Substitution of just five nucleotides at and around the transcription start site of rat β-actin promoter is sufficient to render the resulting transcript a subject for translational control

Yael Biberman, Oded Meyuhas*

Department of Biochemistry, The Hebrew University-Hadassah Medical School, P.O. Box 12272, Jerusalem 91120, Israel

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Abstract Vertebrate mRNAs with a 5′ terminal oligopyrimidine tract (5′ TOP), including those encoding ribosomal proteins and elongation factors, are candidates for translational control in a growth-dependent fashion. The present study was designed to determine the minimal *cis*-regulatory element involved in this mode of regulation. We selected rat β-actin mRNA, a typical translationally uncontrolled transcript, as a subject for gain-of-function analysis. Mutations at and around its cap site leading to the formation of a 7 pyrimidines long 5′ TOP render the resulting transcript translationally repressed upon growth arrest of lymphosarcoma cells. In contrast, growth-dependent translational control of this mRNA in fibroblasts requires, in addition, a GC motif downstream of the 5′ TOP. A similar motif is present in all ribosomal prtein mRNAs shown to be translationally controlled.

Key words: Translational regulation; Ribosomal protein mRNA; Oligopyrimidine tract; Polysome

1. Introduction

The translation efficiency of mRNAs encoding several vertebrate proteins associated with the translational apparatus, such as ribosomal proteins [1] and elongation factors EF1α and EF2 [2,3], is predominantly dependent on the cellular growth status. One feature common to all these mRNAs that have been rigorously analyzed thus far is the 5′ terminal oligopyrimidine tract (TOP). This element is comprised of a cytidine residue at the cap site followed by an uninterrupted stretch of up to 13 pyrimidines [1,4,5]. Our previous attempts to delineate the translational *cis*-regulatory element (TLRE) were based on deletion and point mutations at the 5′ UTR of several ribosomal protein (rp) mRNAs with a special emphasis on rpS16 mRNA. Using this loss-of-function approach we have established the critical role of the 5′ TOP in the translational control of rp mRNAs in mammalian cells [6] and the strict dependence of this mode of regulation on the 5′ terminal location of the oligopyrimidine tract [7].

To delimit more precisely the TLRE, we set out to address this issue by gain-of-function strategy. To this end, we selected actin mRNA, which is efficiently translated in all examined cell types regardless of their growth status, and therefore is routinely used as a negative internal reference in our and others' experiments [7–9,15]. The transcription of rat β-actin gene starts at an A residue surrounded by four pyrimidine residues [10], and thus comprises a typical eukaryotic transcription start site [11]. In the present study we carried out systematic base substitutions at and around the cap site of this gene so that this region became progressively similar to the corresponding one in mouse rpS16. Analysis of the translational control of the various resulting transcripts in two different cell lines has yielded the following information: (a) the minimal TLRE operative in cells of lymphatic origin appears to require no more than a 5′ TOP composed of 6–8 pyrimidines; (b) growth-dependent translational control in fibroblasts, however, requires in addition a GC motif downstream of the 5′ TOP; and finally (c) the selection of a specific C residue as a transcription start site requires its location within an appropriate pyrimidine stretch.

2. Materials and methods

2.1. Cell culture and DNA transfection

P1798.C7 mouse lymphosarcoma cells and NIH 3T3 mouse fibroblasts were grown, transfected and arrested as described [7].

