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Translational reinitiation in the presence and absence of a Shine and Dalgarno sequence

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ABSTRACT

The process of translational reinitiation in *Escherichia coli* was studied in a two cistron system where expression of the downstream reporter gene was dependent on translation of an upstream reading frame. The dependence was almost absolute. Upstream translation increased expression of the downstream gene by two to three orders of magnitude. This large difference allowed us to quantitate restarts in a meaningful manner. In the absence of a Shine and Dalgarno (SD) region reinitiation occurred but its efficiency was about 10% of that found in the SD carrying counterpart. We discuss three ways by which translational coupling between neighboring cistrons can be enforced.

INTRODUCTION

In Eubacteria like *Escherichia coli (E. coli)*, genes are usually expressed from polycistronic messengers. Ribosomes have the potential to initiate at internal cistrons, and by modulating the strength of each individual ribosomal binding-site (RBS) the cistrons present on a messenger can be translated with extremely varying relative rates (1). Nature can modulate the efficiency of a RBS by choosing a particular SD setting or by shielding the RBS in RNA secondary structure of varying stability (2−9; for further references see ref.10).

Sometimes, an internal cistron is inactive in itself and expression depends on translation of the 5′ neighboring cistron, a phenomenon called translational coupling (11). Couplings have been described for ribosomal protein operons and several other systems (12−16) and the cell can exploit this feature to control expression of a polycistronic messenger from a single point, *i.e.* the first RBS. It is assumed that in translational coupling the same ribosome that reads the upstream cistron reinitiates at the second frame.

The inactivity of a distal cistron in *de novo* initiation was in one instance shown to be caused by an inhibitory RNA structure which is melted and activated by the terminating ribosome (17), but there are also claims that inactivity can be an inherent property of the RBS, unrelated to secondary structure (18,19).

Reinitiation efficiency is variable and depends on unidentified context features, but it generally increases when the distance between the stop and the restart codon decreases (20,21). Still unclear in this process is the contribution of the SD region (15,22).

Here, we address this question using the rat interferon (IFN)-α_1 gene as the downstream cistron (23). Our IFN-α_1 clone has an inactive RBS that becomes activated by coupling to an upstream reading frame, *i.e.* the coat protein gene of bacteriophage MS2 (24). Restarts in the absence of an SD sequence are observed but their efficiency is 10-fold lower than when the SD region is present.

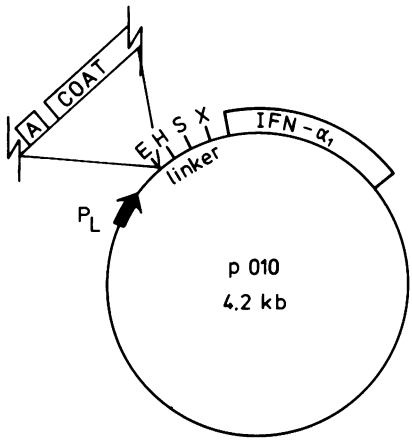

Fig. 1. Basic features of clone p010 containing the rat interferon-α_1 gene under transcriptional control of the lambda promoter p_L (25). The polylinker sequence in p010 is the same as in p13.3 (26). Some derivatives of p010 carry the MS2 coat gene fragment to provide for an upstream reading frame. The coat gene contributes about 100 amino acids.

MATERIALS AND METHODS

Strains and growth conditions

E. coli K-12 strain M5219, which carries a defective lambda prophage encoding the thermosensitive repressor *c*Its857 and the transcription antitermination factor *N*, was used in all experiments (25). Cultures were grown in L Broth, which contains (per liter) 10 g of tryptone, 5 g of yeast extract, 8 g of NaCl and 5 ml of 1 M Tris (pH 7.3). Cells were grown at 28°C to an OD_{650} of 0.2 and then induced at 42°C for 120 min.

Plasmid construction

All plasmids are derivatives of p13.3 (26). Clones designated p0.. were obtained by deletion and/or Klenow filling-in of the *Hin*dIII, *Sal*I and *Xba*I restriction sites in the polylinker sequence upstream of the IFN-α_1 start of clone p010. The upstream reading frame was provided for by an *Eco*RI fragment carrying the 5′ terminal part of the coat gene (MS2 numbers 103−1628 (24)). This fragment was inserted in the *Eco*RI site of p004, p108, p029, p030, p070 and p4. A coat gene derivative with a −1 frameshift, due to a single base insertion (17), was inserted in the *Eco*RI site of p071 and p073. Clone p003 carries the *Eco*RI fragment which lacks the RBS of the coat gene (MS2 sequence 1308−1369 is deleted). The construction of clones p108 (27), p1 and p8 (9) has been described.

