# TOP mRNAs are translationally inhibited by a titratable repressor in both wheat germ extract and reticulocyte lysate

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**Abstract Vertebrate TOP mRNAs contain a 5′ terminal oligopyrimidine tract (5′ TOP), which is subject to selective translational repression in non-growing cells or in cell-free translation systems. In the present study, we monitored in vitro the effect of increasing amounts of a 16 nucleotides long oligoribonucleotide representing the 5′ terminus of mouse ribosomal protein S16 mRNA on the translation of TOP and non-TOP mRNAs. Our results demonstrate that the wild-type sequence (but not its mutant counterparts) derepresses the translation of mRNAs containing 5′ TOP motifs, but failed to stimulate the translation of non-TOP mRNAs, even if the latter differed only by a single nucleotide from their 5′ TOP-containing counterparts. Similar results have been obtained with both wheat germ extract and rabbit reticulocyte lysate. It appears, therefore, that translational repression of TOP mRNAs is achieved in vitro by the accumulation of a titratable repressor rather than by the loss of an activator and that this repressor recognizes multiple TOP mRNAs with a diverse set of 5′ TOP motifs.**



*Key words:* Ribosomal protein mRNA; Oligopyrimidine tract; Translational repressor

## 1. Introduction

TOP mRNAs are vertebrate mRNAs encoding nearly 90 proteins, most of which have been assigned to the translational apparatus. These include ribosomal proteins (rp) [1,2], elongation factors EF1α and EF2 [3,4] and poly(A)-binding protein [5]. The translation of these mRNAs is selectively repressed upon growth arrest and this response is mediated by a translational *cis*-regulatory element (TLRE) included within the first 30 nucleotides of TOP mRNAs. The TLRE is comprised of a cytidine residue at the cap site followed by an uninterrupted stretch of up to 13 pyrimidines [1]. Full manifestation of this mode of regulation seems to require both the 5′ TOP and sequences immediately downstream, yet, the translational control is strictly dependent on the 5′ terminal location of the oligopyrimidine tract [6,7]. It should be noted that selective discrimination against TOP mRNAs can be recapitulated in vitro as is evident when it is translated in rabbit reticulocyte lysate or wheat germ extract, both of which derived from non-growing cells [8].

A simple model to account for the selective growth-dependent translational control of TOP mRNAs assumes the participation of a factor that is a component of the general protein synthesis machinery. If such a factor has a particular low affinity for TOP mRNAs, due to their unique sequence at the 5′ terminus, a decrease in its activity or level of expression might lead to a disproportionate discrimination against these mRNAs [9,10]. A prime candidate for such a factor was the limiting initiation factor eIF-4E, which binds specifically to the cap structure of mRNAs and comprises the α subunit of the three-subunit complex, eIF-4F. This hypothesis was supported by the observation that the translation of mouse rp mRNAs in rabbit reticulocyte lysate is relatively inefficient unless either eIF-4F or eIF-3 are added [11]. However, later studies have demonstrated that the presence of a 5′ TOP motif in TOP mRNAs does not lessen their competitive potential for the cap-binding protein [8,12] and that the decrease in activity of eIF-4E upon growth arrest does not play a key role in the repression of translation of TOP mRNAs [12].

A recent kinetic study of the effect of growth arrest on the translational behavior of a TOP mRNA, in comparison with the effect of other treatments that exert a global translational repression, has suggested the involvement of a specific translational regulator of TOP mRNAs [13]. However, the nature of this modulator, activator or repressor, remains obscure.

The apparent discrimination against TOP mRNAs could reflect a relative deficiency of an activator needed for efficient translation of this class of mRNAs or, alternatively, accumulation of a specific repressor upon quiescence or in cell-free translation systems. To distinguish between these two possibilities, we monitored the effect of a short synthetic RNA oligonucleotide containing the first 16 nucleotides of rpS16 mRNA on the translation of TOP and non-TOP mRNAs in wheat germ extract or rabbit reticulocyte lysate. Our results demonstrate that addition of this oligoribonucleotide, in molar excess, selectively increases the translational efficiency of a wide variety of chimeric TOP mRNAs, but not of subtly mutated counterparts. This observation supports the notion that both wheat germ extract and rabbit reticulocyte lysate contain a titratable repressor, which recognizes a wide range of 5′ TOP and adjacent sequences.

