A ribosome binding site sequence is necessary for efficient expression of the distal gene of a translationally-coupled gene pair

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ABSTRACT

Expression of trpB and trpA of the Escherichia coli tryptophan operon is shown to be "translationally coupled", i.e., efficient translation of the trpA coding region is dependent on prior translation of the trpB coding region and termination of translation at the trpB stop codon. To examine the dependence of trpA expression on the ribosome binding site sequence in the distal segment of trpB, deletions were produced that replaced this trpB sequence. Analysis of trpA expression in these deletion mutants established that the ribosome binding site sequence is required for efficient translation of the trpA segment of trp mRNA. A modest effect of translation over the trpA ribosome binding site on independent initiation at that site was also observed.

INTRODUCTION

Many bacterial and phage operons contain two or more genes that are coordinately expressed by translation of separate coding regions in a single polycistronic messenger RNA. In some polycistronic messages there is an untranslated spacer sequence between adjacent coding regions and this spacer contains the ribosome binding site (Shine-Dalgarno sequence, Ref. 1, 2) for the downstream coding region. In several cases the length of the spacer sequence is short and the translation termination and initiation codons of adjacent coding regions either overlap or are separated by a few bases (reviewed in Ref. 3). In these instances the ribosome binding site sequence for the downstream coding region appears to lie within the upstream coding region.

In the trp operon of Escherichia coli there are two examples of adjacent genes with overlapping stop and start codons, the trpE-trpD and trpB-trpA gene pairs (4, 5). These gene pairs specify non-identical polypeptides that function in corresponding enzyme complexes (6, 7). Bacteriophage lambda also has several gene pairs with overlapping start and stop codons (8). In the trpD-trpC and galT-galK gene pairs of E. coli the stop and start codons of

the adjacent coding regions are separated by 3 bases (9, 10). Mutational studies with the trp operon of E. coli established that chain termination mutations in the first major structural gene of the operon, trpE, are more polar on expression of the adjacent downstream gene, trpD, than on the three distal genes, trpC, trpB and trpA (11). Further analyses suggested that the disparity was due to inefficient translation of trpD mRNA as a consequence of the inability to translate the distal segment of trpE mRNA (12). This phenomenon was termed "translational coupling" (12). Translational coupling has also been observed in studies of the trpB-trpA gene pair (13, this paper), the galT-galK gene pair (14), and the ribosomal protein gene clusters (15, 16).

In an effort to explain translational coupling our first objectives were to examine the dependence on stop codon position, and to determine the extent to which the presumed ribosome binding site sequence of a translationally-coupled gene pair was essential for translation of the downstream gene. To obtain this information we produced insertions and deletions that shifted the trpB translation stop codon or replaced the normal trpA ribosome binding site sequence at the distal end of trpB. We show that relocation of the trpB stop codon or alteration of the ribosome binding site sequence markedly reduces translation of trpA mRNA. Our findings also suggest that translation over the trpA translation initiation site modestly reduces utilization of that site.

MATERIALS AND METHODS

Deletion constructions:

Plasmid pWS1 contains the trpCBA segment of the trp operon of E. coli fused to the tet promoter (17). pWS1 was linearized by digestion at the single HpaI site in trpB, located 66 bp before trpA. Linear plasmid DNA was digested with nuclease Bal 31 for limited periods under appropriate conditions. Digestion was monitored by examining the size of specific restriction fragments. After digestion with Bal 31, the DNA fragment ends were filled in with DNA polymerase I Klenow fragment and ligated with T4 DNA ligase. The ligated plasmid DNA was digested with HpaI and was then used to transform cells of E. coli JA194 to chloramphenicol resistance (17). About 100 independent colonies were picked and the plasmid DNA they contained was isolated by the rapid screen procedure of Birnboim and Doly (18). The extent of each deletion was estimated by examining the size of fragments generated by digestion with the enzymes HinfI and Sau3A. Forty plasmids were analyzed

by sequencing (19) to determine the precise deletion end points.

