupling to occur.

In *Escherichia coli*, three of the four enzymes involved in the conversion of aspartate to threonine are encoded by a single polycistronic message (19). Polycistronic messages encoding several enzymes involved in a single biochemical pathway are common in *E. coli* and presumably help the organism to maintain an appropriate ratio of enzymes for efficient metabolism during changing growth conditions. The different enzymes encoded by a single mRNA need not necessarily be synthesized at the same rate since factors such as sequence of ribosome-binding sites (RBSs) and codon usage will affect both efficiency of translation and rate of polypeptide elongation (16).

In the past few years, it has become clear that the rate of translation of some genes on a polycistronic mRNA depends on translation of the preceding gene on that mRNA. The phenomenon has been termed translational coupling and has been observed in a number of different operons (1, 14, 18, 21, 22). Several theories have been put forward to explain translational coupling, but there are few experimental data to support to contradict any of these models.

Here, we describe an example of translational coupling in the threonine operon and demonstrate that features of the coupling of the translation of *thrB* to *thrA* do not fit easily into any of the current theories for translational coupling.

MATERIALS AND METHODS

Bacterial strains and plasmids. The following strains of *E. coli* K-12 were used: C600 (*thrB1023 leuB6 thi-1*) (11), DH1 (*recA hsdR thi-1*) (7), RV308 (ATCC 31603), A164 (*trpE9851 leu-277 rho-4*) (13), and A165 (*trpE9851 leu-277*) (13). The plasmids pKK223-3 and pDR720 were obtained from Pharmacia Molecular Biologicals (catalog numbers 27-4935-01 and 27-4930-01).

Media and chemicals. Growth media are described by Maniatis et al. (11). Restriction enzymes, DNA polymerase, DNA ligase, and L-[^{35}S]methionine (555 MBq/ml) were obtained from Amersham P.L.C. Calf intestinal phosphatase was supplied by Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Casamino Acids and methionine assay medium were from Difco Laboratories (Detroit, Mich.). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.). Ampicillin was used at a concentration of 100 μg/ml, and tetracycline was used at 20 μg/ml.

DNA manipulations. DNA manipulations were performed as described by Maniatis et al. (11). Plasmid DNA was isolated by the method of Ish-Horowitz and Burke (8). DNA fragments from subcloning were gel purified and isolated by electroelution. Appropriate fragments were ligated and used to transform *E. coli* DH1. Potential recombinant plasmids were analyzed by restriction digestion, and junctions were sequenced when appropriate. Cosmid cloning was performed as described by Grosveld et al. (6).

Induction procedure. Bacterial cultures for induction were grown overnight in L broth (containing ampicillin for plasmid-containing strains) and diluted 20-fold into 10 ml of fresh L broth (plus ampicillin if appropriate). These were allowed to grow to an optical density at 600 nm of approximately 0.4, washed in M9 medium, and resuspended in M9 medium containing 1% Casamino Acids supplement (plus ampicillin) to an optical density at 600 nm of 0.4. For induction of the *trp* promoter, the cells were grown at 37°C for 3 h. For the *tac* promoter, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 10^{-4} M; again the cells were grown at 37°C for 3 h.

Labeling procedure. A volume of induced cells which would give an optical density at 600 nm of 1.00 when suspended in 1 ml was harvested by centrifugation, washed in 1 ml of M9 medium, and suspended in 1 ml of M9 medium supplemented with 1% methionine assay medium. The cells were labeled by incubation with 1 μl of [^{35}S]methionine (specific activity, 30 TBq/mmol) for 2 min at 37°C. The cells were pelleted by centrifugation, suspended in 200 μl of sample buffer (10), and boiled for 3 min. Samples (20 μl) were subjected to electrophoresis through a 10% polyacrylamide gel containing sodium dodecyl sulfate (SDS) (10). Gels were dried onto paper under vacuum and autoradiographed.

