A selective effect of Mpl ligand on mRNA stabilization during megakaryocyte differentiation

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Abstract Megakaryocytes, the platelet precursors, are induced to differentiate in response to Mpl ligand. Here we report that stability of the megakaryocyte-specific platelet factor 4 (PF4) mRNA is substantially augmented in the presence of Mpl ligand. This stabilization requires protein synthesis, but the 3′-untranslated region of PF4 mRNA is not sufficient for granting the effect. This cytokine also significantly or mildly stabilizes Mpl receptor or GAPDH mRNAs, respectively, in contrast to a previously reported lack of effect on $P2Y_1$ receptor mRNA. Our study is the first to suggest that Mpl ligand-induced lineage specification is also determined by message stabilization. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Megakaryocyte; Megakaryopoiesis; mRNA; Message stability; Mpl ligand

1. Introduction

Megakaryocytes are hematopoietic precursors of platelets, which play an essential role in thrombosis and hemostasis [1]. Megakaryocyte proliferation, polyploidization and maturation are primarily regulated by the megakaryocyte growth and development factor (MGDF), also called thrombopoietin, the ligand of the myeloproliferative (Mpl) receptor [2,3]. Several studies have examined the role of Mpl ligand in determining lineage specificity by focusing on transcriptional regulation [4,5]. This route of investigation has necessitated in most cases the use of cell lines because of the rarity of megakaryocytes in the bone marrow (~0.05% of total mononuclear cells) and the difficulties associated with isolating a sufficient number of primary megakaryocytes for study.

The phenotype and function of a cell are defined by the spectrum of its cytoplasmic mRNAs. The level of a specific mRNA is determined by the balance between its rates of synthesis and degradation. Recent studies in a variety of experimental systems highlighted the central role of mRNA stability in the abundance of cellular proteins [6]. Among the genes exclusively expressed in megakaryocytes is the platelet factor 4 (PF4). PF4 is an abundant and stable protein, which was identified as a chemokine component of the α-granules, with potential immunomodulatory and antiangiogenic activity [7]. Hence, the regulation of PF4 mRNA abundance may impact on the control of such processes as angiogenesis as well as on lineage determination.

In the current study, we have shown that the steady state level of PF4 mRNA was increased by 10-fold in the Y10/L8057 murine megakaryocytic cell line in response to stimulation with Mpl ligand. PF4 promoter activity was increased only by about two-fold. Message stability, however, was substantially augmented. Similarly, Mpl ligand considerably augmented Mpl receptor mRNA stability, but significantly less so of GAPDH mRNA. Our study is novel as it indicates that Mpl ligand-induced lineage differentiation is also determined by mRNA stabilization. Such an increase in message stability is important in understanding the major burst in protein synthesis seen during the final phases of megakaryopoiesis.

2. Materials and methods

2.1. Cell culture and materials

The Mpl ligand-responsive Y10 subclone [8] of a murine megakaryocytic cell line L8057 [9] was maintained in F12 medium, supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin (all Gibco BRL, Gaithersburg, MD, USA) in a humidified incubator at 37°C with 5% CO_2. Cells were induced to differentiate by growing in Iscove's modified Dulbecco's medium (IMDM) (Gibco BRL), supplemented as above, in the presence of 25 ng/ml of a recombinant Mpl ligand (PEG-rHu-MGDF, generously supplied by Amgen, Thousand Oaks, CA, USA) as it was shown before to elevate ploidy and lineage-specific markers [5,8]. 5 μg/ml of actinomycin D (ActD), 15 mg/ml of 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB), and 100 μg/ml of cycloheximide (CHX; all Sigma, St. Louis, MO, USA) were used when indicated.