2.2. Plasmid construction

The site-directed mutagenesis at and near the transcription start site of the chimeric gene Act(28)-GH [7] was carried out with the 'megaprimer' method [12]. pAct/m(+1)-GH was constructed through the following steps: (a) an approximately 430-bp fragment was generated by PCR using the plasmid pAct(28)-GH as a template and the two oligonucleotides: Act-1 (5′ CGGACTCGACAGGGGCTGCGCGTTG 3′; positions +13 to −12 of rat β-actin gene, with underlined letters in this and the following oligonucleotides representing substituted nucleotides) and PUC-1 (5′ GTTTTCCCAGTCACGACGTT 3′; positions 361–380 of pUC18) as primers; (b) the resulting double strand DNA fragment ('megaprimer') together with the primer hGH-3 (5′ CGTCCCATCTACAGGTCGCT 3′; positions 123–140 of hGH gene) and the template pAct(28)-GH were used in a second PCR. The resulting ca. 570-bp fragment was cleaved by *Hin*dIII and *Bam*HI and the resulting ca. 400-bp fragment was cloned in between the *Hin*dIII and *Bam*HI sites of the promoterless plasmid p0GH [13]. The reaction conditions in both rounds of the PCR were with 100 ng of plasmid DNA and 60 ng of primers in 50 μl of 10 mM KCl, 20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.2 mM each of the 4 dNTPs, and 1 unit of Vent DNA polymerase (New England Biolabs, Beverly, MA). The second PCR differed from the first one by inclusion of 4% dimethyl sulfoxide and 90 ng of the 'megaprimer'. Both reactions were for 30 cycles each for 1 min at 94°C, 2 min at 40°C and 2 min at 72°C.

The mutant thus obtained was used as the starting material in sequential construction of a series of mutants using the protocol described above. The following oligonucleotides: Act-3 (5′ GACTCGACAGGGAAGGCGCGTTGCGCC 3′; positions +11 to −16 of rat β-actin gene), Act-4 (5′ GCGGACTCGAAAGGGAAGGCG 3′; positions +14 to −7 of rat β-actin gene), Act-5 (5′ GTGGACGCGGACGGAAAAGGGAAGGCG 3′; positions +20 to −7 of rat β-actin gene) were used in the first PCR during the construction of pAct/m(−4 to +1)-GH, pAct/m(−4 to +4)-GH and pAct/m(−4 to +8)-GH, respectively. pAct/m(+12 to +15)-GH was constructed by initially generating an approximately 140-bp fragment by PCR using the plasmid pAct/m(−4 to +8)-GH as a template and the two oligo-

*Corresponding author. Fax: (972) (2) 6784010.
E-mail: meyuhas@cc.huji.ac.il

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nucleotides: Act-6 (5′ CCCTTTTCCGTCAATTTCCACCCGCGAG 3′; positions −1 to +27 of rat β-actin gne]) and hGH-3 as primers. All other details as described for pAct/m(+1)-GH.

2.3. Primer extension and DNA sequencing

Determination of the transcription start site in hGH chimeric transcripts was carried out by primer extension as previously described [7]. Double-stranded plasmid DNA was sequenced by the dideoxy method [14] using a Sequenase kit (U.S. Biochemical Corp., Cleveland, OH).

2.4. Polysomal fractionation and RNA analysis

Harvesting and lysis of cells as well as size fractionation of polysomes by sedimentation through sucrose gradients were performed as described [15]. Northern blot analysis was performed as described [16]. To assess the effectiveness of the growth arrest treatment and the selectivity of the effect on rp mRNAs, we compared in each case (even if not shown) the polysomal association of a chimeric mRNA with that of endogenous rp mRNA and non-rp mRNA from the same polysomal gradient. Only experiments in which both these controls exhibited their typical translational behavior (repressed and unrepressed, respectively) were included.

2.5. Molecular probes

The isolated fragment probe used in the Northern blot analysis were: a 1.15-kb *Pst*I fragment containing mouse α-actin cDNA [17]; a 0.29-kb *Eco*RI-*Hin*dIII fragment containing the cDNA insert of mouse S16 derived from a subclone in pUC18 [18]; and a 0.8-kb *Hin*dIII fragment containing a hGH cDNA (kindly provided by T. Fogel, Bio-Technology General).

