# The use of two-cistron constructions in improving the expression of a heterologous gene in *E.coli*

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## ABSTRACT

**Many heterologous genes when cloned into bacterial expression vectors are poorly expressed because of an inefficient ribosome binding site (RBS). We have constructed a plasmid which expresses human γ-interferon (γ-IF), where the level of expression is limited by the RBS. Expression was increased by placing the γ-IF sequence immediately downstream of a small translated sequence. The production of γ-IF was dependent upon the efficiency of translation of this upstream cistron and could be increased to very high levels. The same upstream cistron would greatly improve the expression of γ-IF in a plasmid where the RBS was very poor due to inhibitory secondary structure at the 5′ end of its mRNA. However, it would not improve the efficiency of a poor RBS containing a weak Shine-Dalgarno sequence. The general utility of the two-cistron expression strategy to diagnose a weak RBS is discussed.**

## INTRODUCTION

When a foreign gene is inserted immediately downstream of the initiating codon in an expression plasmid, expression in *E.coli* is dependent on several factors which are determined by the coding sequence itself. These include (a) the presence or absence of internal transcriptional termination signals, (b) the stability of the transcript, (c) efficiency of the RBS, (d) codon usage, and (e) stability of the product. When expression is poor, it is often difficult to identify the limiting factor, because transcription and translation are coupled in prokaryotes. Demonstration of a low steady state level of specific mRNA may implicate factors (a) or (b) but these can be secondary consequences of a primary translational effect (1, 2). It is therefore desirable to have a method for establishing if translational events are limiting. We have already described how we improved poor expression, when the limiting factor was codon usage (2). In this paper we use the expression of human γ-IF as an example of expression of a heterologous protein where initiation of translation is limiting and we demonstrate how a two-cistron construction can overcome this limitation.

γ-IF is a member of the interferon family of proteins which exhibit a range of anti-viral and anti-tumour activities (3). The gene has been cloned and expressed in various systems including *E.coli* (4, 5). A preliminary account of our initial expression studies of γ-IF in *E.coli* has already been published (6).

In *E.coli* the RBS is defined by an approximately 40 nucleotide segment of the mRNA molecule (7). It contains an initiation codon (usually AUG) preceded by a sequence containing a run of three to six nucleotides which are complementary to the 3′ end of 16S rRNA (8). The nature of an efficient RBS is still not understood. Large changes in expression have been reported when the distance between the Shine-Dalgarno (SD) sequence and the initiation codon has been altered (9). Equally large variation in expression levels has been observed when nucleotide changes have been made upstream of the SD sequence (6, 10, 11), downstream of the SD sequence (12, 13) and downstream of the initiation codon (14, 15). Secondary structure of the mRNA molecule which results in sequestration of the SD sequence and/or initiation codon has often been implicated as a reason for poor RBSs (14, 16–20). However, secondary structure has not been able to account for all of the variation in expression resulting from sequence changes in the RBS (12, 13, 21, 22).

One solution to the problem of a poor RBS is to mutagenize the RBS region and screen for increased expression (12, 15). However, this can be time-consuming and should not normally be considered without some evidence that the RBS is limiting expression. Another approach is to place the gene plus RBS downstream of another cistron which is efficiently translated. Such a two-cistron arrangement has been used successfully for some genes (20, 23). It relies on translational coupling where translation of the downstream cistron is dependent upon translation of the upstream cistron (24, 25) and not merely upon the absence of termination of transcription which often occurs in untranslated mRNA molecules (1, 26).

A number of models have been put forward to explain translational coupling: (i) disruption of inhibitory structure by a ribosome translating the upstream cistron, thereby making the RBS accessible to free ribosomes, (ii) translation of the upstream cistron resulting in local high ribosomal concentration in the vicinity of a weak downstream RBS, (iii) translation of both cistrons by the same ribosome (27, 28). Model (ii) is unlikely because of the finding that a SD sequence is required in the