2.2. Plasmid construction

The plasmids CMV-HGH and CMV-HGH-PF4polyA were constructed from fragments of two different vectors: pcDNA3 (Invitrogen, Carlsbad, CA, USA) containing CMV promoter and neomycin-resistant gene, and pPF4GH containing the rat 1.1-kb PF4 promoter, linked to the human growth hormone gene (HGH) [10]. pcDNA3 was linearized with *Bam*HI/*Eco*RI or *Bam*HI/*Xba*I restriction digest. The HGH fragment was excised from pPF4GH vector by *Bam*HI/*Eco*RI or *Bam*HI/*Bgl*I restriction digest correspondingly. The rat PF4 gene (GenBank accession number M15254) [11] was used to generate the rPF4polyA tail by PCR reaction with a pair of specific primers: sense primer 5′-CTCGAGCCAGGGAGGCACAGAGCCACGCTGAAGAATGG-3′ and antisense primer 5′-TCTAGAAACATATAAACACTGTGTATTCATTC-3′. This PCR product was ligated to TA vector (Invitrogen, Carlsbad, CA, USA) and cut with *Bgl*I/*Xba*I restriction enzymes. This PF4 3′ end spans the region from 640 bp to 1182 bp in the gene (with 0 counted as the transcription start). The

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final plasmids were generated by ligation of gene fragments, which were purified using GeneClean II kit (Bio 101, Vista, CA, USA). Using similar approaches, the PF4 3′-untranslated region (UTR) was subcloned downstream to the $P2Y_1$ gene (instead of its own 3′ end [12]) (GenBank accession number AJ245636).

2.3. mRNA isolation and Northern blot analysis

Total RNA was extracted using TRIzol Reagent (Gibco BRL) according to the manufacturer's instructions. Northern blot analysis was performed as described elsewhere [13]. As indicated, rat PF4, mouse Mpl receptor, GAPDH, HGH or 18S cDNAs were used as probes. The probes, available by cloning or by PCR, were confirmed by DNA sequencing (all sequences are available in GenBank). The data were quantitated using InstantImager (Packard BioScience Company, Downers Grove, IL, USA) or Electrophoresis Documentation Analysis System (Eastman Kodak Company, Rochester, NY, USA) as indicated.

2.4. Transfections and reporter gene assays

Y10/L8057 cells were stably transfected with PF4GH plasmid [10] or vehicle plasmid (100 μg each) by electroporation, along with 20 μg of pcDNA3 (Invitrogen, Carlsbad, CA, USA), which encodes neomycin acetyltransferase that confers resistance to neomycin analog G418, as described elsewhere [5]. Stable clones were selected by culturing in the presence of 400 μg/ml G418 (Gibco BRL) for 4 weeks. Resistant cells were used in the HGH expression assay that was performed on the medium of cells cultured in the absence or presence of 25 ng/ml MGDF for indicated times [10]. The level of HGH produced by cells was determined by HGH kit (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA), as described by the manufacturer, and normalized to the total cell number. CMV-HGH and CMV-HGH-PF4polyA plasmids were stably transfected into Y10/L8057 cells and selected, using the above-described method.

2.5. Nuclei isolation and nuclear run-on assay

Nuclei were prepared from Y10/L8057 cells cultured in the absence or presence of 25 ng/ml MGDF for 24 h as described elsewhere [14]. Isolated nuclei were frozen in liquid nitrogen in 50 mM Tris, pH 8.3, 5 mM $MgCl_2$, 0.1 mM EDTA, and 40% glycerol in aliquots of 10^7 nuclei/100 μl. Nuclear run-on assay was performed essentially as we described before, with minor modifications [5].

2.6. Analysis of mRNA decay

Y10/L8057 cells were plated at a concentration of 5×10^5 cells/ml in IMDM medium with 10% FBS. At the time of seeding, half of the flasks were supplemented with 25 ng/ml of MGDF. 24 h post seeding, 15 mg/ml of DRB was added to each flask. At indicated times, after addition of DRB, cells in each subgroup were harvested for RNA isolation (TRIzol Reagent, Gibco BRL) and Northern blot analysis was performed as described above. The same membrane was stripped by treatment with boiling solution of 0.2% SDS and used for several probes. For graphic representation of the data, the intensity of the band for time 0 was referred to as 100%. Control for efficient inhibition of gene transcription was performed in each experiment by incubating cells in the presence of DRB prior to incubation with MGDF (data not shown).