Plasmid pHS1-BamHI was constructed as follows: plasmid pHS1 (17), after linearization by digestion with HpaI, was ligated to the BamHI linker dCCGGATCCGG (BRL, Inc.). The mixture was digested with excess BamHI, treated with phenol, precipitated with isopropanol, and then circularized by ligation. Cells were transformed to drug resistance and a plasmid with a unique BamHI site at the correct location was isolated and sequenced.

Enzyme Assay:

Plasmids were introduced into strain W3110 tnaA2 trpA14-17 for enzyme assays. This strain has two nonsense mutations in trpA and therefore does not produce tryptophan synthetase α . Cultures (500 ml.) were grown in minimal medium (20) supplemented with 0.2% glucose, 0.5% acid hydrolysed casein, 20 μ g/ml L-tryptophan and 20 μ g/ml chloramphenicol. After growth to Klett 80 (660 filter), cells were harvested, washed in cold 0.85 % NaCl, centrifuged, and resuspended in 1 ml cold 0.1 M Tris-HCl, pH 7.8. Extracts were prepared by sonic disruption followed by centrifugation to remove cell debris. Indoleglycerol phosphate synthetase (trpC polypeptide) and tryptophan synthetase α (trpA polypeptide; indole to tryptophan reaction) were assayed according to Smith and Yanofsky (21). Specific activity is expressed as units of enzyme activity (21) per mg. of protein (22). Under the growth conditions employed the specific activities of the trpC and trpB polypeptides produced from the chromosome of W3110 tnaA2 trpA14-17 should be approximately 0.5 and 1.0, respectively.

Other Procedures:

E. coli strain W3110 tnaA2 Δ trpEA2 was used for trpB⁺ selection. trpB⁺ transformants of this strain grow in media containing indole. The wild type plasmid pHS1 was the only plasmid studied that conferred trpB⁺. For trpA⁺ selection the auxotrophic E. coli strain W3110 tnaA2 trpA14-17 was employed as a transformation recipient. Extracted proteins were analyzed on sodium dodecyl sulfate-polyacrylamide gels (23).

RESULTS

The junction region separating the trpB and trpA coding regions of E. coli consists of overlapping stop and start codons, UGAUG (Table 1). Immediately upstream from the AUG start codon of the trpA coding region (Table 1) there is a purine-rich sequence complementary to the 3' end of 16S RNA, a so-called Shine-Dalgarno sequence (1). A recognition site for the restriction endonuclease HpaI is located ca. 60 bp upstream from this

Table I: Expression of trpC and trpA of Deletion Plasmids

Plasmid	Type of Mutation	bp Deleted	Sequence Preceding the trpA Start Codon	Protein Sp. Act. trpC	Relative trpA protein ^a
pWS1	none	--	<div style="text-align: center;"> -15 -10 -5 A C G A G G G A A A U C U G A U G^b </div>	130	100
Δ81	Shine-Dalgarno deletion	123	C C A C G C C G A A A U C U G A U G	165	7.2
Δ32	Shine-Dalgarno & stop codon deletion	105	G U U G A A A A U G A U G C G A U G - 28n-UGA	155	0.2
pWS1-BamH1	insertion	0	<div style="text-align: center;"> UAA - 48n - A U G (normal sequence) </div>	N.D.	26.8
Δ33 (+1)	frameshift deletions	88	A A A C C G G A A A U C U G A U G - 74n-UGA	120	3.9
Δ40 (+1)		85	A C C C G G G A A A U C U G A U G - 74n-UGA	155	3.2
Δ38 (-1)		86	A C G A G G G A A A U C U G A U G - 801n-UAA	145	37.5
				145	15.6