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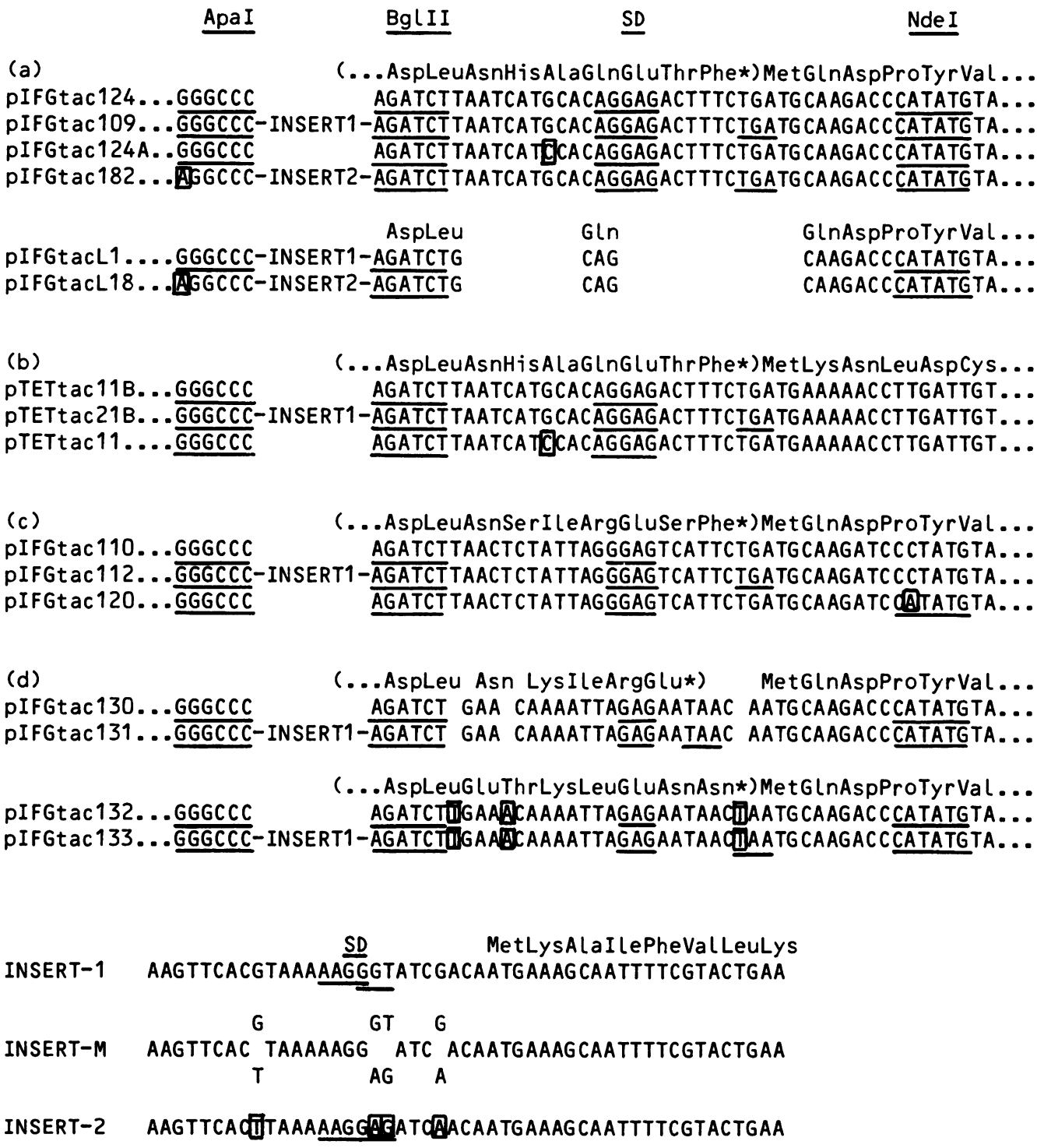


**Fig. 1.** DNA sequences in the RBS region of the expression plasmids. The amino acid sequence at the top of each group is for the N-terminus of γ-IF (a, c, d) or of tetanus toxin fragment C (b). The amino acid sequence in parenthesis is for the predicted C-terminus of the upstream short peptide which initiates in INSERT-1 or INSERT-2. Restriction enzyme sites, SD sequences and (where appropriate) stop codons for the upstream cistron are underlined. Base changes within each group are boxed.

downstream RBS (29, 30). In some examples, translationally coupled proteins are made in an equimolar ratio (24, 31, 32), which for those systems at least would clearly favour model (iii).

We have investigated the two-cistron approach in order to find out (a) if it always overcomes a poor RBS, (b) if it is the most efficient way of overcoming a poor RBS and (c) if it can be used as a diagnostic tool to identify a poor RBS. In order to do this we have adapted the sequence at the 3′ end of the *E.coli trpE* gene which also contains the RBS for the adjacent *trpD* gene to which it is translationally coupled (24, 33).

## MATERIALS AND METHODS

### Strain

*E.coli* strain MM294 (34) was used throughout this work.

### Plasmids

Plasmids pIFGtac124 and 124A have already been described (6), using the cDNA clone of Nishi *et al* (35) as source of γ-IF sequence. A restriction map of the derived plasmid pTETtac1 has been published in Makoff *et al* (36). Plasmid pIFGtac109 was constructed by cloning two oligonucleotides, containing the sequence INSERT-1 (Fig. 1), between the *Apa*I and *Bgl*II sites of pIFGtac124. Plasmid pIFGtacL1 was constructed by cloning two oligonucleotides between the *Bgl*II and *Nde*I sites of pIFGtac109. A mixed oligonucleotide, containing the complementary sequence of INSERT-M (Fig. 1), was cloned as a single strand between the *Apa*I and *Bgl*II sites of pIFGtacL1 to generate a number of possible plasmids, including pIFGtacL18.

