In Vivo Studies of Translational Repression Mediated by the Granulocyte-Macrophage Colony-stimulating Factor AU-rich Element*

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The AU-rich element (ARE) controls the turnover of many unstable mRNAs and their translation. The granulocyte-macrophage colony-stimulating factor (GM-CSF) ARE is known to be a destabilizing element, but its role in translation remains unclear. Here we studied in vivo the role of the GM-CSF ARE on the mRNA and protein expressions of an enhanced green fluorescent protein reporter gene. The GM-CSF ARE had a repressor effect on translation independently of its effect on mRNA levels. In the context of an internal ribosome entry site, the GM-CSF ARE still repressed translation but was no longer functional as a destabilizing element. Gel retardation assays showed that poly(A)-binding protein is displaced from the poly(A) tail when the ARE is present in the 3′-untranslated region. These data suggest that the GM-CSF ARE controls translation and mRNA decay by interfering with poly(A)-binding protein-mediated mRNA circularization.

The mechanisms of post-transcriptional regulation at the level of mRNA degradation and translation are of importance in the control of gene expression, with the 3′-untranslated region (UTR)1 central in this process (1). The adenosine uridine-rich element (ARE) is the most common regulatory determinant found in the 3′-UTR of many unstable mRNAs, and several types have been described (1).

The ARE induces rapid mRNA turnover by first triggering poly(A) shortening, then inducing both 5′-end decapping and RNA body degradation by either 5′ and 3′ exonuclease complexes (1–4). Recent data also suggest involvement of the ubiquitin-dependent pathway in the control of ARE-mediated mRNA turnover (5). Whereas the ARE-mediated cap-dependent deadenylation model is largely accepted in yeast, it remains to be demonstrated in mammalian cells. For this reason, human homologs of the yeast decapping enzyme were cloned (6), and a mRNA-decapping activity was shown to be regulated by ARE in HeLa cells (7). The ARE is also a translational silencer (8–10). The main question is whether ARE works both as an mRNA destabilizer and a translational repressor.

Both termini of an mRNA can associate via an interaction between the cap binding complex (eukaryotic initiation factor 4F) and the poly(A)-binding protein (PABP) (11–13). By facilitating the binding of these proteins on their substrates, this interaction protects the mRNA extremities from the degradation and potentiates the translation of capped mRNAs. Because a poly(A) tail is needed for both mRNA stabilization and efficient translation (1, 14) and because factors associated with the cis-stabilizing element of the highly stable α-Globin mRNA cooperate with PABP (15), AREs may control mRNA decay and translation by targeting the PABP/poly(A) tail association.

In this work we used the granulocyte-macrophage colony-stimulating factor (GM-CSF) ARE as a model. The GM-CSF is a hematopoietic growth factor that influences the commitment, differentiation, and functional activity of myelomonocytic cells (16). This ARE belongs to class IIA and contains five reiterations of the AUUU motifs embedded in an AU-rich sequence. It was the first to be associated with the control of mRNA stability (1, 17). The physiological importance of the ARE in the control of GM-CSF gene expression was demonstrated in transgenic mice bearing GM-CSF gene constructs lacking the ARE that presented severe hematopoietic disorders (18). The control of translation by the GM-CSF ARE is much less documented. ARE-containing mRNAs are poorly translated, and the GM-CSF ARE inhibits translation in vitro (8–9).

To date many ARE-binding proteins (ARE-BPs) have been described (1, 19). These include the embryonic lethal abnormal visual system-like protein family including HuR, CUGBP2, a novel ARE-BP involved in cyclooxygenase-2 post-transcriptional regulation (10), the heterogeneous nuclear ribonucleoprotein D isoforms, T-cell restricted intracellular antigen-1/TIAR proteins, and tristetraprolin (1–2, 19–20). Because they associate with the ARE, these proteins are thought to participate in ARE effects on either mRNA decay or translation. However, further investigations need to be made to understand their exact role in these mechanisms.

We sought whether the GM-CSF ARE could control in vivo the translation of a reporter gene. Using an enhanced green fluorescent protein (EGFP) expression system, we demonstrate that the GM-CSF ARE represses translation independently of its mRNA-destabilizing effect. In a context of cap-independent translation, the GM-CSF ARE is no longer efficient as a destabilizing element, whereas it still functions as a translational...
and its antisense. To generate plasmid pEGFP-A\textsc{m}, eight point muta-
egens using the QuikChange site-directed mutagenesis kit
re-ligated on itself.