3. Results and discussion

3.1. Effect of Mpl ligand on PF4 gene expression in megakaryocytes

It had been previously shown that the Y10/L8057 megakaryocytic cell line responds to Mpl ligand through increases in cell ploidy and abundance of megakaryocytic markers [5,8]. Because of the rarity of megakaryocytes in bone marrow, we utilized this cell system to explore the mechanisms by which PF4 mRNA is elevated by the Mpl ligand. The PF4 gene encodes an extremely abundant protein, which is found only within α-granules of megakaryocytes and platelets, therefore serving as an excellent marker of lineage differentiation [11,15]. The steady state level of PF4 mRNA was elevated by up to 10-fold by treatment with the recombinant Mpl ligand, MGDF (see Section 2) (Fig. 1). To explore the inducibility of the PF4 gene promoter by MGDF, we utilized the Y10/L8057 cell line, stably transfected with the PF4GH plasmid [10] (see Section 2). The PF4 promoter regulatory elements (P1, P2, and P3), identified by transfection of rat bone marrow cells [10], were confirmed to act similarly in Y10/L8057 cells (data not shown). This suggested that the regulation of the PF4 promoter is similar in the cell line and primary cells. In our studies, the activity of the PF4 promoter in Y10/L8057 cells was increased only by 1.6-fold after 24 h of MGDF treatment and by 2.4-fold after 48 h of treatment (Fig. 2A). In addition, nuclear run-on assay indicated that the rate of transcription of the endogenous PF4 gene was not significantly changed by MGDF (Fig. 2B). We recognize, however, that the sensitivity of this assay is too low to reproducibly detect differences in the range of two-fold.

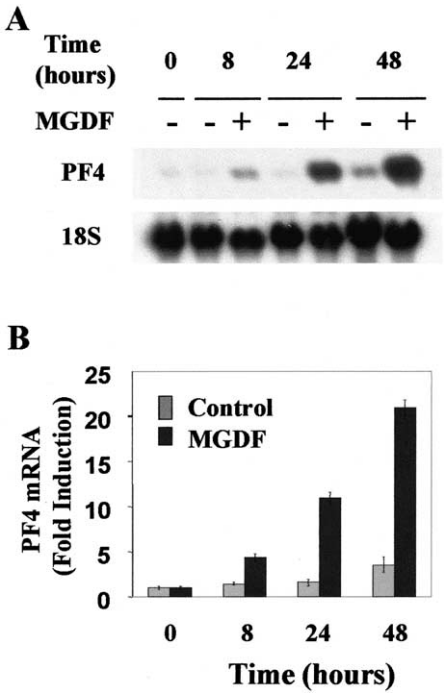

Fig. 1. The level of PF4 mRNA is increased in response to MGDF treatment in the megakaryocytic cell line Y10/L8057. A: Northern blot analysis of PF4 mRNA. Y10/L8057 cells were plated at a concentration of 5×10^5 cells/ml in IMDM medium supplemented with 10% FBS in the presence or absence of 25 ng/ml MGDF. At indicated times, cells were harvested, followed by RNA extraction. Total RNA was electrophoresed, transferred onto a nylon membrane, and hybridized with a ^{32}P-labeled rat PF4 cDNA. 18S ribosomal RNA (rRNA) was determined for loading control. B: Quantitative representation of Northern blot analysis. The data were analyzed by an InstantImager (Packard BioScience), followed by normalization to 18S rRNA. The data presented are representative of three experiments performed.

Based on the results described above, we wished to explore to what extent mRNA transcription and/or protein synthesis was required for MGDF effect on the level of PF4 mRNA in Y10/L8057 cells. As seen in Fig. 3, the blockage of protein synthesis by the addition of CHX [8] or message transcription by the treatment with ActD [16] led to abrogation of MGDF effect on PF4 mRNA. Hence, we concluded that mRNA transcription together with translation was required for the effect of MGDF on PF4 mRNA.