Overlines indicate the wild type sequence homologous to the 3' end of 16S RNA (1). The stop codon in the trpB reading frame of each deletion plasmid is underlined and the trpA start codon is doubled underlined. Enzyme activities were measured according to Smith and Yanofsky (21). A tryptophan synthetase α specific activity of 253 corresponds to 5% of the soluble protein of the extract as this polypeptide. The tryptophan synthetase β2 specific activity of these extracts (not measured) would be expected to be ca. 1.0. Active β2 would be specified by the chromosome of the recipient strain. The bp in trpB deleted in the various plasmids were as follows: Δ81, 1063-1185; Δ32, 1089-1193; Δ33, 1097-1184; Δ40, 1098-1182; and Δ38, 1068-1153. The wild type gene contains 1191 bp. N.D. = not determined; n. = nucleotides.

a the relative tryptophan synthetase α chain specific activity, setting the wild type level at 100 percent. These values were not corrected for the small differences in trpC polypeptide specific activities.

b the predicted ΔG's for the 50 nucleotides centered by each AUG start codon are: pWS1, -6.7; Δ81, -5.2; Δ32, -14.8; Δ33, -5.0; Δ40, -6.7; and Δ38, -6.7.

Shine-Dalgarno sequence, in the distal segment of trpB. A plasmid containing the trpC, trpB and trpA genes of E. coli was linearized at this HpaI site and was then treated with nuclease Bal 31 to remove 75-150 base pairs. After screening the resulting plasmids and sequencing the deletion junctions, 5 deletions of the desired phase and sequence were selected for detailed analysis. These deletion plasmids are described in Table I; the deletion end points in these mutants are given in the legend. The deletions removed 85 to 123 bp and shortened the trpB coding region by 28 to 41 codons. These altered trpBs failed to express trpB⁺ activity. All of the deletions selected gave plasmids with an intact trpA. When tested for the ability to complement a trpA mutation, all five deletion plasmids were able to do so, however the resulting transformants differed in colony size suggesting that trpA expression varied for each of the plasmids.

The specific activity of tryptophan synthetase α was determined in extracts of strains with the various deletion plasmids (Table I). As expected from the trpA⁺ complementation tests, a wide range of tryptophan synthetase α chain specific activities (from 1/500 to 1/4 that of the non-deleted plasmid) was observed in strains with deletion plasmids. Indoleglycerol phosphate synthetase (trpC polypeptide) activity was measured as an internal control and varied only slightly ($\pm 25\%$ of the non-deletion plasmid) among strains with deletion plasmids. Thus the observed range of trpA activity is not due to plasmid copy number variation in the deletion strains.

The constructed plasmids fall into several classes (Table I). Plasmid $\Delta 81$ is an example of a Shine-Dalgarno deletion mutant in which there is no change in the trpB reading frame. The strain with this mutant plasmid has low levels of tryptophan synthetase α chain activity (Table I, line 2). The nucleotide sequence in the Shine-Dalgarno region of $\Delta 81$ reveals little homology to the 3' end of 16S rRNA. Therefore the marked reduction in trpA expression in $\Delta 81$ can be ascribed to the absence of a fully functional ribosome binding site sequence. Deletion $\Delta 32$ is a second example of a Shine-Dalgarno deletion mutation that does not change the trpB reading frame. However since the U of the normal UGA trpB stop codon has been replaced in $\Delta 32$, translation of the trpB coding region terminates within the trpA coding region. This plasmid barely directs trpA protein synthesis (Table I, line 3).

The insertion plasmid pWS1-BamHI was used to examine the quantitative contribution of translational coupling on trpA expression (Table I). This

Fig. 1 Proteins synthesized in strains containing wild type and $\Delta 38$ plasmids. Extracts were prepared as described in "Materials and Methods" and were analysed on sodium dodecyl sulfate-polyacrylamide (10%) gels according to Laemmli (23). Protein bands were visualized by staining with Coomassie Blue. Approximately 5% of the protein in the extract of the strain containing the wild type plasmid is tryptophan synthetase α . Lanes 1 and 4, 40 and 60 μ g of protein from cells harboring the wild type plasmid; lanes 2 and 5, 40 and 60 μ g of protein from cells harboring the $\Delta 38$ plasmid; lane 3, a mixture of bovine serum albumin (3 μ g) and trpA protein (3 μ g).