3. Results

The chimeric Act(28)-GH gene is composed of human GH gene driven by rat β-actin promoter [7]. The resulting transcript starts with the first 28 nucleotides of the actin 5′ UTR followed by the hGH sequence. The cap site of this chimeric mRNA is at the same A residue as in the native β-actin mRNA (Fig. 1a) [10], and like the endogenous counterpart it is efficiently translated, regardless of the growth status of the transfected P1798 or NIH 3T3 cells (Fig. 2, lines 1 and 2) [7]. Interestingly, 12 nucleotides within the rat β-actin gene, spanning positions −4 to +28, also appear at the corresponding positions in the mouse rpS16 gene (Fig. 2, line 2). Hence, a relatively small number of substitutions are required to completely transform this actin sequence into the corresponding rpS16 sequence. Consequently, we set out to progressively perform such a transformation and to monitor at each step both the cap site and the translational behavior of the resulting transcript. A transcript is considered translationally controlled if it is converted from mostly polysome-associated in growing cells to mostly subpolysome-associated in resting cells.

Table 1
GC motifs immediately downstream of the 5′ TOP within mammalian rp mRNAs shown to be translationally controlled in a growth-dependent manner

Protein	Sequence (+1 … 30)	5′ TOP (nucl.)	Accession Number
m rpL13a	CUUUCCCAGGCGGCUGCCGAAG	7	X51528
m rpL30	CCUUUCUCGCUCCCCGGCCGUCUUGGCGGC	8	K02928
m rpL32	CUUCUUCCUCGGCGCUGCCUACGAGGUGGC	10	K02060
m rpS4	CUCUUUCCGUUCCUAGCGCAGCC	8	L24371
m rpS16	CCUUUUCCGGUCGCGGCGCUGCGGUGUGGA	8	M11408
r rpP2	CUUUCGCCGCCGGACGCCGCCGAGGUCGCA	5	X55153
h rpS4Y	CUCUUCCGUCGCAGAGUUUCGCC	7	L24370
h rpS4X	CCUCUUUCCUUGCCUAACGCAGCC	11	L24369
h rpS6	CCUCUUUUCCGUGGCGCCUCGGAGGCGUUC	10	X67309

Sequences presented in this table represent only mRNAs whose cap site has been rigorously determined. Underlined letters, GC motifs; m, mouse; r, rat; h, human.

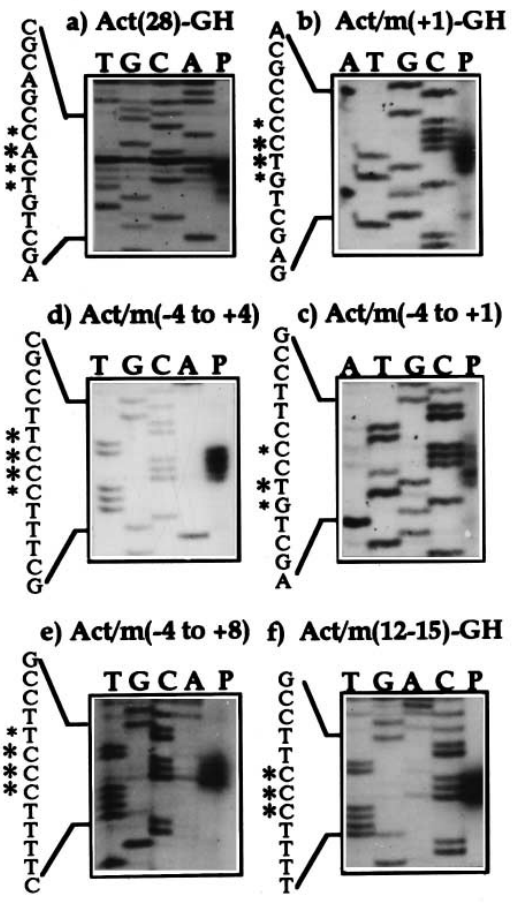

Fig. 1. Determination of the transcription start site of various hGH chimeric mRNAs by primer extension. A 5′ end labeled synthetic oligonucleotide complementary to nucleotides +31 to +12 of hGH gene was annealed to 0.5–5 μg of poly(A)$^+$ mRNA from stably transfected NIH 3T3 and extended with AMV reverse transcriptase. The extended product (P) was analyzed on a 6% acrylamide-urea gel alongside with a dideoxy sequencing reaction (A, C, G, T), in which the same primer (unlabeled) was used. Large and small asterisks indicate major and minor transcription start sites, respectively.