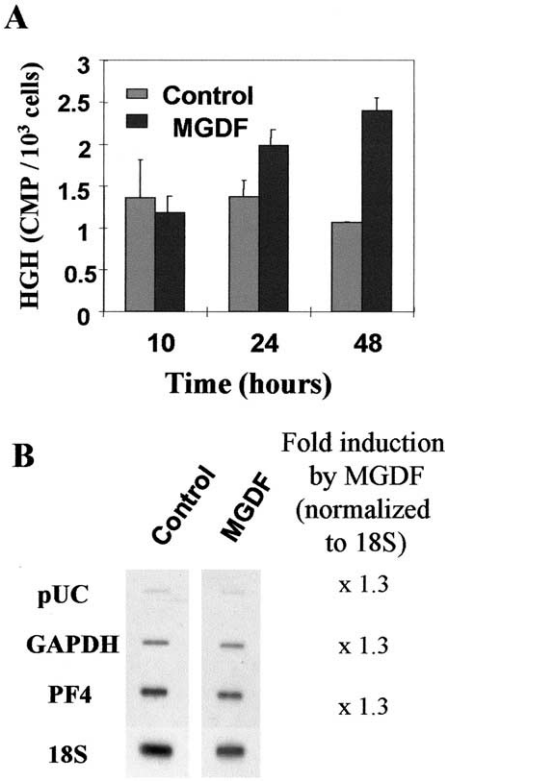

Fig. 2. Increased PF4 promoter activity in response to MGDF. A: PF4 promoter activity. Y10/L8057 cells, stably transfected with PF4GH plasmid, were seeded in six-well plates at a concentration of 10^5 cells/ml in IMDM medium supplemented with 10% FBS in duplicates. At the time of seeding, half of the wells were treated with 25 ng/ml MGDF. After 10, 24, and 48 h in culture, medium aliquots were taken to count cell number and to determine the level of HGH production. HGH concentration was determined by radioimmunoassay as described in Section 2. The data are averages of two experiments performed in duplicates. Error bars represent standard errors. B: Nuclear run-on assay. 10 μg of plasmid containing cDNA for PF4, GAPDH, 18S rRNA or pUC 19 was applied to nitrocellulose. The data presented are of a representative experiment out of three performed. Fold induction by MGDF was calculated with the use of the Electrophoresis Documentation Analysis System (Eastman Kodak Company, Rochester, NY, USA). The exposure time for 18S rRNA was 1/5 of the time for the rest of the bands.

3.2. *PF4 mRNA stability is augmented in Y10/L8057 cells in response to MGDF*

Since MGDF-induced elevation of PF4 promoter activity was not as high as that of PF4 mRNA, we suspected that this cytokine might have an effect on PF4 mRNA half-life. To measure the kinetics of PF4 message decay, we treated Y10/L8057 cells with the transcriptional inhibitor DRB [17] after 24 h incubation in the presence or absence of MGDF. The choice of DRB concentration (15 mg/ml) was based on its effective inhibition of transcription (data not shown) without compromising cell viability (Fig. 4A, lower panel). As shown in Fig. 4, treatment of Y10/L8057 cells with MGDF resulted in a substantial increase in PF4 mRNA half-life (from about 18 h to more than 28 h in the presence of MGDF). The stability of Mpl receptor mRNA, a selectively abundant message, was also enhanced by MGDF (from about 14 h to almost 24 h) (Fig. 4). In addition, we have found that Mpl ligand had a general modest protective effect on mRNAs, as reflected by a small increase in GAPDH mRNA stability (from about 18 h to close to 21 h in the presence of MGDF; Fig. 4B). In a different study in the same cell type we reported that Mpl ligand had no significant influence on the $P2Y_1$ receptor mRNA [18], which has a half-life of 2–3 h, indicating a selective effect of this cytokine on mRNA decay rates. It should be pointed out that the half-life of PF4 mRNA was estimated in the range of 15–18 h when using the transcription inhibitor ActD (data not shown). This was not used for detailed studies because of its toxicity during prolonged incubation.

Sottile et al. reported that human platelets contain large amounts of PF4 mRNA, suggesting a prolonged half-life for this message [19]. We, hence, propose that upon lineage determination, Mpl ligand ensures high abundance of PF4 mRNA in differentiating megakaryocytes by a combined effect on gene transcription and mRNA stability.