BglII site from pEGFP-C3. To generate plasmid p0, the 5\textsuperscript{-}UTR and the

GATCCATTACGGTAAAACATCTTGAATA-3\textsuperscript{H11032}

were inserted in the
generated plasmids pIRES\textsc{m} and pIRES-A, the BglII- and BamHI-digested PCR fragments containing either the whole \(\beta\)-globin 3\textsuperscript{-}UTR (amplified from pFBF template (a gift of Dr. J. Belasco, New York University School of Medicine, New York)
using primers 5\textsuperscript{-}GAGAGTCCTCTATCTCCTGCTGGCAAAAAATATG-3\textsuperscript{H9262}
and 5\textsuperscript{-}GCGATTGCGGTCTGGATGCTC-3\textsuperscript{H11032}
the GM-CSF
ARE (amplified from pG-D\textsc{m} CF template (ATCC) using primers 5\textsuperscript{-}GAGAGTCCTCTATCTCCTGCTGGCAAAAAATATG-3\textsuperscript{H11032}
and 5\textsuperscript{-}GCGATTGCGGTCTGGATGCTC-3\textsuperscript{H11032}

To generate plasmid p0, the 5\textsuperscript{-}UTR and the
total EGFP coding region was cut out by Nhel and BglII, filled-in, and
re-ligated on itself.

To generate the plasmid pEGFP-0, the whole 5\textsuperscript{-}UTR including the start
codon for EGFP was deleted from pEGFP-C\textsc{m} by site-directed
mutagenesis using the QuikChange site-directed mutagenesis kit
(Stratagene) and the primer 5\textsuperscript{-}GTCGACATTACGGTAAAACATCTTGAATA-3\textsuperscript{H11032}
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5\textsuperscript{-}GAAGATCTCTAACAGGCATGGCAGAAGAATG-3\textsuperscript{H11032}

using primers 5\textsuperscript{-}TACTACAGAAAT-

3\textsuperscript{-}TTT-ATTCAAG-3\textsuperscript{H11032}

were inserted in the

A\textsc{m} and pIRES-A, the ScaI/Smal fragment from either pEGFP-A\textsc{m} or pEGFP-A was inserted by blunt-end ligation in the

NotI-cut filled-in pIRES2-EGFP plasmid. When necessary, the constructs were controlled by DNA sequencing.

Cell Culture, DNA Transfection, Cell Extract Preparations—HeLa

cells were cultured in Dulbecco's modified Eagle's medium, 10% fetal

loin A. Steitz (New Haven, CT) as templates (22). Labeled RNA tran-

scription reactions were performed according to the instructions of Promega (Charbonni`eres, France) using T7 RNA polymerase and either the XbaI- or SacI-linear-\n
ated downstream of the EGFP coding region in the pEGFP-C\textsc{m} plasmid. pT7/T3-18 was used as negative control. As shown in

Fig. 2, A and B, when HeLa cells were transfected by pEGFP-A, GM-CSF ARE dramatically decreased the EGFP expression (around 72-fold in flow cytometric measurements), whereas the percentage of EGFP-positive cells was reduced by 40%. The steady-state level of EGFP-A mRNA was also reduced when compared with EGFP-B mRNA (3.4 ± 1.4-fold, average of 4 experiments) but remained easily detectable (Fig. 2C), thus confirming the role of the GM-CSF ARE as a destabilizing element. Reduced EGFP expression in cells transfected with pEGFP-A together with the persistence of the corresponding mRNA pointed to a repressive effect of the GM-CSF ARE on in vivo translation.

Because the nature of the EGFP coding region and the GM-CSF ARE in the plasmid pEGFP-A was controlled by DNA sequencing, the dramatic decrease in EGFP expression observed in the cells could not be accounted for by mutations abolishing the protein expression. However, to demonstrate the ARE specificity in the translational repression in cells transfected by pEGFP-A, further investigations were performed. Two negative controls (plasmids p0 and pEGFP-0) for EGFP expression were made from the backbone of the pEGFP-C\textsc{m} plasmid. The p0 plasmid was obtained by deleting the 5\textsuperscript{-}UTR and the whole EGFP coding region (Fig. 1B) and was used as negative control for both EGFP and mRNA expression in transfected cells. As expected, neither EGFP mRNA nor protein was produced by cells transfected with this plasmid (Fig. 3, A–C, lanes 1). Similarly, the plasmid pEGFP-0 was obtained by deleting the 5\textsuperscript{-}UTR and the EGFP start codon (Fig. 1B). In

transfected cells, this plasmid was used as negative control for EGFP expression and positive control for EGFP-mRNA expres-

sion. As expected, the transfected cells did not produce any EGFP, but a shorter mRNA (deleted of the 5\textsuperscript{-}UTR and the start codon) was easily detectable by Northern blot (Fig. 3, A–C, lanes 2).