Plasmids pTETtac11B and pTETtac21B were made from pTETtac11 (2), pIFGtac124 and 109 using the *Bsm*AI site (GAGAC) which is cleaved between the *Bgl*II site and SD sequence in all three plasmids. Plasmids pIFGtac110, 112, 120, 130, 131, 132, and 133 were made by cloning the appropriate pair of oligonucleotides between the *Bgl*II and *Nde*I sites of pIFGtac124 or 109.

All synthetic oligonucleotides were made on a Pharmacia Gene Assembler and the resulting plasmids confirmed by sequencing as previously described (2).

### Induction of cultures

Cultures were induced and analysed as described previously (36, 37). Levels of expression of γ-IF or tetanus toxin fragment C were quantified by densitometer scanning of SDS-polyacrylamide gels using a Joyce-Loebl Chromoscan 3. Western blots were visualized by a mouse monoclonal antibody (D7, a gift from Celltech) and goat anti-mouse IgG conjugated to horseradish peroxidase (Biorad) followed by colour development reagent and hydrogen peroxide. Expression was quantified after calibration of the blot by a range of dilutions of an extract of an induced culture of pIFGtac124 which has been estimated for expression levels as already described. The calibration curve was able to discriminate over approximately a twenty-fold range of γ-IF concentrations.

### Northern blots

RNA was prepared, separated on a gel, blotted, hybridized, autoradiographed and quantified as described previously (2). Similarly treated RNA size markers (BRL) were included in the gel. For hybridizing to γ-IF specific transcripts a DNA probe covering 424bp of γ-IF sequence just downstream of the *Nde*I site was used. For hybridizing to tetanus toxin C transcripts, an oligonucleotide which had been synthesized for the construction of pTETtac7 (2) was used.

### mRNA secondary structure

Prediction of secondary structure was carried out as described previously (37).

## RESULTS

### Expression of γ-IF

We have previously described three plasmids pIFGtac128, 124 and 124A, which each express different levels of γ-IF in *E.coli*

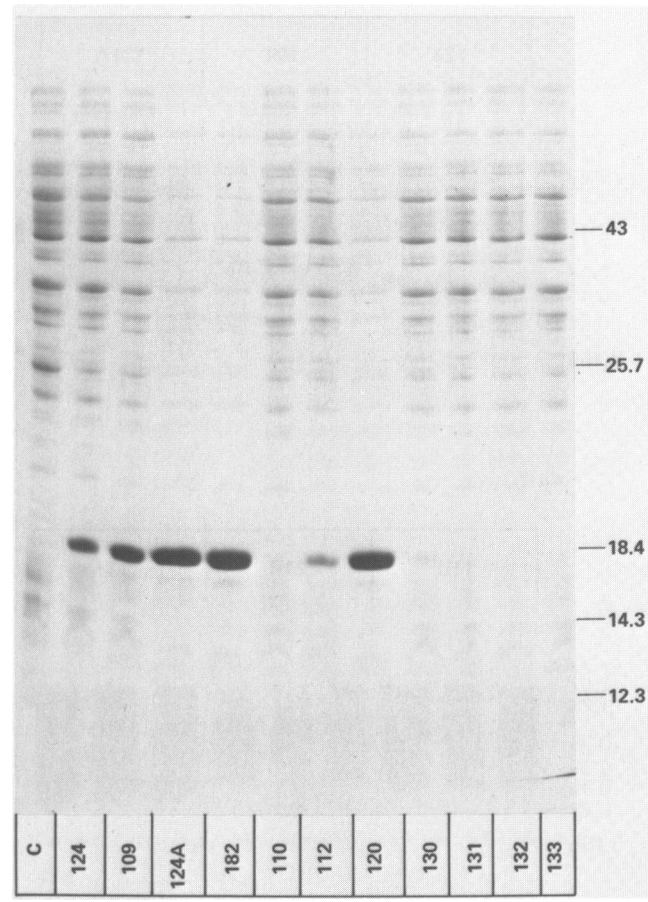


**Fig. 2.** Coomassie blue stained SDS-polyacrylamide gel analysis of induced extracts. The numbers of each track refer to the pIFGtac plasmid present in the culture; C refers to the control track. The size markers are given in Kdaltons.

(6). They all use the *trpD* SD and flanking region (33) and differ only in the sequence immediately upstream of the SD sequence, suggesting that their RBSs have different efficiencies.

In order to determine whether a two-cistron arrangement would improve the efficiency of initiation of translation of pIFGtac124, we constructed pIFGtac109 (Fig. 1). This plasmid differs from pIFGtac124 by the presence of the sequence INSERT-1 (Fig. 1) which contains the *trpL* RBS including the first eight codons of the *trpL* polypeptide (33). Initiation of translation at this RBS would be predicted to lead to translation of a short peptide comprising the first eight residues of the *trp* leader, the last six residues of the *trpE* gene product and a three amino acid linker. This arrangement mimics the *trpE/trpD* junction in the *trp* operon where the *trpD* gene is translationally coupled to the *trpE* gene (24).