3.3. *The role of the 3′ end of the PF4 gene in conferring message stability*

Stabilities of specific eukaryotic mRNAs can vary markedly (reviewed in [16,20]). The 5′-UTR and more frequently the 3′-UTR within mRNAs, as well as sequences within a coding region were shown to contain binding sites for a number of

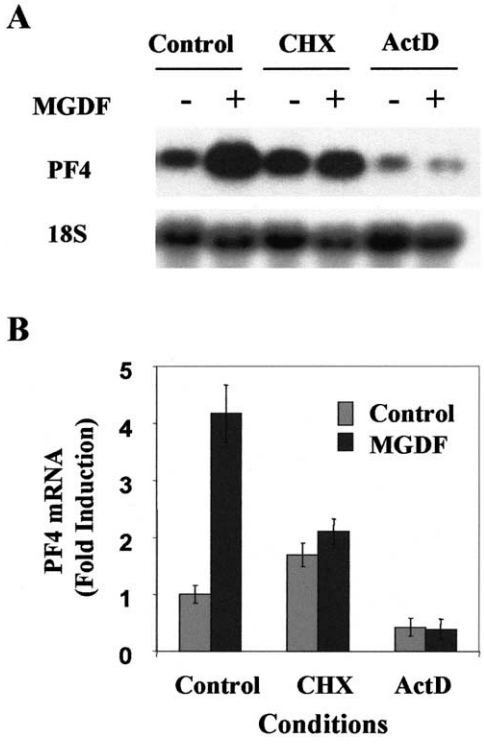

Fig. 3. PF4 mRNA is regulated on multiple levels in Y10/L8057 megakaryocytes. A: Northern blot analysis. Y10/L8057 cells were plated at a concentration of 8×10^5 cells/ml in IMDM medium supplemented with 10% FBS. At the time of seeding, cells were treated with 100 μg/ml CHX or 5 μg/ml ActD, as indicated, and 30 min later 25 ng/ml MGDF was added, also as indicated, and the incubation was continued for 24 h. Next, cells were harvested and RNA was extracted. Total RNA was electrophoresed, transferred onto a nylon membrane, and hybridized with a ^{32}P-labeled PF4 cDNA. 18S rRNA probe was determined as a loading control. B: Quantitative representation of Northern blot analysis. The data were analyzed by an InstantImager (Packard BioScience), followed by normalization to 18S rRNA. The data presented are representative of two experiments performed.