To assess the specificity of the ARE-mediated translational repression we made eight point mutations into the GM-CSF ARE producing plasmids as used for the negative control to gate the EGFP- and DaRed-positive cell populations.

To avoid interference between EGFP and DaRed detectors, a lower amount of pDsRed2-N1 plasmid was used for the co-transfections in comparison with the other tested plasmid, and we adjusted the compensation settings provided with the Expo 32 analysis software. So that the flow cytometry and Western blotting data could be compared, the
global EGFP expression of the cell population was determined by taking into account the mean fluorescence intensity of the EGFP-positive population and the percentage of the EGFP-positive cells (%EGFP\textsc{+}) obtained from data plots and expressed as an arbitrary unit. This global EGFP expression was normalized as \([\text{mean fluorescence intensity (MFI) of the test} \times \text{MFI of negative control}}]/\text{MFI of negative control}}\times \%\text{EGFP}^+\text{ of the test} \times \%\text{EGFP}^+\text{ of the negative control}}\). The transfection efficiency in each condition was compared on the basis of the percentage of DaRed-positive cells (Figs. 3 and 5).
expression (around 42-fold), the percentage of EGFP-positive cells (around 2-fold), and the steady-state level of its transcript (2.5 H11006 0.3-fold, average of 2 experiments) compared with pEGFP-A, which contains the functional ARE (Fig. 3, A–C; compare lanes 3 and 4). These results demonstrated that the translational repression was ARE-specific. The ARE-mediated inhibition on EGFP translation from pEGFP-Am was around 1.7-fold less marked when compared with the inhibition on translation from pEGFP-B. It is likely that the eight point mutations inserted in the GM-CSF ARE (plasmid pEGFP-Am) were not sufficient to totally abolish the GM-CSF ARE effects on EGFP expression.

To assess transfection efficiency during these experiments cells were co-transfected with the pDsRed2-N1 plasmid constitutively expressing a red fluorescent protein (see "Experimental Procedures" for details). As shown in Fig. 3A, top line, a similar amount of HeLa cells expressed only the DsRed protein in the different conditions. Therefore, the difference in EGFP expression (around 42-fold), the percentage of EGFP-positive cells (around 2-fold), and the steady-state level of its transcript (2.5 ± 0.3-fold, average of 2 experiments) compared with pEGFP-A, which contains the functional ARE (Fig. 3, A–C; compare lanes 3 and 4). These results demonstrated that the translational repression was ARE-specific. The ARE-mediated inhibition on EGFP translation from pEGFP-A blanks was around 1.7-fold less marked when compared with the inhibition on translation from pEGFP-B. It is likely that the eight point mutations inserted in the GM-CSF ARE (plasmid pEGFP-A blanks) were not sufficient to totally abolish the GM-CSF ARE effects on EGFP expression.

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and Northern blots and mRNA quantitation were the same as in Fig. 2. The loading controls used for Western percentage/H11006 bottom part/cells. The panel A 2. In pEGFP-Am plasmid, flow cytometric results showed that the experiments performed in Figs. 3 and 4 using the panel C as described in Fig. 2. In panel A the top line shows the percentage of the DsRed-positive cells. The bottom part shows the EGFP expression ± S.D. and the percentage ± S.D. of the EGFP-positive cells as described in Fig. 2 (average of three experiments). The loading controls used for Western and Northern blots and mRNA quantitation were the same as in Fig. 2.

Fig. 3. In vivo translational repression is ARE-specific. HeLa cells were co-transfected with 2.5 µg of plasmid pDsRed2-N1 used as control for transfection efficiency and 5 µg of plasmids pEGFP-Am (lane 3), pEGFP-A (lane 4), or the control plasmids p0 (lane 1) and pEGFP-0 (lane 2). Analysis was performed by flow cytometry (panel A), Western blotting (panel B), and Northern blotting (panel C) as described in Fig. 2. In panel A the top line shows the percentage of the DsRed-positive cells. The bottom part shows the EGFP expression ± S.D. and the percentage ± S.D. of the EGFP-positive cells as described in Fig. 2 (average of two experiments). The loading controls used for Western and Northern blots and mRNA quantitation were the same as in Fig. 2.

expression could not be accounted for by a variation in the transfection efficiency.