plasmid was constructed by inserting a 10 bp BanHI linker at the HpaI site within trpB (see Materials and Methods). This insertion causes a frameshift in trpB resulting in chain termination 17 codons upstream from the authentic stop codon. This premature termination creates a 48 bp spacer between the truncated trpB coding region and trpA. Expression of trpA in strains with this plasmid is 27 percent that of wild type (Table I, line 4).

The third class of deletions causes frameshifts in the trpB reading frame. The +1 frameshift deletions, $\Delta 33$ and $\Delta 40$, lack the wild type trpA Shine-Dalgarno sequence. From the known sequence of trpA (4) these deletions are predicted to result in termination of translation 25 codons downstream from the authentic trpB stop codon. Both mutants exhibit a low level of tryptophan synthetase α activity (Table I, lines 5 and 6). A third example of a +1 frameshift was also analyzed. The nucleotide sequence in the Shine-Dalgarno region of this deletion mutant differed from that of $\Delta 33$ and $\Delta 40$, however the trpA polypeptide level was comparable (data not shown). The -1 frameshift deletion, $\Delta 38$, results in an in-phase fusion of the truncated trpB coding region and the trpA coding region. Since the deletion endpoint in $\Delta 38$ is 40 bp upstream from the trpA start codon, the environment of the

trpA initiation codon is the same as in the wild type plasmid (Table I). Analysis of tryptophan synthetase α levels directed by this plasmid is complicated by the fact that both the trpA polypeptide and a trpB'-trpA fusion polypeptide are produced (Fig. 1). The fusion protein, M_r = ca. 69,000 daltons, migrates slightly slower than bovine serum albumin (M_r = ca. 67,000) in sodium dodecyl sulfate - polyacrylamide gels (Fig. 1, lanes 2, 5) as expected from its calculated size. The fusion protein band is not present in extracts of cells containing the wild-type plasmid (Fig. 1, lanes 1, 4) and the trpB polypeptide band present in wild type extracts is missing from extracts of cells containing the mutant plasmid. The trpA polypeptide is visible in both extracts. To determine if the fused trpB'-trpA polypeptide has tryptophan synthetase α activity, the fusion product was separated from the trpA polypeptide by Sephadex G-25 column chromatography. The fusion product did not have enzymatic activity when assayed in the indole $\rightarrow$ tryptophan reaction in the presence of excess tryptophan synthetase β_2 . The trpA polypeptide prepared from the same extract was fully active. We therefore can attribute the 16 percent trpA protein activity in strains with the $\Delta 38$ plasmid (Table I) to normal length wild type trpA polypeptides.

DISCUSSION

The polypeptides specified by trpB and trpA of *E. coli* form a tetrameric enzyme complex of composition $\alpha_2 \beta_2$. The trpE and trpD polypeptides also form an $\alpha_2 \beta_2$ complex. In recent studies it was concluded that synthesis of the trpE and trpD polypeptides of *E. coli* was translationally coupled, i.e., translation of the distal segment of the trpE coding region was required for efficient translation of the trpD coding region (11, 12). The recent studies of Askoy et al. (13) and those reported here indicate that synthesis of the trpB and trpA polypeptides also is coupled. It is thought that translational coupling is a device used in the translation of segments of some polycistronic messengers to ensure proportionate production of functionally related polypeptides or equimolar production of polypeptides that are constituents of one enzyme complex. Overlapping stop-start punctuation regions, such as the UGAUG that separates both trpE and trpD, and trpB and trpA, may facilitate this coupling.