As shown in Fig. 2, induction of pIFGtac109 led to increased expression of γ-IF when compared to pIFGtac124. The expression levels were quantified by densitometer scanning (Table 1), showing that the two-cistron arrangement approximately doubled expression. However a single base change in the RBS of the one-cistron plasmid to give pIFGtac124A (Fig. 1) increased expression further.

### Effects on mRNA levels

Although the sequence changes are in the appropriate region to affect initiation of translation, they could be increasing expression

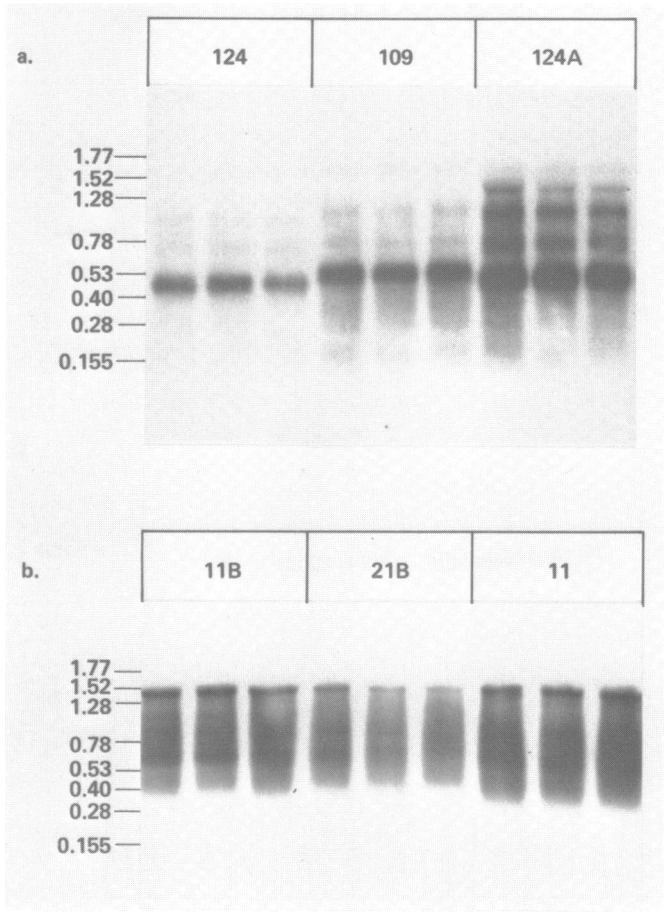


**Fig. 3.** Northern blots of RNA isolated from induced cultures. Each track represents an individual culture. The number on each group of three tracks refers to the plasmid in the three cultures. The sizes are given by RNA markers and are in kilo nucleotides. (a) pIFGtac plasmids; (b) pTETtac plasmids.

by a different route, e.g., by preventing premature termination of transcription or by stabilizing the transcript against degradation. In order to investigate these possibilities we looked at the steady state mRNA levels. The induced cultures of pIFGtac124, 109 and 124A which were used to provide protein samples for SDS-polyacrylamide gels were also used to provide RNA samples for Northern blotting (Fig. 3a). The relative levels of mRNA were estimated by densitometer scanning of the autoradiograph (Table 1) which shows that they closely parallel the protein levels. We ruled out the possibility that the differences in mRNA levels were due to loading differences by hybridizing a similar blot with a probe for β-lactamase (data not shown).

The observed close parallelism of mRNA and protein levels may imply that the three plasmids led to different expression levels by a direct effect on mRNA levels. Alternatively, it may result from different efficiencies of translation, as has been reported for expression of other sequences (1, 2). In order to distinguish between these two explanations, we examined expression of tetanus toxin fragment C in similar plasmids. We have made extensive studies of expression of fragment C in *E.coli* using plasmids based on pIFGtac124A and have no evidence that the RBS limits expression. In the case of plasmid pTETtac11, expression is clearly limited by mRNA levels (2) and consequently is sensitive to any changes which increase mRNA. We constructed from it the plasmid pTETtac11B which is analogous to pIFGtac124 and the two-cistron plasmid pTETtac21B which is analogous to pIFGtac109 (Fig. 1). Induced cultures of the three plasmids were used to produce protein samples for SDS-polyacrylamide gel electrophoresis and RNA samples for Northern blotting. The levels of expressed fragment C were very similar in all three cases (Table 1).