Finally, we examined the ARE-mediated translational repression by transfecting cells with increasing amounts of either pEGFP-Am or pEGFP-A (Fig. 4). In cells transfected by pEGFP-Am, EGFP mRNA and protein expressions increased in parallel to the amounts of plasmid used for transfections (Fig. 4, A–C, lanes 2–4). In this case, EGFP remained hardly detectable by either flow cytometry or Western blotting when cells were transfected with increasing amounts of pEGFP-A, whereas the EGFP-A mRNA accumulated (Fig. 4, A–C, lanes 5–7). In the experiments performed in Figs. 3 and 4 using the pEGFP-Am plasmid, flow cytometric results showed that the cap-dependent translation was inhibited around 40-fold by the GM-CSF ARE whatever the amount of DNA used for the transfection. Together, these data clearly demonstrate that EGFP translation was efficiently repressed by the GM-CSF ARE in vivo independently of its destabilizing effects on mRNAs.

GM-CSF ARE Represses Encephalomyocarditis Virus (ECMV) Internal Ribosome Entry Site (IRES)-dependent Translation—To investigate the repressive role of the ARE on cap-independent translation, we investigated the effects of the GM-CSF ARE when EGFP translation was initiated from the IRES. Cells were transfected by either the pIRES-0 plasmid as the negative control (obtained by deletion of the EGFP coding region in the plasmid pIRES2-EGFP, Fig. 1B) or the pIRES-A and pIRES-Am plasmids containing the 136 nucleotides GM-CSF ARE or its mutated counterpart, respectively, downstream of the EGFP coding region in the pIRES2-EGFP plasmid (Fig. 1B).

When we performed transfection experiments under the conditions described in Figs. 2 and 3 (with 5 µg of plasmid), we observed that EGFP was not detectable in the cells, especially those transfected with the positive-control pIRES-0 plasmid, which contains the non-functional ARE. Therefore, we performed dose-response experiments by transfecting the cells with increasing amounts of either pIRES-Am or pIRES-A, as for pEGFP-Am and pEGFP-A (Fig. 4). Fig. 5A shows that in cells transfected by pIRES-Am, EGFP expression increased in parallel to the amounts of plasmid used for transfections. However, the level of EGFP was almost undetectable with 5 µg of either pIRES-A (Fig. 5A), thus explaining why in the same conditions no EGFP was detected in our previous experiments. By comparing the dose-response results in Figs. 4A and 5A, it may be seen that the level of EGFP expression in cells transfected by pIRES-A was much lower than in cells transfected with pEGFP-Am (around 180-fold less when using 20 µg of DNA).
were the same as in Fig. 2. Controls used for Western and Northern blots and mRNA quantitation of EGFP expression/H11006 the percentage of the DsRed-positive cells. The bottom part containing mRNA but can no longer mediate its decay. Panel A/f5/H9262 efficiency and 20/f5/H9262 cells infected with 5/f5/H9262 plasmid. Analysis was performed by flow cytometry as described in Fig. 2. In each transfection the total amount of DNA. EGFP-positive cells were strongly decreased (around 28- and 5-fold, respectively, from flow cytometric data), thus suggesting that IRES-dependent translation, like cap-dependent translation, was inhibited by the GM-CSF ARE. As in the experiments shown in Fig. 3, transfection efficiency was assessed by co-transfecting the cells with the pDsRed2-N1 plasmid. Here again, a similar amount of HeLa cells expressed the DsRed protein in the different conditions (Fig. 5B, top line), thus demonstrating that the difference in EGFP expression could not be accounted for by a variation in the transfection efficiency of the cells. Interestingly, Northern blot analysis showed that GM-CSF ARE was no longer functional as a destabilizing element when translation was initiated from the ECMV IRES, since the steady-state level of the IRES-EGFP mRNAs was the same whether the transcript contained the GM-CSF ARE or its mutated counterpart (Fig. 5D).

The GM-CSF ARE Intervenes in PABP Binding on the Poly(A) Tail—Because ARE-mediated mRNA decay begins by 3'-end poly(A) shortening (1–2, 19), the GM-CSF ARE can suppress both cap- and IRES-dependent translation (see above), and the PABPs have been shown to participate in both translations (11–13, 23), we investigated whether the ARE may intervene in PABP binding on the poly(A) tail. We performed gel mobility shift assays using HeLa cytoplasmic extracts, two polyadenylated RNA probes (probes AU-UTR(A+) and GC-UTR(A+)) with a poly(A) tail 100 nucleotides long and their deadenylated counterparts (probes AU-UTR(A−) and GC-UTR(A−)) (Fig. 6A). The AU-UTR probes contained the minimal functional part of the GM-CSF ARE, whereas the GC-UTR probes contained a mutated GM-CSF ARE (Fig. 6A). The polyadenylated RNA probes were transcribed in vitro using the T7 RNA polymerase and the SacI-linearized AT-UTRp(A+) and GC-UTRp(A+) plasmids (kindly provided by Joan A. Steitz) as templates (22). In parallel, the deadenylated RNA probes were transcribed in vitro as before using the Xbal-linearized AT-UTRp(A+) and GC-UTRp(A+) plasmids as templates.