Translation of the trpA coding region is not absolutely dependent on prior translation of the trpB coding region since nonsense and frameshift mutations throughout trpB, although exerting a polar effect on trpA expression, nevertheless permit up to ca. 30% of normal trpA expression (24,

I. Crawford, personal communication). Our analyses with the insertion mutant pWS1 BamHI support this conclusion. The insertion in this plasmid introduces a frameshift that causes trpB polypeptide synthesis to terminate 17 codons upstream from the authentic trpB stop codon. Translation termination at the new upstream stop codon would leave a short, ca. 50 nucleotide, untranslated segment of mRNA between the new stop codon and the trpA start codon. This segment would seem to be too short to serve as a site of rho binding and action (25) and it does not contain sequences resembling a rho-independent termination signal. Therefore it is unlikely that transcription would be terminated in the corresponding DNA region. The finding that trpA expression in strains with pWS1·BamHI is only one-fourth that obtained with the wild type plasmid (Table I) therefore suggests that at least three-fourths of trpA expression in wild type results from translational coupling. In agreement with our results, Aksoy et al. (13) have found in studies with a trpA-lacZ gene fusion that only 20 percent of the wild type level of β -galactosidase is produced when trpB polypeptide synthesis is prematurely terminated about 1 Kb upstream from the trpA start codon. They have also shown that this reduction is not due to lowered messenger RNA levels.

The contribution of coupling appears to vary in different messengers. Schumperli et al. (14) report that in the galK-galT translationally coupled system, 30-40 percent of galK expression is contributed by coupling and the remainder by independent translation. A possible explanation for the reduced contribution of coupling in this case is the 3 base spacer between the galT stop codon and galK start codon. However other features of the structure of the translation initiation region, or ribosome behavior during translation initiation and termination, may also influence coupling.

The deletion mutants described in this report establish the requirement of the ribosome binding site in the distal portion of trpB for high level trpA expression. Replacement of the ribosome binding site sequence in $\Delta 81$ reduces trpA expression to 7.2 percent of the wild type level. (Table I). A second deletion mutant, $\Delta 32$, that has lost both the ribosome binding site sequence and the stop-start overlap, and in which translation of trpB terminates in trpA, produces only 0.2 percent of the wild type trpA level (Table I). The low level of trpA expression in both mutants demonstrates dependence on the trpA ribosome binding site sequence within trpB for efficient translation of the trpA coding region.

The fact that trpA expression in $\Delta 32$ is 36 fold lower than that in $\Delta 81$ requires additional explanation. It is conceivable that this difference is

due to the changes in the Shine-Dalgarno region, with the $\Delta 81$ sequence functioning more effectively than the $\Delta 32$ sequence. Alternatively or in addition, masking of the $\Delta 32$ start codon could be responsible for the reduced translation initiation. In the plasmids examined only the deletion in $\Delta 32$ creates a sequence around the AUG start codon that could form a moderately stable secondary structure, $\Delta G = -14.8$ (see footnote to Table 1), in which the start codon is paired with a complementary sequence. An additional consideration is that the overlapping stop and start codons of trpB and trpA of $\Delta 81$, the same configuration as in wild type, may promote initiation at trpA by permitting translational coupling. This may not be possible in $\Delta 32$ because trpB translation presumably terminates within trpA. A further contribution to the discrepancy in trpA expression in the two deletions may be an inhibitory effect of translating ribosomes traversing over the trpA initiation region of $\Delta 32$. These ribosomes could inhibit the attachment of free ribosomes at the trpA initiation region. However, the following findings suggest that this effect would be small. Analyses of the trpB'-trpA fusion polypeptide produced by the frameshift mutant $\Delta 38$ demonstrate that it lacks trpA function. Thus the 16% trpA enzymatic activity detected in extracts of strains with the $\Delta 38$ plasmid must result from independent translation of trpA mRNA. This level of trpA expression is about half that observed with pWS1-BamHI. Since both plasmids have the wild type trpA initiation sequence, inhibition of trpA mRNA translation by ribosomes translating over the trpA initiation region of $\Delta 38$ could at most reduce trpA mRNA translation two-fold.