## 2. Materials and methods

### 2.1. RNA isolation, cell-free translation and analysis of products

Poly(A)$^+$ RNA used for in vitro translation was prepared by the PolyATract System 1000 (Promega). Preparation of cell-free extracts from wheat germ (General Mills) and the conditions for the translation reaction were as previously described [8]. Rabbit reticulocyte lysate (Flexi) was from Promega. Total protein synthesis was measured by monitoring the incorporation of [$^{35}$S]methionine (Amersham) into trichloroacetic acid-precipitable material [14]. To obtain growth hormone (GH) mRNAs with the desired 5′ termini, we initially constructed GH genes driven by various promoters. These chimeric genes were stably transfected into NIH 3T3 cells and the transcription start site for each construct was identified by primer extension analysis [6,7,15,16]. The translation reaction of chimeric GH mRNAs was

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carried out in the presence of an unlabelled mixture of all 20 amino acids and the amount of the resulting protein was assessed by a radioimmunoassay with a commercial kit (Nichols Institute, San Juan Capistrano, CA, USA).

*2.2. RNA oligonucleotides*

wtS16, CCUUUUCCGGUCGCGG; CM5, CGAGUGACCGGUCGCG and m(7-16) CCUUUUAGCUGAAGUC were synthesized in the Oligonucleotide Synthesis facility of the Hebrew University-Hadassah Medical School, Jerusalem, Israel.

## 3. Results

We have previously shown that both rabbit reticulocyte lysate and wheat germ extract selectively discriminate against TOP mRNAs [8]. One plausible explanation for the relative inefficient translation of TOP mRNAs in vitro is the presence of a specific repressor, as previously shown for reticulocyte lysate which failed to translate ferritin mRNA [17]. To directly address this possibility, we monitored the effect of 16 nucleotides long RNA oligonucleotides on the in vitro translation of GH mRNAs containing a variety of 5′ termini. Fig. 1a shows that increasing amounts of an oligoribonucleotide corresponding to the first 16 nucleotides of rpS16 mRNA (wtS16) progressively relieved the translational repression of a mRNA initiating with the first 29 nucleotides of the same mRNA (S16(1-29)-GH). In contrast, mutant oligoribonucleotides, with either substitution of five out of the eight pyrimidines by purines (CM5) or random replacement of nucleotides spanning positions 7–16 (m(7-16)), had no effect on the translational efficiency of this mRNA. It should be noted that similar mutations in S16-GH mRNA render this mRNA refractory to translational control in vivo [7,15]. Moreover, we have previously shown that wheat germ extract translates S16(1-29)-GH mRNA about 2.5 times less efficiently than S16CM3-GH mRNA. Hence, it appears that the wtS16 completely relieves the translational repression of the former.

S16CM3-GH mRNA is not subject to translational regulation in vivo, even though it only differs by a single base substitution (a C to A at the cap site) from its wild-type counterpart, S16(1-29)-GH [6]. Accordingly, wtS16 as well the two mutant oligoribonucleotides (CM5 and m(7-16)) did not stimulate the translational efficiency of S16CM3-GH mRNA (Fig. 1b).

To examine whether the stimulatory effect of wtS16 is confined only to an mRNA with a homologous sequence, we monitored its effect on the translational efficiency of L32(1-34)-GH, a TOP mRNA containing the first 34 nucleotides of mouse rpL32. The results showed that wtS16 can derepress the translation of this mRNA, even though its 5′ terminal sequence bears no homology with that of the oligoribonucleotide, and that the two mutant oligoribonucleotides lack such a capacity (Fig. 1c). L32(-$1_{C\rightarrow A}$)-GH mRNA differs from its wild-type counterpart, L32(1-34)-GH mRNA, by an extra A residue preceding an intact 5′ TOP. This mRNA is not subject to translational control in vivo [7] and, accordingly, is not affected by the wtS16 or by its mutant forms (Fig. 1d). Notably, a highly selective relief of the translational repression has also been observed for L13a-GH mRNA, which