A Northern blot was made using RNA from three independent cultures of each plasmid and hybridized with a probe common to all three plasmids (Fig. 3b). As before, a β-lactamase probe was hybridized to a similar blot to show that any differences in mRNA levels were not due to loading differences (data not

**Table 1.** Levels of expressed protein and mRNA

| Plasmid | No of Cistrons | Expressed Protein (γ-IF or fragment C) % Cell Protein | Relative Levels* | mRNA Relative Levels |
|---|---|---|---|---|
| pIFGtac124 | 1 | 9.5 ± 0.8(5) | 1.0 ± 0.08 | 1.0 ± 0.2(3) |
| pIFGtac109 | 2 | 16.9 ± 0.8(5) | 1.8 ± 0.08 | 2.2 ± 0.3(3) |
| pIFGtac124A | 1 | 32.0 ± 4.0(5) | 3.4 ± 0.4 | 3.7 ± 0.3(3) |
| pIFGtac182 | 2 | 37.9 ± 3.6(3) | 4.0 ± 0.4 | ND |
| pTETtac11B | 1 | 5.8 ± 0.1(3) | 1.0 ± 0.02 | 1.0 ± 0.2(3) |
| pTETtac21B | 2 | 4.3 ± 0.2(3) | 0.7 ± 0.03 | 0.4 ± 0.1(3) |
| pTETtac11 | 1 | 6.5 ± 0.6(3) | 1.1 ± 0.1 | 1.1 ± 0.2(3) |
| pIFGtac110 | 1 | 0.4 ± 0.1(3)# | 1.0 ± 0.3 | ND |
| pIFGtac112 | 2 | 7.2 ± 3.0(3) | 18.0 ± 7.5 | ND |
| pIFGtac120 | 1 | 31.7 ± 2.0(3) | 79.3 ± 5.0 | ND |
| pIFGtac130 | 1 | 0.5 ± 0.2(3)# | 1.0 ± 0.4 | ND |
| pIFGtac131 | 2 | 0.2 ± 0.04(3)# | 0.4 ± 0.07 | ND |
| pIFGtac132 | 1 | 0.3 ± 0.03(3)# | 0.6 ± 0.06 | ND |
| pIFGtac133 | 2 | 0.1 ± 0.01(3)# | 0.3 ± 0.02 | ND |

* Levels are relative to the first member of each group.
\# Levels were estimated by Western blotting.
Number of determinations are shown in brackets.
ND=not determined.

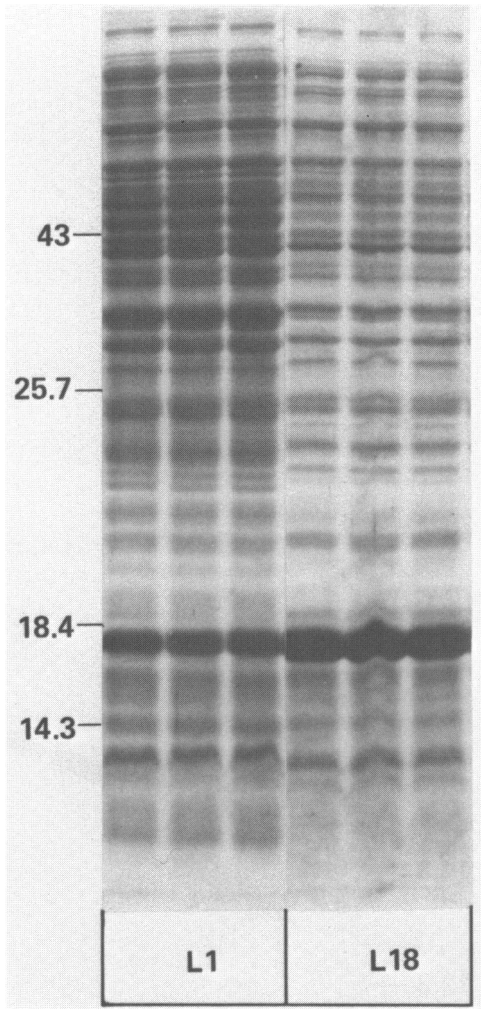


**Fig. 4.** Coomassie blue stained SDS-polyacrylamide gel analysis of three induced cultures each of pIFGtac L1 and L18. The γ-IF fusion protein is the major band in each case.