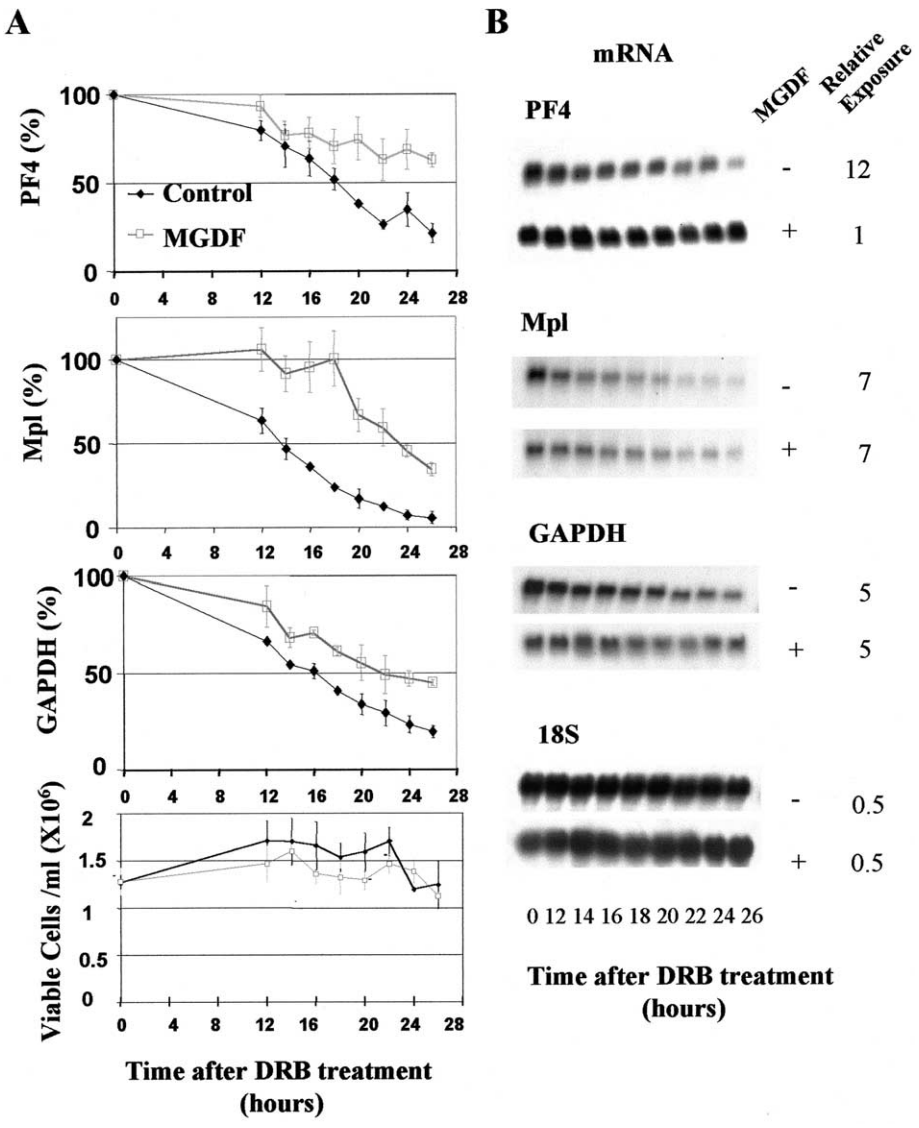

Fig. 4. MGDF treatment of Y10/L8057 cells results in increased stability of PF4 and Mpl receptor mRNAs. Y10/L8057 cells were plated at a concentration of 5×10^5 cells/ml in IMDM medium supplemented with 10% FBS, in the presence or absence of 25 ng/ml MGDF. 24 h post seeding, 15 mg/ml DRB was added to each flask. At indicated times after DRB treatment, the cells were harvested, followed by RNA extraction. Total RNA was electrophoresed, transferred onto a nylon membrane, and hybridized with ^{32}P-labeled cDNAs as indicated. A: Graphic representation of the data. The data were analyzed using an Electrophoresis Documentation Analysis System (Eastman Kodak Company, Rochester, NY, USA). All data were normalized to 18S rRNA and the intensity of the band for time 0 was used as 100%. Shown are averages ± standard deviations. Since DRB specifically inhibits transcription initiation by RNA polymerase II, the level of 18S rRNA did not decrease over time and has not been plotted. B: Northern blot analysis of a representative experiment, out of three performed. '+' and '−' each indicates the presence and absence of MGDF. PF4 mRNA band appears as 0.9 kb, GAPDH mRNA as 1.5 kb, Mpl receptor mRNA as 3 kb, and the 18S rRNA as 1.9 kb. Relative exposure time of the films is indicated. For each experiment, the probes with 18S cDNA were also subjected to shorter exposure times to avoid quantitation of saturated bands.

RNA-binding proteins, which determine the fate of the transcript [20–23]. PolyA sequences have multiple functions affecting nuclear processing of pre-mRNA, transport to the cytoplasm, translation, and cytoplasmic mRNA stability. Hence, we investigated the role of the 3′ end of the PF4 gene in conferring message stability by utilizing chimeric mRNAs.

To this end, we used the HGH gene to generate constructs for stable transfections into the megakaryocytic Y10/L8057 cell line (Fig. 5A). We wished to examine whether the replacement of the gene's 3′-UTR with the 3′ end of the PF4 gene would further enhance the stability of HGH message or would confer the stabilizing effect of MGDF. The 542 bp 3′-UTR of the PF4 gene, used in our study, contained two polyA sites. HGH mRNA has a half-life of approximately 9 h [24], as also confirmed in our system. As shown in Fig. 5C, MGDF treatment increased the level of HGH produced in the stably transfected cells. This was not surprising since the expression of these messages was driven by the CMV promoter, which we have found before to be activated by Mpl ligand [5]. Nevertheless, the decay rate of HGH mRNA in the presence or absence of MGDF was comparable (Fig. 5B,C). Similar results were obtained for an ADP receptor gene $P2Y_1$ chimeric construct (see Section 2), containing the PF4 3′-UTR (data not shown). This message has a half-life of about 2.5 h [18]. Thus, the PF4 mRNA 3′-UTR is not sufficient for granting message stability in response to Mpl ligand.