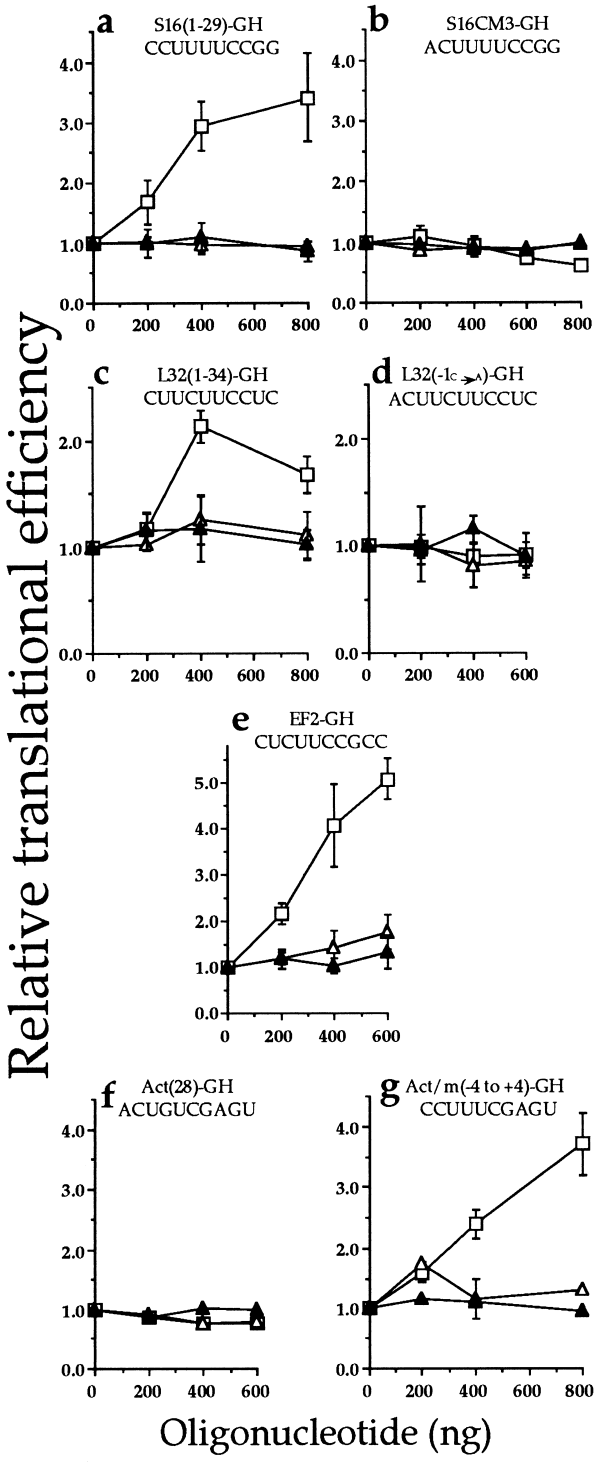


Fig. 1. The first 16 nucleotides of rpS16 mRNA can derepress the translation of various TOP mRNAs in wheat germ. 100 ng of poly-$(A)^+$ RNA from NIH 3T3 cells, expressing various chimeric mRNAs (a, S16(1-29)-GH; b, S16CM3-GH; c, L32(1-34)-GH; d, L32(-$1_{C\rightarrow A}$)-GH; e, EF2-GH; f, Act(28)-GH; g, Act/m(−4–+4)-GH), was translated in wheat germ extract (total volume of 25 μl) in the absence or presence of RNA oligonucleotide (□, wtS16; Δ, CM5; ▲, m(7-16)). The oligoribonucleotides were added 10 min prior to the addition of the poly$(A)^+$ RNA. The amounts of GH produced in the presence of an oligoribonucleotide were normalized to that produced in its absence, which was arbitrarily set at one for each poly$(A)^+$ RNA preparation. Results are presented as averages of at least two experiments and the vertical bars represent S.E.M. calculated for 3–9 experiments. The 5′ terminal 10 nucleotides of each chimeric GH mRNA are indicated.

contained the first 27 nucleotides of mouse rpL13a mRNA (data not shown).

To further explore the range of the derepression capacity of wtS16, we monitored its effect on EF2-GH mRNA. This mRNA contains the first 29 nucleotides of Chinese hamster EF2 mRNA in its 5′ terminus and is a translationally regulated TOP mRNA [15]. The results show that the effect of wtS16 is not confined to rp mRNAs, as it also derepresses very efficiently the translation of EF2-GH mRNA (Fig. 1e).