Initially, we replaced the A residue at the cap site by a C residue [Act/m(+1)-GH]. This mutation did not render the resulting transcript translationally controlled (Fig. 2, line 3), even though the major transcription start sites were at a C and a T residue at positions +2 and +3, respectively (Fig. 1b). Next, we substituted the three nucleotides at positions −4 to −2 by the corresponding nucleotides from rpS16 sequence [Act/m(−4 to +1)-GH]. However, this alteration neither led to the selection of any of the C residues at positions −1 to +2 as the major transcription start sites (Fig. 1c), as in the case of rpS16 gene, nor did it confer translational regulation on the respective transcript (Fig. 2, line 4). Surprisingly, a substitution of only one additional nucleotide, a G to T at position +4 [Act/m(−4 to +4)-GH], sufficed to render the resulting transcripts translationally repressed upon growth arrest of both cell lines (Fig. 2, line 5). The milder repression of these transcripts in comparison with that displayed by the endogenous

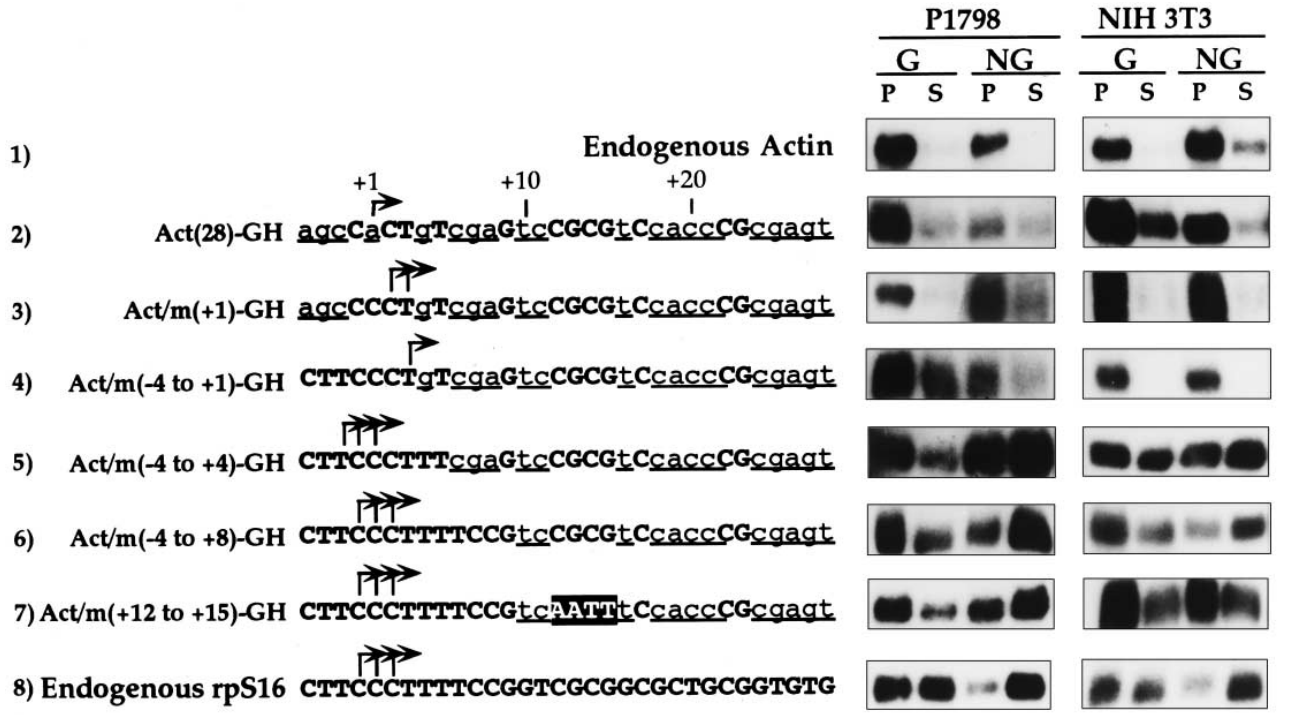

Fig. 2. The effect of the number of pyrimidines within the 5′ TOP and of the downstream GC sequence on the translational control in different cell lines. P1798 and NIH 3T3 cells were transiently and stably transfected, respectively, with the indicated hGH chimeric genes. Cytoplasmic extracts were prepared from growing (G) cells (untreated P1798 and NIH 3T3 cells) and from nongrowing (NG) cells (24-h dexamethasone-treated P1798 cells and 24-h aphidicolin-treated NIH 3T3 cells), centrifuged through sucrose gradients and separated into polysomal (P) and subpolysomal (S) fractions. Poly(A)$^+$ mRNA from equivalent aliquots of these fractions was analyzed by Northern blot hybridization. Analysis of the endogenous rpS16 mRNA or actin mRNA and the chimeric hGH mRNA was performed with the respective probes. The sequences at the left of the autoradiograms represent the nucleotides around the major transcription start sites (marked by arrows). Actin sequences which are identical with the corresponding nucleotides within the rpS16 sequence appear in underlined lowercase letters, whereas actin-specific sequences appear in bold uppercase letters. Shadowed letters depict substituted nucleotides which do not correspond to any of these mRNAs. Results obtained with P1798 cells and presented in lines 1–3 and 5–8 represent at least two independent experiments. Results obtained with NIH 3T3 cells and presented in lines 1 and 5–8 represent at least two independent experiments.

rpS16 mRNA (Fig. 2, line 8) might reflect the fact that third of the former starts at a T residue rather than a C residue (Fig. 1d). In Act/m(−4 to +8)-GH actin sequence spanning positions −4 to +8 was converted to the corresponding rpS16 sequence by the appropriate base substitutions. The corresponding transcripts displayed both selection of the same three C residues as cap sites (Fig. 1e) and a similar translational behavior as exhibited by endogenous rpS16 mRNA (Fig. 2, line 6).

The Act/m(−4 to +4)-GH transcript contains a 6–8 residues long 5′ TOP, which appears to provide the requirement both for the selection of the appropriate transcription start sites and for the TLRE. Nevertheless, we have previously shown that sequences residing downstream of the 5′ TOP of rpS16 are necessary for translational control, at least in NIH 3T3 cells [7]. Close inspection of the sequence of actin mRNA between positions +5 to +28 has disclosed 8 positions in which it shares the same nucleotides with rpS16 mRNAs, with a notable identity of the sequence CGCG at positions +12 to +15. To verify whether this tetramer plays any role in the translational control, we substituted it in Act/m(−4 to +8)-GH by an AATT sequence to yield the mutant Act/m(+12 to +15)-GH. The resulting chimeric mRNA exhibited the typical growth-dependent translational control in P1798 lymphosarcoma cells, but remains mostly in polysomes in growth arrested NIH 3T3 cells (Fig. 2, line 7).