Assay of HSK activity. Homoserine kinase (HSK) activity was assayed as described by Burr et al. (2), except that to

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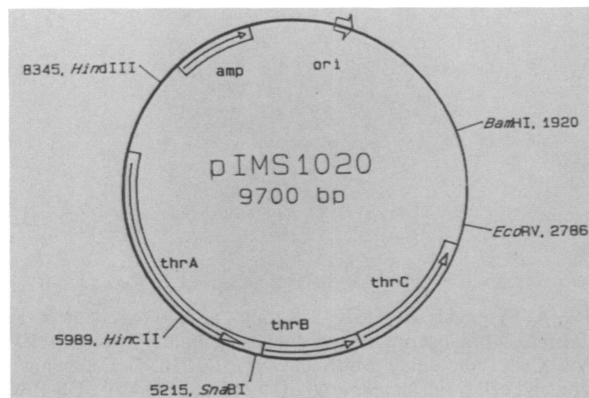

FIG. 1. Diagram of the plasmid pIMS1020 which was derived from pAT153 by the insertion of a 6.7-kilobase *Hin*dIII-to-*Bam*HI fragment carrying the threonine operon.

reduce nonspecific activity, a 2-h centrifugation at 100,000 × *g* was performed prior to assay of the cell extract and 0.1% sodium azide was added to the reaction mixtures.

RESULTS

Cloning of the threonine operon. The threonine operon, comprising *thrA*, *thrB*, and *thrC*, was cloned from the *E. coli* chromosome. *E. coli* RV308 DNA was partially digested with *Sau*3A and ligated into the *Bam*HI site of the cosmid pIMS6026 (a derivative of pLAFR1 [5]). Several recombinant cosmids were obtained which complemented the *thrB* lesion in *E. coli* C600. Previous reports (3) indicated that the threonine operon in *E. coli* K-12 is contained on a 6.7-kilobase region of DNA between a *Hin*dIII and a *Bam*HI site. Analysis of one of the complementing cosmids, pIMS1019, showed that the insert DNA did indeed include such a fragment. This fragment was inserted into plasmid pAT153 to form the plasmid pIMS1020 (Fig. 1), which could also complement the *thrB* lesion in C600. Further restriction analysis and partial sequencing failed to reveal any differences between pIMS1020 and the plasmid pIP3 described and sequenced by Parsot et al. (15).

Expression of *thrB* and *thrC*: transcriptional fusions. An attempt was made to express the two distal genes of the threonine operon, *thrB* and *thrC*, under the control of either the *E. coli tac* or *trp* promoter (4, 17). pIMS1020 was digested with *Hin*cII, which cuts within *thrA*, and *Eco*RV, which cleaves after *thrC* (Fig. 1). The resulting 3.1-kilobase DNA fragment was isolated. This fragment contains the last 809 base pairs (bp) of *thrA*, the complete *thrB* and *thrC* coding regions, and the threonine operon transcription terminator (15). The isolated DNA was ligated into the *Sma*I site of pKK223-3 (*tac* expression vector) to form the plasmid pIMS1021 and also into the *Sma*I site of pDR720 (*trp* expression vector) to form pIMS1023 (Fig. 2). C600 strains containing these constructs were induced, and the proteins produced were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE); no major differences were observed between plasmid-carrying and plasmid-free cells (Fig. 3, lanes 2, 3, and 4). Furthermore, no increase in HSK activity encoded by *thrB* could be measured (Table 1). These results showed that the plasmids did not express either *thrB* or *thrC*.

In these fusions there is approximately 800 bp between the transcription start site and the *thrB* RBS. It has been shown previously that a long untranslated region can, in some