Endogenous actin mRNA or the chimeric Act-GH mRNA are efficiently translated regardless of the growth status of cells and have therefore previously been used as an invariant negative control in our studies of the translational control of TOP mRNAs. Accordingly, wtS16 displayed no stimulatory effect on the translational efficiency of Act(28)-GH mRNA (Fig. 1f). However, three base substitution at and adjacent to the cap site of this mRNA converted it into a TOP mRNA, which is translationally regulated in vivo [16] and is a subject for relief of the translational repression in vitro (Fig. 1g).

Finally, we have examined whether the selective derepression potential of wtS16 is also applicable for reticulocyte lysate. Fig. 2 shows that this oligoribonucleotide relieves the translational repression of S16(1-29)-GH mRNA, but has no effect on S16CM3-GH mRNA. The selectivity is further underscored by the lack of any effect of the mutant oligoribonucleotide, CM5, on either of these two mRNAs.

## 4. Discussion

We have previously shown that the translation efficiency of S16(1-29)-GH mRNA is approximately 2–3-fold lower than a subtly mutated mRNA, S16CM3-GH, in both wheat germ extract and rabbit reticulocyte lysate [8]. In the present report, we have shown that the stimulatory effect of the oligoribonucleotide wtS16 on the translation of S16(1-29)-GH mRNA in both these cell-free translation systems seems to relieve exactly the specific translational repression of this mRNA. It appears therefore that the translation of TOP mRNAs is selectively repressed in both the mammalian- and plant-derived extracts due to the presence of a titratable repressor, rather than the relative absence of a general translation factor or a specific activator. The apparent variation in the extent of stimulation displayed by individual TOP mRNAs, upon titration of the repressor, is likely to reflect their relative affinity to the latter.

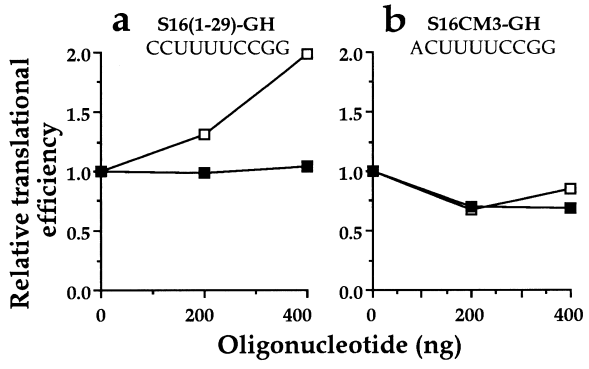


Fig. 2. The first 16 nucleotides of rpS16 mRNA can derepress the translation of a TOP mRNA in reticulocyte lysate. 100 ng of poly-(A)$^+$ RNA from NIH 3T3 cells expressing S16(1-29)-GH (a) or S16CM3-GH (b) was translated in reticulocyte lysate (total volume of 25 μl) in the absence or presence of RNA oligonucleotides (□, wtS16; ■, CM5). The oligoribonucleotides were added 10 min prior to the addition of the poly(A)$^+$ RNA. Due to a non-specific inhibitory effect of both oligoribonucleotides on the global translational efficiency in reticulocyte lysate, their effect on the incorporation of [$^{35}$S]methionine and on the production of GH was measured in parallel. To calculate the net effect of the oligoribonucleotide on the translation of the chimeric mRNAs, the amount of GH produced was divided by the radioactivity incorporated under the same conditions. The effect of each dose of oligoribonucleotide was normalized to that calculated for translation in its absence, which was arbitrarily set at one for each poly(A)$^+$ RNA preparation. The results represent two experiments. The 5′ terminal 10 nucleotides of each GH mRNA are indicated.

The presence of a translational repressor in the reticulocyte lysate is consistent with the fact that this cell-free translation system originates from non-growing cells and that translational repression of TOP mRNAs has been demonstrated upon quiescence of any of the examined cell types [1]. The physiological role of the repressor in wheat germ, however, is still obscure, even though at least two 5′ TOP-containing rp mRNAs have already been identified in plants [18,19].