4. Discussion

In the present report we have demonstrated that the first 6–8 pyrimidine residues suffice for a 5′ TOP to be functional in translational regulation. The requirement for such a relatively short pyrimidine stretch is not unprecedented, as rat rpP2 mRNA which contains a five pyrimidines long 5′ TOP [19] is translationally regulated to the same extent as rp mRNAs with a longer pyrimidine stretch [20]. More surprising, however, is our present observation that a functional TLRE in lymphosarcoma cells appears to need no more than the minimal 5′ TOP (Act/m(+12 to +15)-GH in Fig. 2). Conceivably, the difference in the mode of transfection into NIH 3T3 cells (stable) or P1798 cells (transient) cannot account for the loss of translational control of this mRNA in NIH 3T3 cells, as we have recently observed a selective loss of translational regulation of a similar mutant in fibroblasts regardless of their mode of transfection (Avni et al., manuscript submitted). Nevertheless, these results are consistent with those obtained in recent loss-of-function experiments, in which we deleted nucleotides spanning positions 11–29 or replaced nucleotides spanning positions 7–16 within the 5′ UTR of rpS16. These modifications led to the differential loss of translational control of the resulting transcripts in fibroblasts but not in lymphosarcoma cells (Avni et al., manuscript submitted). Based on these two complementary approaches, it appears that our

previous conclusion that sequences immediately downstream of the 5′ TOP are essential for growth-dependent translational control in fibroblasts [7] should not be extended to cells of lymphatic origin.

The conserved nucleotides within the 5′ UTR of mRNAs encoding mouse rpS16 and rat β-actin have enabled us to identify a CG motif, within the region immediately downstream of the 5′ TOP, which is essential for translational control in fibroblasts. Table 1 shows that similar motifs are present in comparable locations of other mammalian rp mRNAs, known to be translationally controlled in a growth-dependent fashion. Clearly, except for the general composition neither the 5′ TOPs nor the GC motifs are conserved among different rp mRNAs. Conceivably, optimal TLRE composition can be achieved by different combinations of the pyrimidine stretch and the GC motif(s). This notion is supported by our previous observation that sequences downstream of rpS16 5′ TOP failed to replace the corresponding missing sequences in truncated rpL32 TLRE [7]. It should be pointed out, however, that none of the TLREs examined so far contains a significantly stable hairpin structure involving the GC motif.

Our current working hypothesis is that the 5′ TOP interacts with a *trans*-acting factor and the avidity of this interaction depends on the structure of the TLRE which varies among different mRNAs. In accordance with this assumption, any structural modification in the TLRE and/or in the abundance of the *trans*-acting factor might affect the intensity of the interaction between the two and hence the sensitivity to alterations in the growth status. This notion is supported by the differential behaviour of Act/m(+12 to +15)-GH mRNA (Fig. 2, line 7). Thus, if the abundance of the *trans*-acting factor differs between hematopoietic cells and fibroblasts and if downstream mutations affect the affinity of this factor to the 5′ TOP, then the respective base substitution will be more critical in one cell line than in the other.

Many of the mammalian TOP genes examined so far lack a well defined TATA box, yet they initiate transcription with a high precision within an 'initiator' element, which is composed of an uninterrupted stretch of pyrimidines [1]. It should be emphasized that initiation of transcription at a C residue is a relatively rare event (about 17% [21]) among eukaryotic genes, which in most reported cases is at an A residue [11]. We have previously shown that C to A replacements at the cap site of rp promoters or at its immediate vicinity dictated the selection of the purine residue as the transcription start site, and consequently to the loss of translational control of the resulting mRNA [7]. The promoter of rat β-actin gene contains a typical TATA box, yet replacement of the A residue at the cap site by a C residue does not attract RNA polymerase II to initiate at this residue, even though it is surrounded by four pyrimidines. It is not before this residue is followed by five pyrimidines that it is selected as a transcription start site (Fig. 2, line 5). Moreover, selection of three consecutive C residues as cap sites, as in the case of endogenous rpS16 mRNA, is observed only when the eight-pyrimidine 5′ TOP of rp S16 mRNA is generated in the chimeric construct (Fig. 2, line 6). It appears, therefore, that the definition of the initiator element as "a DNA sequence that overlaps a transcription start site and which is sufficient for determining the start site location in a promoter that lacks a canonical TATA box" [22] should be extended to include promoters that do contain such a box.

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