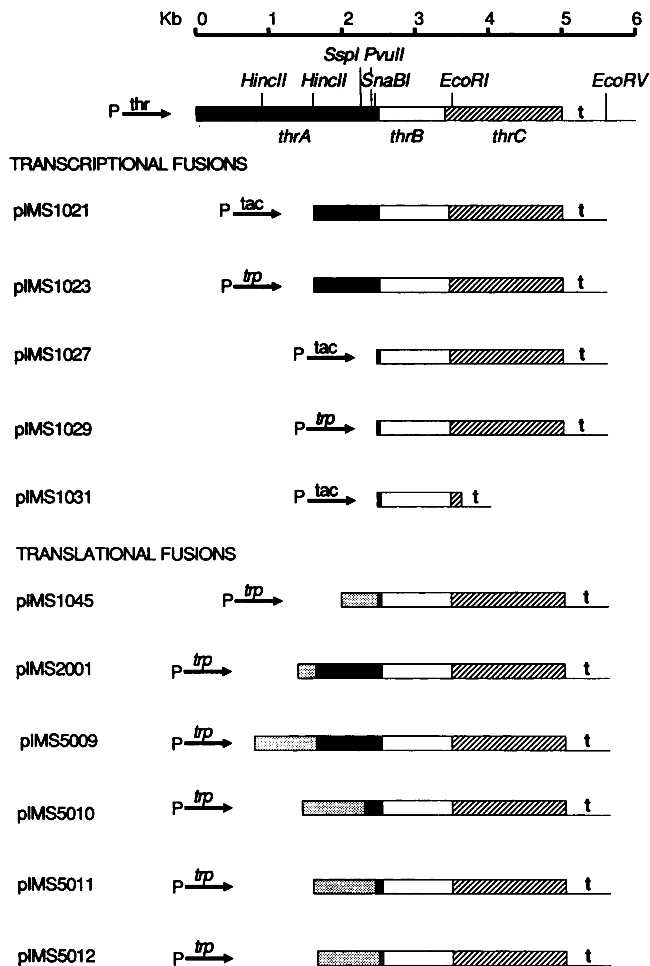

FIG. 2. Diagram of the transcriptional and translational gene fusions used in this study. The extent of the *thrA* gene is indicated by the filled box, *thrB* by the open box, *thrC* by the hatched box, and the *cat* gene by the stippled box. Also shown are the promoters used (*trp* or *tac*), restriction sites, and positions of transcription termination sequences (t). Kb, Kilobases.

circumstances, permit premature transcription termination (13). To overcome this potential problem, we reconstructed the fusions to remove most of the untranslated *thrA* region. Details of these shorter transcriptional fusions, pIMS1027 and pIMS1029, are shown in Fig. 2. A unique *Sna*BI site 30 bp before the 3′ end of *thrA* was used to fuse the *thrB* RBS

TABLE 1. HSK activity of *E. coli* C600 containing the various transcriptional and translational fusion plasmids

Strain[a]	HSK activity (μmol/min per mg)[b]
C600	0.02
C600(pIMS1021)	0.02
C600(pIMS1023)	0.02
C600(pIMS1027)	0.02
C600(pIMS1029)	0.02
C600(pIMS1045)	0.063 ± 0.01
C600(pIMS2001)	0.41 ± 0.02
C600(pIMS1038)	0.02

[a] The strains were induced for 3 h prior to assay.
[b] The results shown are the average of two assays.

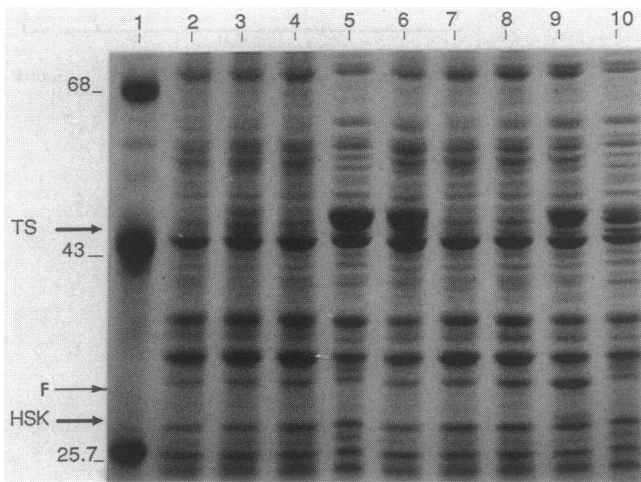

FIG. 3. Synthesis of HSK, TS, and the chloramphenicol acetyltransferase-homoserine dehydrogenase I fusion protein (F) by the various transcriptional and translational gene fusions. *E. coli* C600 strains carrying the plasmids listed below were induced and processed as described in Materials and Methods. Samples (20 μl) were analyzed by SDS-PAGE followed by staining with Coomassie blue. Lanes: 1, molecular weight markers (10^3); 2, *E. coli* C600; 3, C600(pIMS1021); 4, C600(pIMS1023); 5, C600(pIMS1027); 6, C600(pIMS1029); 7, C600(pIMS1031); 8, C600(pIMS1038); 9, C600 (pIMS2001); 10, C600(pIMS1045).

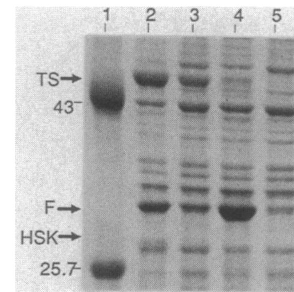

FIG. 4. Synthesis of HSK, TS, and a chloramphenicol acetyltransferase-homoserine dehydrogenase I chimeric protein (F) by *E. coli* C600 containing pIMS2001 or pIMS1038. Following induction and cell lysis, samples (20 μl) were analyzed by SDS-PAGE and visualized by staining with Coomassie blue. Lanes: 1, molecular weight markers (10^3); 2, *E. coli* C600(pIMS2001), induced; 3, C600(pIMS2001), uninduced; 4, C600(pIMS1038), induced; 5, C600 (pIMS1038), uninduced.