In four deletion plasmids, $\Delta 32$, $\Delta 33$, $\Delta 38$ and $\Delta 40$, trpB mRNA translation stops within or at the end of trpA mRNA. Expression of trpA of $\Delta 32$, $\Delta 33$, $\Delta 38$ and $\Delta 40$ is 0.2, 1.7, 16 and 1.4 percent of wild type, respectively. Expression of $\Delta 38$ is the highest of the four; only this deletion does not alter the normal Shine-Dalgarno sequence. Even if we correct for the possible inhibition by traversing ribosomes, $\Delta 38$ expresses trpA at only ca. 1/3 the level of wild type. Since in all four plasmids translation of trpB presumably terminates at least 30 nucleotides beyond the trpA start codon, trpA expression is probably not due to reading by the same ribosome that translates trpB mRNA. The ribosome binding site sequences in the four mutants therefore probably direct independent initiation at the trpA start site at different efficiencies.

Our findings suggest that efficient translation of trpA mRNA depends on prior translation of trpB mRNA and termination of translation at the normal

trpB stop codon. We do not know how translation termination at this stop codon facilitates initiation of trpA protein synthesis. Two possibilities are being considered. First, ribosome translation of the distal segment of trpB mRNA could expose a partially masked trpA translation initiation site, allowing it to function efficiently as a site of independent ribosome binding and initiation. Computer analysis of possible secondary structures involving the wild type trpA initiation region (29) suggest that a structure with a $\Delta G = -6.7$ could conceivably form and partially block ribosome access to the Shine-Dalgarno region. This same structure could form in pWS1-BamHI, $\Delta 40$ and $\Delta 38$ (see footnote b to Table I). Whether this structure does form and inhibit translation initiation is not known. Translational activation of ribosome binding sites has been suggested by several studies (3, 26, 27 and 28). Second, when a ribosome terminates translation at the trpB stop codon the 30S ribosomal subunit may remain engaged on the transcript and reinitiate translation at the trpA ribosome binding site. This reinitiation may be more efficient than independent initiation by free ribosomes. Studies *in vitro* of ribosome behavior at the translation termination codon of the coat protein gene of the RNA phage f2 suggest that the 30S ribosomal subunit remains transiently attached to the RNA following termination (30). It was suggested that when translation termination occurs near a translation initiation site, the 30S ribosomal subunit that participated in translation of the upstream coding region may reinitiate translation on the downstream coding region. The studies of "phaseless wandering" in rII mutants of bacteriophage T4 (31), translation reinitiation in T4rIIB (32) and in lacI mutants of *E. coli* (33), and lysis protein synthesis in phage MS2 (26), are consistent with this explanation. Since the position of the trpB stop codon appears to be crucial to translational coupling of trpB and trpA expression we feel that the latter possibility best explains our findings.

In these studies it was assumed that trpA polypeptide synthesis directed by deletion plasmids was initiated at the normal trpA start codon rather than at some neighboring normally unused start codon. We believe this to be the case for several reasons. First, activation of a restart site often requires premature termination of translation upstream from or very near the restart site (26, 31, 33). With the exception of the insertion in pWS1-BamHI, our plasmids have trpB terminators at their normal location or well within trpA. Second, in ribosome binding site protection experiments with trpA mRNA only the natural start site was protected against nuclease attack (5). [However, an inefficient ribosome binding site in trpA would not have been detected.]

Third, a trpA ochre mutant altered at codon 15, and its amber derivative, are non-leaky (34). This suggests that there are no restart sites at or near codon 15 that can be used to initiate synthesis of a functional trpA protein. We also believe that it is extremely unlikely that the absence of equimolar levels of the tryptophan synthetase β_2 subunit from strains with deletion plasmids is in any way responsible for low tryptophan synthetase chain activities. Extensive studies of α chain stability in vivo in the presence and absence of the β_2 subunit have given no indication of α chain lability (35, 36, Yanofsky, unpublished).

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