Antiviral activity of rat IFN-α_1

10 ml samples of induced cultures were centrifuged and the bacterial pellet was re-suspended in 1ml 6M guanidine-HCl (pH 4.7), 1% β-mercaptoethanol. After overnight incubation at 4°C, the supernatant was tested for antiviral activity by measuring protection of RATEC cells against Vesicular Stomatitis Virus infection (26). Cells withstand up to 6 mM guanidine-HCl and the supernatants must accordingly be diluted at least 1000 times. For this reason the activity can generally not be measured below 10^5-10^4 units.

Table I. Leader sequences and IFN-α_1 yields of clones used in this study.

Clone	*Hin*dIII	*Sal*I	*Xba*I (SD)	D.	units
p1	AAGC	UUAGUUGUCGACUGUUACAGGAGGUCUAGACAUG		-	$<1.6 \cdot 10^4$
p4	AAGC	UUAGUUGUCGACUGUUACAGGAGGUCUAGACAUG		5	$1.5 \cdot 10^7$
p8	AAGC	UUAGUUGUCGACUGUUACAGGAGAUCUAGACAUG		-	$1.0 \cdot 10^7$
p010	AAGC	UUAGUUGUCGA	CCAAGGAGGUCUAGACAUG	-	$2.7 \cdot 10^5$
p108	AAGC	UUAGUUGUCGA	UAAAGGAGGUCUAGACAUG	16	$3.4 \cdot 10^7$
p029	AAGCUAGCUUAGUUGUCGA		CCAAGGAGGUCUAGACAUG	26	$1.1 \cdot 10^7$
p001	AAGC	UUAGUUGUCGAUCGACCAAGGAGGUCUAGACAUG		-	n.m.
p004	AAGC	UUAGUUGUCGAUCGACCAAGGAGGUCUAGACAUG		5	$3.3 \cdot 10^7$
p030	AAGCUAGCUUAGAAGUCGAUCGACCAAGGAGGUCUAGACAUG			30	$1.2 \cdot 10^7$
p003	AAGC	UUAGUUGUCGAUCGACCAAGGAGGUCUAGACAUG		-	n.m.
p064	AAGC	UUAGUUGUCGAUCGAC	UAGACAUG	-	$<2.3 \cdot 10^5$
p073	AAGC	UUAGUUGUCGAUCGAC	UAGACAUG	20	$1.2 \cdot 10^6$
p001	AAGC	UUAGUUGUCGAUCGACCAAGGAGGUCUAGACAUG		-	n.m.
p004	AAGC	UUAGUUGUCGAUCGACCAAGGAGGUCUAGACAUG		5	$3.3 \cdot 10^7$
p063	AAGCUAGCUUAGUUGUCGA	C	UAGACAUG	-	$<2.3 \cdot 10^5$
p071	AAGCUAGCUUAGUUGUCGA	C	UAGACAUG	5	$3.4 \cdot 10^6$
p070	AAGCUAGCUUAGUUGUCGA	C	UAGACAUG	16	$1.5 \cdot 10^6$
p029	AAGCUAGCUUAGUUGUCGA		CCAAGGAGGUCUAGACAUG	26	$1.1 \cdot 10^7$

Sequences downstream from the underlined IFN-α_1 start codon are identical in all clones. Gaps are introduced to align identical sequences. Stop codons, when in use by upstream translation, are underlined. In clone p003 the RBS of the coat cistron is deleted. The G -> A substitution in clone p4 which yields p8 is indicated by an arrow.
D = distance between stop and restart codon in nucleotides. A dash means that no upstream reading frame is present. U = units IFN activity in bioassay (see **MATERIALS AND METHODS**). Clones p001 and p003 were not measured (n.m.) in the bioassay but inspection of Western blots indicated that they produced not more than 10^5 units. The yield of p1, p063 and p064 was estimated from immunoblots to be lower than 10^5 units.