much closer to the *trp* or *tac* promoter. The short transcriptional fusions were induced in C600, and the proteins produced were analyzed by SDS-PAGE. Following induction, one novel protein band accounting for approximately 10% of total cell protein was observed with both pIMS1027 and pIMS1029 (Fig. 3, lanes 5 and 6). The size of this new protein was estimated to be 46 to 48 kilodaltons (kDa), in good agreement with the expected size of threonine synthetase (TS) encoded by *thrC* (47 kDa) and considerably different from the predicted size of HSK encoded by *thrB* (33.5 kDa) (3). A derivative of pIMS1027 in which *thrC* had been deleted but which still contained *thrB* (pIMS1031, Fig. 2) no longer encoded the new protein (Fig. 3, lane 7). Upon induction of C600 harboring pIMS1027 or pIMS1029, there was no increase in HSK activity (Table 1). Taken together, these data show that *thrC* but not *thrB* is efficiently expressed from the *trp* or *tac* promoter in these short transcriptional fusions and, as *thrB* is upstream of *thrC*, suggest that *thrB* is transcribed but not efficiently translated.

Transcription of the 3′ end of *thrA* in the absence of translation does not appear to be sufficient to allow efficient expression of the *thrB* gene. This was demonstrated by transforming the long transcriptional fusion plasmids pIMS1021 and pIMS1023 into the otherwise isogenic *rho*$^+$ and *rho* mutant strains A165 and A164. Upon induction, the A164 strains synthesized large amounts of TS, but HSK was barely detectable even after [^{35}S]methionine pulse-labeling (data not shown).

Expression of *thrB* and *thrC*: translational fusions. To determine whether translation of *thrA* could affect expression of *thrB*, we constructed a translational fusion joining the beginning of the *cat* gene (isolated from pCT135 [16]) in frame with the end of the *thrA* gene (9). The details of the fusion plasmid pIMS2001 are shown in Fig. 2. Plasmid pIMS2001 contains the *trp* promoter followed by the first 112 bp of the *cat* coding sequence fused in phase to the final 809 bp of the *thrA* gene and the complete *thrB* and *thrC* coding sequences. The *cat* gene is highly expressed from the *trp* promoter on the plasmid pCT135 and is known to be efficiently transcribed and translated (16).

Strain C600(pIMS2001) was tested for *thrB* expression initially by HSK activity assays. The results (Table 1) show that strains containing the translational fusion plasmid expressed high levels of HSK. Further evidence of *thrB* expression was obtained from SDS-PAGE of the proteins produced by C600(pIMS2001). The results (Fig. 3, lane 9, and Fig. 4, lane 2) show that upon induction, three new proteins of approximately 46, 34, and 29 kDa are synthesized. Under the electrophoresis conditions used, the 34-kDa protein (marked F in Fig. 3 and 4) comigrated with a major *E. coli* protein. However, the band marked F in Fig. 3 in the lanes containing protein prepared from C600(pIMS2001) and C600(pIMS1038) is more intense than the equivalent band in the protein prepared from the C600 control strain (lane 2). Similarly, in Fig. 4 it can be seen that induction of the *trp* promoter in strains carrying pIMS2001 or pIMS1038 resulted in an increase of the 34-kDa protein (marked F in Fig. 4, lanes 2 and 4 are induced). The 34-kDa protein and the 46-kDa protein accumulated to approximately 5% of total cellular protein, which is about 10 times higher than the accumulation of the 29-kDa protein. By comparison with earlier inductions (Fig. 3, lanes 5 and 6), the largest of the novel proteins is TS, encoded by *thrC*. The sizes of the *cat-thrA* fusion gene product and HSK as calculated from the DNA sequence are 34 and 33.5 kDa, respectively, whereas the observed sizes of the novel proteins were 34 and 29 kDa, respectively. Consequently, experiments were performed to determine which of the two smaller novel proteins was encoded by *thrB*. A derivative of the plasmid pIMS2001 was constructed in which a *Pvu*I fragment of known sequence from the *E. coli metL* gene (nucleotides 195 to 1056 [23]) was inserted in the *thrB* coding sequence at nucleotide 272. This placed a termination codon close to the start of *thrB*. C600 containing this plasmid, pIMS1038, no longer gave detectable levels of HSK activity (Table 1), and analysis of SDS-PAGE (Fig. 4, lanes 4 and 5) revealed that the smaller protein was no longer produced, whereas the 34-kDa protein was still efficiently synthesized. This suggests that the protein with an apparent molecular size of 29 kDa is HSK and that the 34-kDa protein is the product of the *cat-thrA* fusion. Further evidence was obtained by reconstructing the 5′ end and RBS of *thrB* by using oligonucleotides. The nucleotide sequence of the 5′ end of