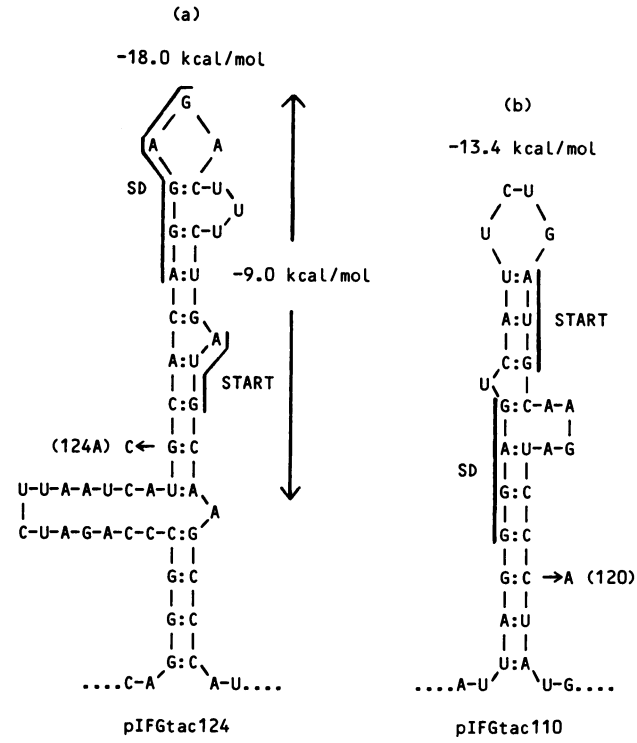


**Fig. 5.** Predicted secondary structure of the 5′ end of mRNA from plasmids pIFGtac124 and 110. Only the central part (−9.0kcal/mol) of the structure for pIFGtac124 is likely to have biological significance (see text). The SD sequence and start codon are indicated by the solid line. The single base change which destabilizes either structure are shown by the arrows.

shown). The levels of the full length fragment C transcript were estimated by scanning densitometry and are shown in Table 1. As can be seen, there are no significant differences in the levels of mRNA between pTETtac11B and 11. The level of mRNA from the two-cistron plasmid pIFGtac21B was actually significantly lower. Consequently, it is unlikely that either the G to C base change between pIFGtac124 and 124A or the upstream cistron insertion in pIFGtac109 directly increase mRNA levels.

### Effect of the Efficiency of the Upstream RBS

In the two-cistron plasmid pIFGtac109, the upstream cistron uses essentially the native *trpL* RBS. As shown in Fig. 1, it has an identical 52 nucleotide sequence flanking the AUG codon, including INSERT-1 (33). In order to screen for a stronger RBS we first constructed from pIFGtac109 the plasmid pIFGtacL1 which expresses a fusion protein between the N-terminal half of the *trpL* gene product and γ-IF. Between the *Apa*I and *Bgl*II sites of pIFGtacL1 we cloned a mixed single stranded oligonucleotide containing the complementary sequence of INSERT-M (Fig. 1). [The particular ambiguities in this sequence were chosen partly in order to decrease the base-pairing potential of the SD sequence and partly to reduce the G content, in view of the discrimination against Gs in natural RBSs (38) and the evident negative influence of Gs on several RBSs (12, 13, 21).] Induced cultures from a number of the resulting transformants were analysed by SDS-polyacrylamide gel electrophoresis which showed that many plasmids expressed higher levels of fusion protein than pIFGtacL1 (data not shown). Three independently induced cultures of one of these high expression plasmids (pIFGtacL18) were compared with three induced cultures of pIFGtacL1 and shown in Fig. 4. Sequencing of pIFGtacL18 showed that there were differences from pIFGtacL1 at all four ambiguous positions within the mixed oligonucleotide and also that a G from the *Apa*I site had been deleted (Fig. 1).

The sequence upstream of the *Bgl*II site of pIFGtacL18 was used to construct the two-cistron plasmid pIFGtac182 from pIFGtac124 (FIg. 1). As can be seen in Fig. 2 and Table 1, the improved upstream RBS increased expression of γ-IF still further and to levels approximately the same as the high expressing one-cistron plasmid pIFGtac124A.

### Two-cistron Stimulation of a Weak RBS Inhibited by Secondary Structure

The RBS of pIFGtac124 is not particularly weak, and so, of necessity, the lifting of such partial inhibition of initiation of translation by a two-cistron arrangement has only a relatively small effect on expression. Our preliminary report (6) presented data which rule out any inhibition of RBS in pIFGtac124 by the −18.0kcal/mol structure shown in Fig. 5a, but cannot establish whether or not the smaller −9.0kcal/mol structure within the larger structure is involved.

In order to construct a plasmid with a much weaker RBS we made a number of changes to pIFGtac124 which were likely to

lead to more stable secondary structure at the 5′ end of its mRNA. As shown in Figs. 1 and 5b, the plasmid (pIFGtac110) was designed so that its predicted secondary structure would be destabilized by a single nucleotide change (pIFGtac120). A two-cistron version of pIFGtac110 (pIFGtac112) was constructed (Fig. 1) and induced cultures of all three plasmids were analysed by SDS-polyacrylamide gel electrophoresis as before (Fig. 2). As can be seen, pIFGtac120 gave substantially more γ-IF than pIFGtac110. The two-cistron plasmid pIFGtac112 gave intermediate levels. Gel scanning could be used to quantify the levels of expression only in cultures of pIFGtac120 and 112 as no band could be detected on a stained gel for pIFGtac110. However, cultures of pIFGtac110 would give a detectable band on a Western blot (Fig. 6) and therefore, when calibrated with extracts of known γ-IF content, Western blotting could be used to estimate expression (see Materials and Methods). As shown in Table 1, the presence of an upstream cistron increased expression by a factor of between 10 and 20.

