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cis-Acting Elements Involved in the Alternative Translation Initiation Process of Human Basic Fibroblast Growth Factor mRNA

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Four forms of basic fibroblast growth factor (bFGF) are synthesized from the same mRNA, resulting from alternative initiations of translation at three CUG start codons and one AUG start codon. The CUG- and AUG-initiated forms have distinct intracellular localizations and can modify cell phenotypes differently, indicating that control of the alternative expression of the different forms of bFGF has an important impact on the cell. In this study, we investigated the roles of the mRNA 5′ untranslated region and the alternatively translated region located between the CUG and AUG codons in the regulation of alternative translation of the different forms of bFGF. Deletions and site-directed mutagenesis were carried out in bFGF mRNA leader, and translation was studied in vitro and in vivo. The results enabled us to identify five *cis*-acting RNA elements (two in the 5′ untranslated region and three in the alternatively translated region) involved in the regulation of either global or alternative initiation of translation. Each of these elements had a specific effect on the level of synthesis of the different forms of bFGF. Furthermore, we showed that the 5′ untranslated region regulatory elements had different effects on bFGF translation, depending on the translation system used. These results suggest that bFGF translation is modulated by *cis*-acting elements corresponding to secondary or tertiary RNA structures, which could be the targets of cell-specific *trans*-acting factors.

Basic fibroblast growth factor (bFGF) belongs to a family of potent mitogens and promotes proliferation and differentiation of neuroectodermal, mesenchymal, and epithelial cells. It is also implicated in angiogenesis and wound-healing processes (13, 30). The biological activity of bFGF is mediated through a family of high-affinity cell surface receptors but also requires a low-affinity heparinlike receptor (36). bFGF is synthesized by a wide variety of cells, including capillary endothelial cells (33); nevertheless, the mechanism by which the molecule is secreted is still unknown. Primary subcultures of adult bovine aortic endothelial cells synthesize a significant amount of bFGF; however, they require exogenous bFGF to proliferate (6), indicating that endogenous and exogenous bFGF have distinct roles.

In most producing cell types, several forms of bFGF are detected (24, 29). It has been shown by mutagenesis of bFGF cDNA that these different forms result from alternative initiation of translation at an AUG codon or at three in-frame upstream CUG codons, leading, respectively, to synthesis of a small form of 18 kDa or of large forms of 21, 21.5, and 22.5 kDa (12, 27). In different cell lines, the relative amounts of the different forms are substantially different, suggesting that this alternative initiation process is highly regulated.

The process of alternative initiation of translation has many consequences for the fate of bFGF. In SK-Hep1 cells, the 18-kDa form is found to be primarily cytosolic, whereas the high-molecular-weight bFGFs are recovered in the nucleus (29). Furthermore, it has been shown that in COS cells transfected with human bFGF cDNA, the signal sequence responsible for the nuclear localization of bFGF is contained in the 37 residues upstream of the AUG codon (7). Recent studies have demonstrated that the intracellular distribution of bFGF plays a key role in cell behavior. In adult bovine aortic endothelial cells infected by recombinant retroviruses expressing one, two, or all forms of bFGF, constitutive expression of the AUG-initiated form (18 kDa) leads to cell transformation whereas expression of the CUG-initiated forms leads to cell immortalization (8).

Alternative initiation of translation, using noncanonical codons, involves not only viral mRNAs, such as those of Sendai virus with an ACG codon (9) and murine leukemia virus with a CUG codon (26), but a growing number of cellular mRNAs, including, in addition to the bFGF mRNA (27), mRNAs encoding c-*myc* (15) and *int-2*, another member of the FGF family (1). In the cases of murine leukemia virus, bFGF, and *int-2*, the choice of initiation codon determines the subcellular fate and the function of the protein. These observations indicate that the process of alternative initiation of translation not only increases gene diversity but also regulates the relative expression of proteins with different locations and functions but encoded by the same gene.