a) natural sequence

5' ATGGAAGTTAGGAGTCTGACATGGTTAAA

b) modified sequence

5' CACGTAAAAAGGGTATCGATATGGTTAAA

FIG. 5. (a) Sequence around the start of the *thrB* gene in the *E. coli* threonine operon. (b) Sequence of the 5' end of the *thrB* gene in pIMS1047. The initial codon of the *thrB* gene and the termination codon of the *thrA* gene are underlined.

the reconstructed *thrB* gene is shown in Fig. 5. Upon induction, strains carrying this plasmid (pIMS1047) synthesized a large amount of a novel protein which comigrated with the protein believed to be HSK encoded by pIMS2001. HSK appears to migrate abnormally in SDS-PAGE (data not shown).

The HSK expression studies described in the previous paragraph suggested that efficient expression of the *E. coli thrB* gene is coupled to translation of at least part of the upstream *thrA* gene. To investigate *thrB* expression further, we constructed a shorter translational fusion plasmid, pIMS1045, which fused the first 474 bp of *cat* to a *Sna*BI site in *thrA* just 30 bp upstream of *thrB* (Fig. 2). The sequence of the complete junction region in pIMS1045 from *cat* into the *thrB* coding sequence was checked and found to be correct. Upon induction, C600 containing this plasmid gave expression of *thrC* but barely detectable expression of *thrB* (Fig. 3, lane 10, and Table 1).

This result showed that translation of the extreme 3' end of the *thrA* gene was not sufficient to ensure efficient initiation of translation at the *thrB* RBS. However, the two translational fusions encoded by pIMS1045 and pIMS2001 contained different regions of the *cat* gene. To check that this was not affecting *thrB* expression and to further characterize the phenomenon, we constructed four additional plasmids. These plasmids carried gene fusions consisting of the first 98% (217 codons) of the *cat* gene fused in frame with the 3' end of the *thrA* gene 30 bp (pIMS5012), 57 bp (pIMS5011), 220 bp (pIMS5010), or 800 bp (pIMS5009) from the termination codon. The details of these constructs are shown in Fig. 2.

Protein synthesis in C600 strains carrying these plasmids was examined by pulse-labeling induced cells with [^{35}S] methionine. The strains carrying the plasmids encoding the two fusions containing 800 and 200 bp of the *thrA* gene (pIMS5010 or pIMS5009) produced a HSK-sized protein as a major product of protein synthesis (Fig. 6). In contrast, the strain containing the fusions in which only the last 57 or 30 bp of the *thrA* gene are translated (pIMS5012 or pIMS5011) synthesized much lower levels of HSK. Densitometric scanning of Fig. 6 showed that all four strains produced the chloramphenicol acetyltransferase fusion protein and TS in the same proportion, suggesting that transcription was not interrupted in these plasmids.

DISCUSSION

Translational coupling has been reported in several different operons (1, 14, 18, 21, 22), and here we present evidence for translational coupling between the *thrA* and *thrB* genes of the *E. coli* K-12 threonine operon. However, certain features of this coupling cannot be explained by current models of translational coupling.

When most of the *thrA* coding sequence was removed and a strong promoter (*trp* or *tac*) was placed 30 bp upstream of the initiation codon for *thrB*, transcription passed through the *thrB* gene and resulted in efficient translation of the promoter-distal *thrC* gene. However, SDS-PAGE and enzyme assays showed that there was very little translation of the *thrB* gene. Sequencing data confirmed the data of Parsot et al. (15) and showed that just prior to the start of the *thrB* gene there are two potential RBSs (the junction sequence is shown in Fig. 5). Although these initiation signals failed to allow efficient translation of the *thrB* gene in a transcriptional fusion, we found that the *thrB* gene was efficiently expressed if a translational fusion was constructed with the upstream *thrA* gene such that the last 800 bp of this gene were efficiently translated. Translation of the *thrA* gene terminated 1 bp before the initiation codon of the *thrB* gene (Fig. 5).