When we performed gel mobility shift assay using the deadenylated non-ARE GC-UTR(A−) RNA probe, no specific RNP complexes formed (Fig. 6B, left panel), suggesting that no HeLa cytoplasmic protein was able to associate with the GC-UTR RNA core. When we performed gel mobility shift assay using the deadenylated ARE-containing AU-UTR(A−) RNA probe, we observed the formation of three major RNP complexes (Fig. 6B, right panel) corresponding to the RNP pattern obtained with the full-length GM-CSF ARE probe (data not shown). To assess the ARE specificity of the complexes, we added antibody against HuR and TIAR, two potent ARE-BPs (1–2, 19). As expected, supershifted complexes appeared either with anti-

This clearly demonstrates that the translation initiated from the ECMV IRES was much less efficient in the HeLa cells than the cap-dependent translation and that flow cytometry was sensitive enough to allow the detection and quantification of low amounts of fluorescent EGFP. When cells were transfected with increasing amounts of pIRES-A, which contains the functional ARE, the EGFP expression level was always lower than that obtained when using the control pIRES-Am plasmid (Fig. 5A, around 12-fold with 20 μg of DNA and around 5-fold with 50 μg of plasmid), undetectable in cells transfected with 5 μg of DNA, and remained low even with high amounts of DNA.

These data point to a repressive role of the GM-CSF ARE on IRES-dependent translation. However, to further demonstrate this repressive effect, we performed transfection experiments using 20 μg of either pIRES-Am, pIRES-A, or the negative pIRES-0 plasmid and evaluated the expression level of both EGFP and mRNA as before by flow cytometry and Western and Northern blotting. Flow cytometric and Western blot results (Fig. 5, B and C) showed that in cells transfected with the pIRES-A, the EGFP expression level and the percentage of the EGFP-positive cells were strongly decreased (around 28- and 5-fold, respectively, from flow cytometric data), thus suggesting that IRES-dependent translation, like cap-dependent translation, was inhibited by the GM-CSF ARE.

Regulation of Translation by the GM-CSF ARE

![Graph](image-url) Fig. 5. The GM-CSF ARE represses translation of an IRES-containing mRNA but can no longer mediate its decay. Panel A, HeLa cells were transfected with increasing amounts (5–50 μg of DNA) of the plasmids pIRES-Am or pIRES-A and 50 μg of pIRES-0 plasmid as the negative control. In each transfection the total amount of DNA was kept constant at 50 μg by adding appropriate amounts of the pIRES-0 plasmid. Analysis was performed by flow cytometry as described in Fig. 2. The EGFP expression ±S.D. and the percentage of EGFP-positive cells ±S.D. is shown as described in Fig. 2 (an average of two experiments in duplicate was performed). Panels B–D, HeLa cells were transfected with 5 μg of plasmid pDsRed2-N1 used as control for transfection efficiency and 20 μg of the plasmids pIRES-Am (lane 2), pIRES-A (lane 3), or the control plasmid pIRES-0 (lane 1). Analysis was performed by flow cytometry (panel B), Western blotting (panel C), and Northern blotting (panel D) as described in Fig. 2. In panel B, the top line shows the percentage of the DsRed-positive cells. The bottom part shows the EGFP expression ±S.D. and the percentage of EGFP-positive cells ±S.D. as described in Fig. 2 (average of 4 experiments). The loading controls used for Western and Northern blots and mRNA quantitation were the same as in Fig. 2.
HuR or anti-TIAR antibodies (arrowheads in Fig. 6B, right panel). However, no supershift was observed with the anti-PABP antibody (Fig. 6B, lane 10) or when using the GC-UTR(−) RNA probe with any of these different antibodies (Fig. 6B, left panel). These results showed that some proteins associated specifically with the AU-UTR (but not with the GC-UTR(−) RNA probe with any of these different antibodies (Fig. 6B, left panel). These results showed that some proteins associated specifically with the AU-UTR (but not with the