### Failure of Two-cistron Stimulation of a RBS Containing a Weak SD Sequence

The *trpE* RBS has a SD sequence which is predicted to interact very weakly with the 3′ end of 16S rRNA. In our hands, although it functioned sufficiently well to express high levels of the *trpE* gene product or its fusion proteins (37), it did not function well as a hybrid RBS with heterologous genes (unpublished observations). We constructed a plasmid (pIFGtac130) with such a hybrid RBS involving the *trpE* SD and flanking sequence (Fig. 1). As can be seen in Fig. 2, when an induced extract was analysed by SDS-polyacrylamide gel electrophoresis no stained band corresponding to γ-IF could be seen. Unlike the previous poor or moderate expressing plasmids no stable secondary structure could be predicted for the 5′ end of its mRNA. We made a two-cistron variant (pIFGtac131) of this plasmid, as before, (Fig. 1). Again, on induction, no γ-IF could be detected as a stained band on a SDS-polyacrylamide gel (Fig. 2). Comparison between expression levels required Western blotting (Fig. 6) and quantification was carried out as for pIFGtac110 (Table 1). As can be seen, on this occasion the two-cistron construction decreased expression. The failure of translation of the upstream cistron to stimulate expression could be due to the presence of the rare codon AGA (Fig. 1). Another possibility might be the increased spacing between the stop codon of the upstream cistron and the start codon of the downstream cistron. We investigated these possibilities by constructing the plasmids pIFGtac132 (one-cistron) and pIFGtac133 (two-cistron). These differ from pIFGtac130 and 131 by the insertion of three nucleotides which alter the reading frame for a short distance, thereby removing the rare codon from the upstream cistron and restoring the spacing between the cistrons (Fig. 1). Expression of γ-IF from induced cultures of these plasmids was also very low (Fig. 2), and again required Western blotting (Fig. 6) for quantification. As with the previous pair of plasmids, the two-cistron construction (pIFGtac133) gave lower expression than the one-cistron plasmid (Table 1).

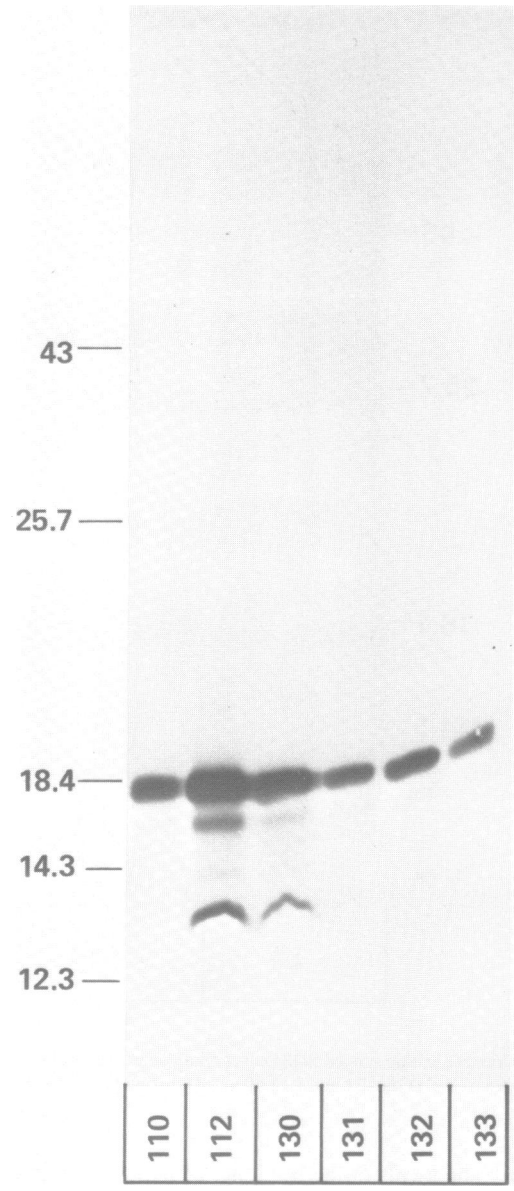


**Fig. 6.** Western blot of induced extracts. The numbers on each track refer to the pIFGtac plasmid present in the culture.

## DISCUSSION

In order to demonstrate translational coupling in a two-cistron system it is necessary to show that (a) stimulation of expression is due to translation of the upstream cistron, and (b) in the absence of the upstream cistron, expression is limited by the downstream RBS and not by premature termination of transcription. In practice it is difficult to show that both criteria have been satisfied because, in prokaryotes, transcription and translation are tightly coupled. Poorly translated mRNAs are often less abundant either because of degradation or premature termination of transcription (1, 2, 11, 39).