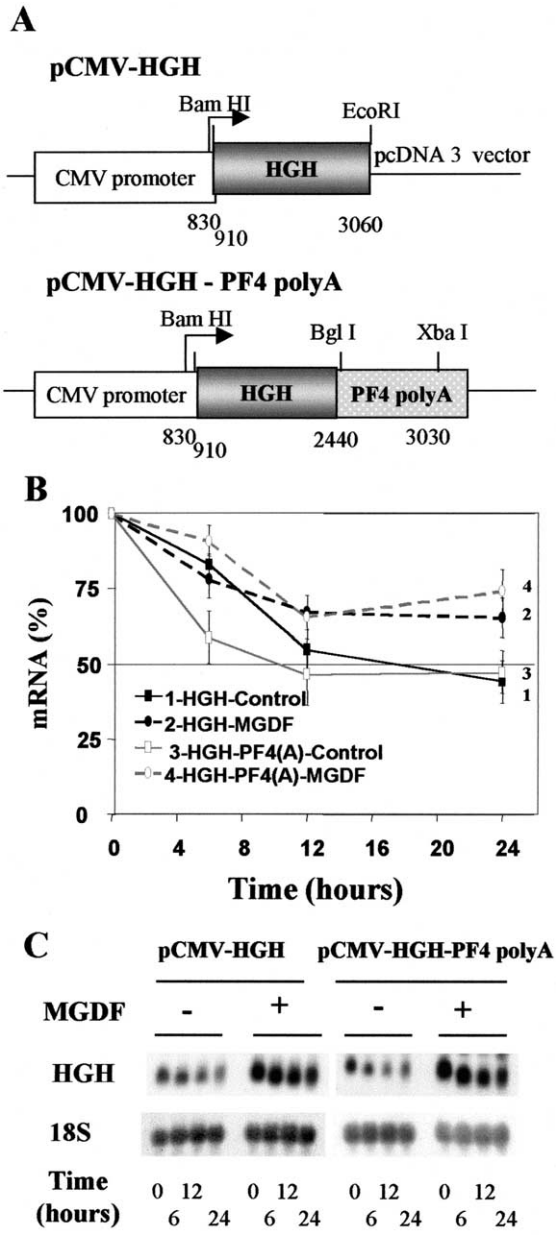

Fig. 5. The role of the 3′-UTR of the PF4 gene on message stability of HGH. A: Schemes of the HGH constructs used for transfection. pCMV-HGH consists of 2150 bp of the HGH gene down to the stop codon, with its native 3′ end and polyA signal. pCMV-HGH-PF4polyA consists of the HGH gene and 542 bp of PF4 3′-UTR and polyA signal, replacing the native HGH 3′ end. The numbers in the schemes denote bp positions, with 1 being the 5′ end of the CMV promoter. B,C: Y10/L8057 cells, stably transfected with CMV-HGH or CMV-HGH-PF4polyA plasmids, were used for mRNA stability determination as described in Fig. 4. B: Graphic representation of the data. The intensity of the band for time 0 was used as 100% and data were normalized to 18S rRNA. C: Northern blot analysis of a representative experiment, out of two performed. To avoid quantitation of saturated bands the filters were also subjected to a shorter exposure (than the one shown here for the purpose of illustration).

In summary, we propose that the maintenance of a megakaryocytic phenotype partially depends on stabilization of selective mRNAs. Mpl ligand effect on the level of PF4 mRNA reflects combined influences on gene transcription and message stabilization. Although our report is the first to link maintenance of a megakaryocyte differentiation phenotype to mRNA stability, this phenomenon was reported before in regard to globin mRNAs stabilization and erythropoiesis [25]. As indicated above (Fig. 3), mRNA transcription and protein synthesis were both required for Mpl ligand effect on the level of PF4 mRNA in Y10/L8057 cells, indicating that the mechanism of PF4 mRNA stabilization involves synthesis of specific proteins. Future studies should identify common or unique sequences within a variety of megakaryocyte-selective mRNAs that are essential for stabilization in response to Mpl ligand.

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