**Fig. 6. GM-CSF ARE intervenes on PABP/poly(A) tail interaction.** Ribonucleotidic sequences of RNA probes used in the gel mobility shift assay are as shown in panel A. The XbaI site is in bold (obtained from Svitkin et al. (22)). The 32P-labeled deadenylated GC-UTR(−) (panel B, on the left), deadenylated AU-UTR(−) (panel B, on the right), polyadenylated GC-UTR(+) (on the left, panels C and D), or polyadenylated AU-UTR(+) (on the right, panels C and D) RNA probes were incubated in a crude HeLa cytoplasmic extract and treated as described under “Experimental Procedures.” Antibodies (Ab) against HuR (lanes 3 and 8, panels B and C; lanes 3 and 7, panel D), TIAR (lanes 4 and 9, panels B and C), or PABP (lanes 5 and 10, panels B and C; lanes 4 and 8, panel D) were added to the mixture for 20 min before loading on a non-denaturing low ionic strength 6% (panels B and C) or 5% (panel D) polyacrylamide gel. RNP complexes are as indicated. Double arrowheads indicate PABP-containing supershifted complexes. Single arrowheads indicate HuR- and TIAR-containing supershifted complexes. Open double arrowheads show the decrease in PABP-containing supershifted complexes. In panels C and D, dashed arrows show poly(A)-specific RNP complexes. As shown by the arrow at the bottom right panel C, the C1 complex just ran out of the gel during this experiment.
Regulation of Translation by the GM-CSF ARE

GC-UTR core) and that HuR and TIAR, two well known ARE-BPs, but not PABP, figure among these proteins. Therefore, we performed experiments using the polyadenylated RNA probes.

When resolving the polyadenylated GC-UTR(A+) RNA probe, two major RNP complexes (C4 and C5 complexes) formed specifically with the poly(A) tail (Fig. 6C, left panel, lanes 2–4), since no protein could associate with the GC-UTR core (Fig. 6B, left panel). None of these complexes was supershifted with the antibodies raised against the two ARE-BPs, HuR and TIAR (Fig. 6C, lanes 3 and 4), but supershifted PABP-containing RNP complexes were observed when the monoclonal antibody against PABP was added to the reaction binding (double arrowheads in Fig. 6C, lane 5).

When using the polyadenylated AU-UTR(A+) RNA probe, the RNP complex pattern was similar to that obtained with the deadenylated AU-UTR(A–) probe (cf. Fig. 6, B and C, right panels), except that during this experiment, the C1 complex just ran out of the gel as shown by the arrow at the right bottom of Fig. 6C. The C4 and C5 poly(A)-specific complexes were also detectable just downstream and upstream of the C3 ARE-specific complex, respectively (see the dashed arrow, Fig. 6C).

Here again, supershifts containing HuR or TIAR appeared with the addition of their corresponding antibody (arrowheads in Fig. 6C, lanes 8 and 9, respectively), demonstrating the specificity of the RNP complexes for the ARE core when using the AU-UTR(A+) RNA probe. However, when the anti-PABP monoclonal antibody was added to the reaction mixture the PABP-containing supershifted RNP complexes were hardly detectable compared with the same experiment using the polyadenylated GC-UTR(A+) RNA probe (compare lanes 5 and 10 in Fig. 6C). Because the upper PABP-containing supershifted complex was close to the loading well, we repeated the experiment using a 5% polyacrylamide gel. The results in Fig. 6D are similar to those in Fig. 6C and support the hypothesis that the GM-CSF ARE interferes in vitro on the association between PABP and the poly(A) tail.

**DISCUSSION**

In this report we used flow cytometry as a very sensitive tool to investigate in vivo the effects of the ARE from the GM-CSF mRNA on the expression level of an EGFP reporter gene. The Western blot findings validate our flow cytometric data and demonstrate the exactness of the latter in investigating in vivo the role of regulatory elements such as AREs in the control of gene expression.

Our findings show that in mammalian cells, the GM-CSF ARE dramatically decreases cap-dependent EGFP translation in vivo, independently of its effect on mRNA decay, thereby suggesting that the ARE is a translational repressor (8). Interestingly, this ARE-mediated translational repression was also observed when translation was initiated from an IRES, but in this condition, the ARE was no longer functional on mRNA decay. Finally, we found that the GM-CSF ARE intervenes in vitro in PABP binding with the poly(A) tail. ARE-mediated translational repression has been reported in vitro in Xenopus oocytes and rabbit reticulocyte extract (8–9) but also in somatic cells (10). Our results confirm these data and show that GM-CSF ARE represses translation in vivo in HeLa cells.