RESULTS

Translation of the IFN-α_1 messenger depends on upstream translation

In all of our clones transcription is under control of the p_L promoter which in turn is regulated by the thermosensitive repressor *c*Its857. As an upstream reading frame we used the coat gene of bacteriophage MS2 (Fig. 1). Translation from this gene ends at various positions ahead of the IFN-α_1 start depending on deletions or insertions introduced in the polylinker sequence. In Table I the sequences of the IFN-α_1 leader regions in various clones are presented. Whenever the upstream reading frame is in use its termination codon is underlined and the distance between that codon and the IFN-α_1 start is shown as the number of nucleotides under D. If upstream reading is absent this is indicated by a dash

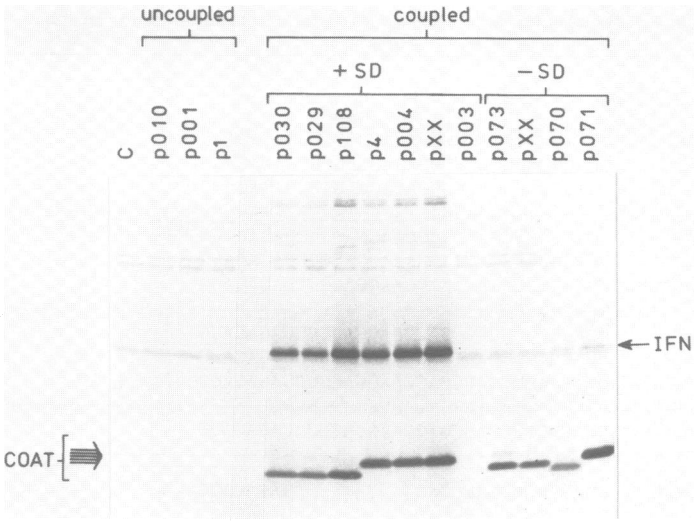

Fig. 2. Western blot (28) showing rat IFN-α_1 and MS2 coat protein synthesis in various clones. The antiserum used to develop the blot was raised against a coat protein-IFN-α_1 fusion protein. Note that there is a background band just below the IFN-α_1 position. The upper band is IFN-α_1. The control in the left lane (C) contains a plasmid without the coat and IFN-α_1 gene. 'Coupled' means that the upstream reading frame is present. Clones marked pXX have another IFN-α_1 leader sequence. Their discussion is not relevant in this publication.

under D. IFN-α_1 expression is measured in a bioassay (see MATERIALS AND METHODS) and the protein is also visualized in immunoblots.

In plasmids p1, p010 and p001, which differ in their leader sequence, the IFN-α_1 gene is not preceded by the MS2 coat gene and expression is very low ranging from 10^4 to $2.7 \cdot 10^5$ units (Table I, Fig. 2). This result illustrates that the RBS of these particular IFN-α_1 clones is almost completely inactive in *de novo* initiation. Whenever we insert the upstream reading frame there is a dramatic increase in IFN-α_1 activity. For instance, between the couple p4 and p1 there is a 1000 fold increase and p004 synthesizes 100 times more IFN-α_1 than its uncoupled counterpart p001 (see Fig. 2). Essentially the same stimulation is seen in the series p010, p108 and p029.

Comparison of all clones in the upper part of Table I yields the general pattern that the restart event proceeds with higher efficiency when stop and restart codons lie closer together. Clone p003 is an additional control showing that the coupled IFN-α_1 activity indeed depends on upstream translation. In this clone, the RBS of the coat gene is deleted and as expected IFN-α_1 activity returns to a very low level (Fig. 2).

We have shown elsewhere (9) that the RBS of the IFN-α_1 gene in the clones discussed above is inactive due to the presence of a hairpin in which the SD region is basepaired to residues 7−15 of the coding region (Fig. 3). We assume therefore that the activation is based on melting of this structure by the terminating ribosome followed by random lateral diffusion along the mRNA which will eventually bring the AUG codon in register with the ribosomal P-site. This point of view explains the correlation between restart efficiency and the distance to be bridged. (A mechanism involving a second ribosome taking advantage of the melted RBS is unlikely since the physical space to place two ribosomes is lacking.)

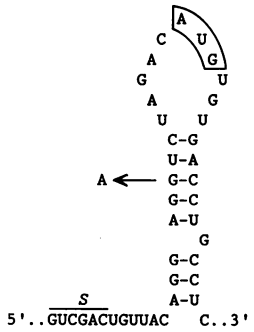

Fig. 3. The IFN-α_1 initiator hairpin present in p1 and related clones. The structure is based on RNAse sensitivity of nucleotides, mutational analysis and computer predictions (9). The G→ A substitution yields clone p8 which produces about 1000 times more IFN-α_1 activity than clone p1 (Table I). The start codon is boxed. *S* is *SalI*.