The structural features within the RNA which are required for its recognition by the repressor in vitro are those previously defined as necessary for growth-dependent translational control in vivo. These include: (a) an intact stretch of pyrimidines as exemplified by both the inability of the oligoribonucleotide CM5 to derepress the translation of the chimeric TOP mRNAs and the lack of activation of S16CM3-GH or Act(28)-GH mRNAs by the wild-type oligoribonucleotide, (b) a GC-rich sequence immediately downstream of the 5′ TOP, as judged by the failure of the oligoribonucleotide m(7-16) to exert a derepressive effect on TOP mRNAs and (c) location of the pyrimidine stretch at the 5′ terminus, as evidenced by the lack of an activation effect of the wild-type oligoribonucleotide on the translation of L32(-1$_{C\rightarrow A}$)-GH mRNA. It is noteworthy that the wild-type oligoribonucleotide, wtS16, used in the present study perfectly matches the first 16 nucleotides of mouse rpS16 mRNA, yet the former, due to inherent technological limitations of the automated oligonucleotide synthesis, lacks the cap structure. It appears, therefore, that interaction of the repressor with its cognate sequence, although strictly dependent on its location at the 5′ terminus, does not display an absolute requirement for the cap structure. However, we cannot exclude the possibility that the relatively high concentration of the wild-type oligoribonucleotide needed for the derepression simply reflects its deviation from the structure of an authentic 5′ TOP.

An intriguing question is how many different repressor molecules there are required to recognize and inhibit all TOP mRNAs (at least 85 species are known to date [1]) which vary in both their 5′ TOP and the adjacent sequences. Our present results show that the first 16 nucleotides of mouse rpS16 mRNA are sufficient to titrate out, in the wheat germ, a repressor which recognizes any of the examined TOP mRNAs. We have previously shown that a selective discrimination against TOP mRNAs is similarly displayed by wheat germ extract and non-growing cells [8]. Taken together, it is conceivable that quiescent cells, like the wheat germ, utilize one or, at the most, a few different repressor molecules to regulate the translation of the diverse TOP mRNA species.

Establishing the role of the 5′ TOP in the translational control of TOP mRNAs has led to the hypothesis that this

motif might bind a specific translational *trans*-acting factor. Indeed, a cytoplasmic protein from mouse T-lymphocytes was shown to specifically bind the first 34 nucleotides of mouse rpL32 mRNA [20,21]. Similarly, a sequence containing the first 52 nucleotides of *Xenopus* rpL1 mRNA directs binding of the La autoantigen to the oligopyrimidine tract and a cytoplasmic nucleic acids-binding protein to sequences immediately downstream [22–24]. It should be noted, however, that no direct relationship in vivo or in vitro has been demonstrated so far between the binding of these proteins to the TLRE and the growth-dependent translational control of TOP mRNAs.

The involvement of pyrimidine-binding proteins in translational control has recently been reported for 15-lipoxygenase mRNA. This mRNA is translationally repressed in reticulocytes but becomes active during erythroid differentiation. This mode of control is mediated through a tandem repeat of a pyrimidine-rich sequence residing at the 3′ untranslated region of this mRNA [25]. Two proteins have been shown to bind this *cis*-regulatory element, hnRNP K and hnRNP E1, and by doing so, they act as translational silencers of 15-lipoxygenase mRNA [26].

Despite intensive efforts, we have failed to show, by an electrophoretic mobility shift assay or UV-cross linking, any specific binding of a protein to the wtS16 oligoribonucleotide. This failure is further underscored by the fact that binding to the 240 nucleotides long pyrimidine-rich *cis*-regulatory element of 15-lipoxygenase mRNA was readily demonstrated under the same conditions (data not shown). Moreover, we could not monitor binding in the wheat germ extract or the reticulocyte lysate, even under the conditions used for the translation, in which the oligoribonucleotide exhibited its ability to titrate out the repressor. It should be pointed out, however, that our inability to show binding to the 16 nucleotides long wtS16 might simply reflect a very weak RNA-protein complex (due to the relative short length of oligoribonucleotide?) which does not survive the experimental manipulations required for its identification on the gel. Nevertheless, the possible involvement of various candidate proteins in the translational control of TOP mRNAs is currently being studied by functional assays, both in vitro and in vivo, in our lab.

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