As in yeast, ARE-mediated mRNA decay in mammalian cells begins by poly(A) shortening and is controlled by trans-acting factors through direct interactions with either the ARE or the poly(A) tail (1, 19). Here, although the PABP bound on a short polyadenylated RNA probe, its in vitro binding was significantly reduced when it bore the GM-CSF ARE in its 3′-UTR (Fig. 6, C and D, right panels). This result suggests that the ARE might prevent PABP binding on the poly(A) tail. The mechanism by which ARE might interfere on the PABP/poly(A) tail association is unknown and needs further investigation, but it is likely that factors associated with the GM-CSF ARE are involved.

A recent study using the human Papillomavirus type-1 h1ARE as the model showed in vitro a direct interaction between a recombinant GST-PABP and the viral ARE (24). We did not observe any PABP-supershifted complex when using the anti-PABP antibody and the deadenylated ARE-containing probe (Fig. 6B, right panel). However, formation of the C3 RNP complex was reproducibly reduced when the anti-PABP antibody was added to the binding reaction (Fig. 6, B–D, right panels). This result may suggest that the PABP is part of the ARE-specific complex and may participate to its formation and/or stability. In this way, PABP has been shown to interact with either the SMUG mucin mRNA ARE-BPs in trypanosomes (25) or the AUF1/eIFG4 complex in HeLa cells (26) and to belong to a protein complex purified by affinity chromatography using the GM-CSF ARE as probe (27). From our observations we can speculate that the ARE, by binding either directly or indirectly the PABP, may tend to displace the PABP from the poly(A) tail. This could provide a model for how the ARE mediates both mRNA degradation and translational inhibition. Such a model is reminiscent of the model proposed by Wilusz et al. (1) in which the ARE-protein complexes alter interactions between the PABP and the poly(A) tail.

Both mRNA stability and translational efficiency depend on the association of the PABP with the poly(A) tail (13, 28). Therefore, the displacement of PABP from the poly(A) tail by the ARE may tend to decircularize (or linearize) the mRNA, allowing poly(A) shortening to operate at the 3′-end by recruiting the specific enzyme, poly(A) ribonuclease (PARN/DAN) (1). In parallel, at the 5′-end, the uncoupling of PABP from the poly(A) tail may weaken the interaction between eukaryotic initiation factor 4F and the cap, allowing the binding of the decapping complex and/or poly(A) ribonuclease (PARN) and the decapping (20). Because PARN can bind on both ends of the mRNA, it may participate simultaneously in the deadenylation and decapping processes.

In fibroblast cells ARE-mediated translational repression by unloading of the ribosomes from the mRNA has been suggested for the tumor necrosis factor-α, but this effect remains specific to the tumor necrosis factor-α and was not observed for the interleukin-1β, interleukin-6, or GM-CSF mRNAs (29). This repression is controlled by the ARE-BP T-cell restricted intracellular antigen-1 protein. However, other reports showed that the ARE-containing mRNAs are associated with the polysomes and that the ARE destabilizing function depends on ongoing translation (29–32). In view of these data and our results, we speculate that the ARE-mediated PABP removal from the poly(A) tail does not impede ribosome loading or scanning on the ARE-containing mRNAs but, rather, decreases the efficiency of its loading, thus explaining why the translational rate of these mRNAs is very low. This model is in accordance with the low EGFP expression measured by flow cytometry in cells transfected with the pEGFP-A plasmid (Figs. 2–4).

To evaluate the inhibitory effect of the GM-CSF ARE on cap-independent translation, we performed transfection experiments using two plasmids containing the EGFP reporter gene under the translational control of the ECMV IRES and either the functional GM-CSF ARE or its mutated counterpart (Fig. 5). We first observed that in HeLa cells, the EGFP expression level from pIRES-Aw was very low compared with the expression obtained with the pEGFP-B or pEGFP-Aw plasmids. This low efficiency was due to the IRES itself, since EGFP expression increased together with the amount of pIRES-Aw plasmid
used in the transfections (Fig. 5A). Second, our results (Fig. 5) clearly showed that the GM-CSF ARE could also inhibit translation initiated from an IRES. However, the ARE-mediated inhibition on IRES-dependent translation was less marked than on cap-dependent translation. Because the translation initiation is much less efficient from the IRES than from the cap, we cannot, however, conclude that this less marked ARE-mediated inhibition on IRES-dependent translation is related to the IRES.