An SD sequence stimulates reinitiation efficiency by an order of magnitude

Clone p063 does not contain an SD region nor an upstream reading frame and its activity is consequently low ($<2.3 \cdot 10^5$ units) (Table I). In the equivalent clones p070 and p071 upstream translation is imposed and we notice at least a 10-fold increase in IFN-α_1 activity. It is probably significant that also here restarts occur more efficient when the distance between termination and restart site gets shorter. In clone p071 IFN-α_1 synthesis is twice as high as in clone p070. We can also compare clone p070 to p029 which is its SD(+) equivalent. The restart dependent activity in this clone is 10 times higher, which we ascribe to the presence of the SD sequence.

A second set of clones has as its SD(−) prototype clone p064. This clone as well as its SD(+) counterpart clone p001, produce background levels of IFN-α_1 activity ($\approx 10^5$ units). However, clone p001 can be activated about 100-fold by upstream translation (clone p004). In contrast, clone p064 is only stimulated one order of magnitude by upstream translation (clone p073).

Our conclusion is that restarts do not need an SD sequence *per se* but are about 10-fold stimulated by its presence. This is in good agreement with results presented by Das and Yanofsky (15) working with the coupled *trpB-trpA* genes. When the SD sequence in front of the *trpA* gene was removed, trpA levels were only 7% of that when the SD region was present.

We propose here that the mechanism of reinitiation in SD(−) and SD(+) clones is basically the same. The terminated ribosome is thought not to immediately leave the messenger, and its eventual alignment with the AUG codon may result in a restart event. It seems plausible that in the absence of an SD sequence the sticking time to the mRNA, and thus the reinitiation probability, is drastically reduced. We feel that these results are relevant because they show that translational coupling can be enforced by an inadequate SD region (see below).

DISCUSSION

In this paper we analyze translational restarts at varying stop to restart distances, in the absence or presence of an SD sequence. Our results indicate that shorter distances are

more efficient irrespective of the presence of an SD region. We also find that an SD sequence increases the translational restart frequency by an order of magnitude.

An important aspect of translational coupling is the question which structural feature renders the downstream RBS inactive in *de novo* initiation. We can distinguish three ways to achieve this feat.

The first is RNA secondary structure. This was shown for the MS2 lysis cistron whose activity depends on upstream translation. All nucleotide substitutions that led to a decreased stability of the hairpin which masks the initiation signals of the lysis gene resulted in uncoupled expression (8). An inhibitory structure was also found in the IFN-α_1 clones used in this study and indeed the IFN-α_1 cistron can also be activated by a simple G → A substitution that breaks up a G-C base pair in the initiator hairpin (clone p8 in Table I and Fig. 3) (9).

The second possibility to keep a downstream RBS silent is the absence of an adequate SD region. As we show here, an SD-less RBS can, to a certain degree, be activated by close by termination. High expression levels can probably not be reached in these instances. This mechanism may be applicable to gene VII in phage f1 whose expression is coupled to translation of the upstream gene V (19). Deletion studies ruled out a role for a repressive RNA folding (29) but we note that gene VII has a poor SD sequence.

The third possibility is the use of an improper initiation codon at the distal gene. The lysis gene in RNA phage fr starts with a UUG codon and its expression is dependent on translation termination of the upstream cistron. Deletion studies did not reveal an inhibitory RNA structure. In addition, uncoupled translation was achieved when the UUG codon was replaced by GUG or AUG (30). It was speculated that *de novo* initiation at UUG codons is suppressed by IF3 mediated proofreading of the codon-anticodon interaction (31). Restarts would not be scrutinized since a free 30S ribosome·IF3 complex is not necessarily an intermediate in the restart pathway. In fact, there is some evidence that ribosomes cross the boundary between translationally coupled cistrons as 70S particles (32) and IF3 has no affinity for 70S ribosomes.

From the relative amounts of coat protein fragment and IFN-α_1, present in clone p004 after radiolabeling, we calculate a reinitiation efficiency of about 2% (data not shown). Accordingly, in our SD(−) clones restarts occur with a probability of 0.2%.

All of our results are consistent with a model in which restarts (at least those occurring over short distances) are mediated by a fraction of the terminating ribosomes that remains messenger-bound, and is potentially capable to assemble into an initiation complex when an initiation codon is hit. The probability to reach a start codon obviously increases with decreasing distances between stop and restart and with the presence of an SD region.

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