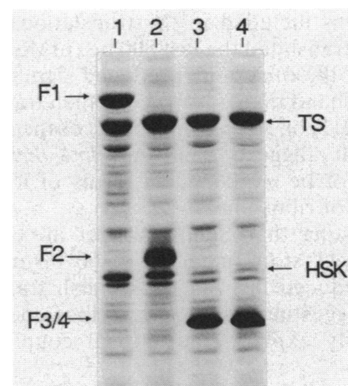

FIG. 6. Autoradiograph showing the synthesis of HSK, TS, and various chloramphenicol acetyltransferase-homoserine dehydrogenase I fusion proteins (F1 to F4) by four translational fusions. *E. coli* C600 strains carrying the plasmids listed below were induced and pulse-labeled, and samples (20 μl) were analyzed by SDS-PAGE followed by autoradiography. Lanes: 1, C600(pIMS5009); 2, C600 (pIMS5010); 3, C600(pIMS5011); 4, C600(pIMS5012).

In both the transcriptional and translational fusions described in the previous paragraph, there appeared to be approximately equal amounts of TS produced, suggesting that equivalent levels of transcription pass through the *thrB* gene. However, the translational fusion encoded by pIMS2001 produced at least 20 times as much HSK as the transcriptional fusions (as assessed by enzyme assays). Transcriptional fusions which inserted a powerful promoter in the *thrA* gene such that the last 800 bp of this gene should be transcribed but not translated failed to produce bands of either HSK or TS. This is probably due to *rho*-dependent termination of transcription in the untranslated portion of *thrA*. Experiments in a *rho* mutant indicate that long transcriptional fusions produce TS efficiently but only very low levels of HSK (data not shown). Efficient translation of the *E. coli thrB* gene seems to require translation of the 3' end of the preceding *thrA* gene.

Translational coupling has been reported in several operons, and it has been suggested that translation of the promoter-proximal gene ensures a high localized concentration of ribosomes around the RBS of the second gene (14). To test this theory in *thrA-thrB* translational coupling, we made a new set of fusions such that a series of chimeric proteins were produced which contained increasing amounts of the C terminus of the *thrA* gene product. In all cases, the sequence of the 3' end of the *thrA* gene was left intact such that translation terminated at the wild-type sequence and translation of HSK should initiate in response to natural signals. The fusions differed only in the amount of the *thrA*

gene which was included in the translational fusion. Constructs which translated the last 800 bp (pIMS5009) or 220 bp (pIMS5010) of the *thrA* gene produced significantly more of an HSK-sized band than constructs which translated the last 57 bp (pIMS5011) or 30 bp (pIMS5012) of the *thrA* gene (Fig. 6). This result suggests that the *thrA-thrB* translational coupling cannot be explained in terms of a localized high concentration of ribosomes.

It is interesting that Schumperli et al. (18) found that moving the *galT* stop signal 40 to 50 bp upstream of its normal site reduced but did not abolish translational coupling, again suggesting that localized ribosome concentration may not totally explain translational coupling in the *gal* operon.

A second model for translational coupling suggests that a polycistronic message, in the absence of translation, forms a secondary structure which sequesters the RBS of the coupled gene (14). Computer-aided (Beckman Microgenie program) analysis of the DNA sequence in the region from 250 bp upstream of the start of *thrB* to 20 bp downstream from the start of *thrB* failed to detect any potential secondary structure involving the *thrB* translation signals with a G value of less than −5 kcal/mol. Furthermore, the type of structure postulated by this model would act in a negative fashion. In contrast to the data presented here, the short translational fusion carried on pIMS1045, pIMS5011, and pIMS5012 would be expected to express *thrB* at least at efficiently as the long fusions and potentially more efficiently owing to removal of regions involved in forming the secondary structure.

Thomas et al. (20) have reported that in the *E. coli* α operon the ribosomal protein gene L17 is translationally coupled to upstream ribosomal protein genes despite the presence of an intervening separately regulated gene. This observation and the data presented here are difficult to reconcile with current models of translational coupling. McCarthy et al. (12) have identified a sequence close to (within 30 bp) of the Shine-Dalgarno sequence of the *atpE* gene which enhances the translational initiation of several genes. It is possible that other sequences act over longer distances (possibly by modifying the translational machinery), resulting in the form of translational coupling seen in the threonine operon. Since a fusion 220 bp from the end of the *thrA* gene results in efficient expression of the *thrB* gene, any such sequence must occur in this region. It is hoped that further analysis will allow a more exact localization of the region involved.

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