The ARE-mediated repression of the IRES-dependent translation also supports our model, since formation of the initiation complex in this context necessitates both the binding of the initiation factors eIF4G and eIF4A on the IRES and the direct interaction between PABP and eIF4G (23). Because eIF4G/PABP association seems to be as critical for initiation of the cap-dependent translation, ARE-induced displacement of PABP from the poly(A) tail may explain translation repression in both cases. Therefore, we postulate that the main target for the control of either cap- or IRES-dependent translation by cis-acting elements, such as the ARE, is the eIF4G/PABP/poly(A) tail association, which may condition the efficiency of the ribosome loading on the mRNA, the rate of the translational initiation, and the re-recruitment of the ribosome ending the translation.

Surprisingly, when we evaluated IRES-EGFP mRNA expression, the steady-state level was the same whether the mRNA contained the GM-CSF ARE or not, thus supporting the idea that the IRES-A mRNA is very stable even though it contains an ARE. This suggests that the ARE no longer functions in mRNA turnover when translation is initiated from the IRES. To our knowledge this is the first report of the functional inefficiency of an ARE in vivo in conditions where no inhibitor of translation elongation was added or no ARE-associated regulatory element was ectopically overexpressed. Indeed, several authors have reported that cycloheximide treatment stabilizes unstable mRNA and that an ARE-containing mRNA needs to be translated to be degraded (1, 20, 33). Moreover, ectopic expression of many ARE-BPs had a stabilizing effect on ARE-containing mRNA (Ref. 34; for reviewed, see Ref. 19). Blocking of ARE-mediated mRNA decay because translation initiates from an IRES suggests that the ARE-distabilizing function may be linked to events occurring at the 5′-end of the transcript, such as decapping. This is in accordance with recent data reported in mammalian cells, where the ARE stimulated decapping in HeLa cell extracts (7), and in vitro experiments, where the GM-CSF 5′-UTR was able to suppress the translational repression mediated by the GM-CSF ARE located in the 3′-UTR (9). Concerning this last point, we performed cell experiments with a plasmid containing both GM-CSF 5′ and 3′-UTRs and repeated our analysis of EGFP expression by flow cytometry and Western and Northern blots. In our hands the presence of the GM-CSF 5′-UTR did not modulate the ARE functions on either mRNA decay or translation. This discrepancy could be explained by the use of very different systems. However, in their report, Jarzembowski et al. (9) show that the GM-CSF ARE could specifically inhibit translation of a polyadenylated transcript independently of its effect on the mRNA decay. Our in vivo results support these in vitro results since we showed that EGFP expression initiated by either cap- or IRES-dependent translation is dramatically inhibited by the GM-CSF ARE, whereas ARE-containing EGFP mRNAs persisted in the cells (Figs. 2–5).

Whereas blocking the cap-dependent translation initiation of ARE-containing mRNAs by either mutations or hairpin loop inclusion in the 5′-UTR does not inhibit mRNA decay, inhibition of translation elongation by inhibitors leads to mRNA stabilization (1, 31, 33). Because the ARE is not functional as a destabilizer when translation initiates from an IRES, we speculate that the ARE-distabilizing function is closely coupled to the cap-dependent translation process (for review, see Refs. 1, 20, and 33). Our data support the theory of Mitchell and Tollervey (20), i.e., the initiation of cap-dependent translation could be a “window of opportunity” for the ARE-mediated decay mechanism to proceed. One possibility could be that the cap is accessible to the decapping complex only during cap-dependent 40 S ribosome loading. When an ARE is present with its associated factors, the decapping frequency may be highly increased because the decapping complex is more active and/or the protective cap/eukaryotic initiation factor 4F/PABP/poly(A) tail association is weakened by a less efficient binding of the PABP on the poly(A) tail. When translation is initiated through an IRES, the ARE might no longer be able to induce decapping (and, therefore, mRNA body degradation) since 40 S ribosome loading might no longer depend on the cap and its associated factors, but it still could affect the translation efficiency by intervening in the IRES/eIF4F/PABP/poly(A) tail association. On this basis, the stabilization of ARE-containing mRNAs by inhibitors of translation elongation may also be explained by a reduction of the decapping, because 40 S ribosomes are no longer loaded on the transcript (35).

In conclusion, mRNA decay may occur through a similar mechanism in both yeast and mammalian cells, where decapping immediately occurs after deadenylation and precedes RNA body degradation. Further investigations need to be done to assess the dependence of the decapping step in the mechanism of the ARE-mediated mRNA decay and to identify the regulatory factors involved in this process.

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