We believe that translational coupling occurs in both pIFGtac109 and 182 since, for the following reasons both criteria (a) and (b) have been satisfied. (i) Expression by pIFGtac124 was increased in the two-cistron plasmid pIFGtac109 and increased still further in pIFGtac182 where the upstream RBS is stronger (Table 1). (ii) Expression by pIFGtac124 could be increased by a single nucleotide change just upstream of the SD sequence to give pIFGtac124A (Table 1). This base change is in a region known to be involved in ribosome binding and may destabilize the central part (−9.0kcal/mol) of the putative

secondary structure (Fig. 5a). We have good evidence that the larger structure (−18.0kcal/mol) is not stable enough to influence expression in pIFGtac124 but when stabilized by a deletion also just upstream of the SD sequence, to give pIFGtac128 (6), a large decrease in expression results. (iii) Although mRNA levels of pIFGtac124, 109 and 124A closely paralleled expression levels, they did not parallel the mRNA levels of the analogous tetanus toxin fragment C plasmids (Table 1). This makes it most unlikely that the variation in expression levels between the three γ-IF plasmids is due to a transcriptional terminator upstream of the AUG codon of pIFGtac124.

Cho and Yanofsky (11) also observed parallel changes in mRNA and expression levels, after alterations upstream of the SD sequence. Their experiments suggested that the different mRNA molecules were being degraded at different rates but they could not distinguish between the sequences either directly influencing mRNA stability or exerting their influence via translation. Our experiments can rule out direct modulation of a degradation site upstream of the AUG, although they cannot rule out a degradation site which is dependent on sequences both sides of the AUG codon. The variation in mRNA levels is probably a consequence of variation in the translation of γ-IF due to ribosomal protection of either a transcriptional terminator or degradation site(s) within the γ-IF coding sequence itself. We observed a similar phenomenon when the level of tetanus toxin fragment C mRNA levels increased as a result of increased translation following alteration of the codon bias of the heterologous gene (2).

Because expression of pIFGtac124 was reasonably high, and therefore not greatly stimulated in the two-cistron construction, we designed two plasmids with poor RBSs. One of these (pIFGtac110) was designed to achieve this by means of the strong secondary structure while the other (pIFGtac130) was to achieve this by means of a weak SD sequence. In both cases, expression was very poor (Table 1). Plasmid pIFTtac110 could be modified by a single base change to give pIFGtac120, which completely destabilized the predicted secondary structure (Fig. 5b). This change brought about an 80-fold increase in expression (Table 1), which is consistent with mRNA secondary structure limiting expression in pIFGtac110 by inhibiting its RBS. The two-cistron arrangement (pIFGtac112) gave a 10−20 fold stimulation of expression over pIFGtac110 (Table 1). This is a much greater stimulation than that obtained with pIFGtac109 as compared to pIFGtac124, although the absolute level is lower. This may be because of the close proximity to the initiating codon of two rare codons: AGG in the upstream cistron and CCC in the downstream cistron.

By contrast, the two-cistron variant (pIFGtac131) of the other plasmid with a poor RBS (pIFGtac130), lowered expression still further (Table 1). This was apparently not due to either the rare codon AGA in the upstream cistron or the increased spacing between the cistrons, because essentially the same result was obtained with the similar plasmids pIFGtac132 and 133 which lacked both these features (Table 1). The high stimulation of translation in pIFGtac112 by translational coupling compared to the failure of any stimulation of translation in pIFGtac131 is therefore likely to be due to their different RBSs. In the former case, the RBS is undoubtedly inhibited by secondary structure, whereas the RBS of pIFGtac130 is devoid of any predicted stable secondary structure. This RBS is probably limited by its weak SD sequence, although it apparently functions adequately when translating *trpE* or gene fusions involving *trpE* (37). [It is possible that there is a feature in the native *trpE* RBS which is absent in our constructs and which compensates for the weak SD sequence.] It is very unlikely that secondary structure is a necessary part of the mechanism involved in translational coupling, since there are several published examples where secondary structure is not involved (21, 22, 40, 41). It would therefore appear that translational coupling requires a good SD sequence. This is in agreement with Schoner *et al* (30) who could detect no two-cistron stimulation of expression of bovine growth hormone in the absence of a SD sequence. However, Spanjaard and van Duin (41) detected weak two-cistron stimulation of rat α1-IF expression in the absence of a SD sequence, but they did not address the possibility that a transcriptional terminator was limiting expression in their one-cistron constructions.

Although, it is clear from our results that a two-cistron plasmid will not always overcome a poor RBS, the exception which we have found probably relates to the avoidable situation where a poor SD is used. Furthermore, it is unclear whether translational coupling will always provide the most efficient way of overcoming a poor RBS. However, our experiments with tetanus toxin fragment C show that there was a small two-cistron induced decrease in expression when the RBS was not limiting. This suggests that two-cistron plasmids should not be used routinely, although we cannot tell whether this decrease is a general problem or one that is due to a particular feature of our two-cistron plasmids. It is likely that two-cistron stimulation of expression would provide a good diagnosis for expression which is limited by the RBS. We have recently used this application to diagnose a poor RBS in a plasmid expressing very low levels of the P69 surface antigen of *Bordetella pertussis* (Makoff *et al*, submitted to Bio/Technology). Because of this, we mutagenized the RBS region and isolated a plasmid which expresses this recombinant protein at levels greater than 30% of total cell protein.

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