In eukaryotic cells, translation is usually initiated according to the ribosome scanning model (18). That is, the 40S ribosomal subunit and translation initiation factors bind to the 5′ end of mRNA and scan the RNA molecule in the 3′ direction until they reach an AUG codon in the appropriate context; then the 80S ribosome assembles and begins protein synthesis. It follows that structural features within the 5′ untranslated region (UTR), such as secondary structures and open reading frames (ORFs) (16), can strongly influence the efficiency of translational initiation. The compilation of sequence information from 699 vertebrate mRNAs has indicated that more than 90% of the sequenced 5′ UTR is less than 200 nucleotides (nt) in length and devoid of AUG triplets (17). However, most mRNAs coding for proto-

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oncogenes and other factors related to cell proliferation possess long G+C-rich 5′ UTRs with one or more AUGs, suggesting that expression of these genes is translationally regulated (20). In recent years, a number of examples of translational regulation by the 5′ UTR have been reported. Expression of oncoprotein c-Myc and of platelet-derived growth factor 2 is translationally inhibited by secondary structures present in the mRNA 5′ UTR (25, 28), whereas the biosynthesis of FGF-5, another member of the FGF family, is influenced by an ORF located in the 5′ UTR (4). In the cases of ornithine decarboxylase and of transforming growth factor β3 (TGF-β3) mRNAs, translation is jointly regulated by secondary structures located in the 5′ part of 5′ UTR and by small ORFs located in the 3′ part of 5′ UTR (2, 14, 23). However, translational regulation by the mRNA 5′ UTR can also be positive; for example, the *Xenopus* β-globin mRNA leader can dramatically improve the translation of a variety of different coding sequences (10).

The bFGF mRNA (6,774 nt) possesses a 483-nt-long leader sequence, a 468-nt-long coding region downstream of the AUG codon, referred to as the common translated region (CTR), and a 5,823-nt-long 3′ UTR. Like leader sequences of all mRNAs having alternative initiation codons, the leader of bFGF mRNA can be divided into two regions: the 5′ UTR (318 nt), located upstream of the first initiation codon, and the alternatively translated region (ATR; 165 nt), located between the initiation codons. As expected for a growth factor mRNA (20), the bFGF mRNA leader is very G+C rich, especially in the ATR, which contains 90% G and C residues. One can predict the existence of strong secondary structures able to regulate translation initiation. However, there is a major difference between an mRNA translated from one initiation codon and an mRNA translated from several alternative codons. In the latter case, translational regulation by the leader sequence may have qualitative as well as quantitative effects; by modulating the relative initiation of translation at the different codons, *cis*-acting elements may govern the relative expression of the different proteins encoded by the mRNA.

In this report, the role of bFGF mRNA leader in the regulation of translation initiation was studied in vitro and in vivo. Deletion of the entire 5′ UTR plus ATR resulted in overexpression of the small bFGF form, indicating that the 5′ leader was responsible for a strong inhibition of translation. However, mutagenesis of the three upstream CUG initiation codons showed no competition between CUG and AUG codons. Deletion mutagenesis was performed in the 5′ UTR and ATR and was followed by RNA translation in rabbit reticulocyte lysate and wheat germ extract or by transfection of COS cells. These experiments allowed the identification of five *cis*-acting elements in the mRNA leader, each having a specific effect on global or alternative translation initiation. Moreover, the two elements located in the 5′ UTR had different effects on translation initiation in wheat germ extract than in reticulocyte lysate and COS cells. These results suggested the existence of *trans*-acting factors involved in the control of bFGF expression.

MATERIALS AND METHODS

Plasmid construction. The starting plasmids used to make the constructs were pRF12 and pRF11, which had the first 1,179 nt of bFGF cDNA sequence cloned in the *Hin*dIII site of vector Bluescript KS+ (Stratagene) under the control of T7 and T3 RNA polymerase promoters, respectively. Vector pRF40, possessing the entire bFGF cDNA cloned in vector pSP65 (27), was used to subclone the complete 3′ UTR. For construction of eukaryotic vectors, the starting plasmid was pSCT-CAT, containing the chloramphenicol acetyltransferase (CAT) gene under the control of cytomegalovirus and T7 promoters. This vector contains the simian virus 40 replication origin and polyadenylation site (31).

The authentic 5′ end of bFGF mRNA was reconstructed by polymerase chain reaction (PCR) amplification of the 5′ UTR fragment with a 5′ primer, giving the new 5′ end of the leader (5′-GGGTCTAGACCGCCGAACTCAGGCCGGCC CCAGAAAACCCGAG-3′) (34). The template was vector Bluescript KS+ containing the 5′ UTR alone, allowing us to use the T3 primer as the 3′ primer. The newly created 5′ UTR was reinserted into vector pRF12, using *Xba*I (5′ end) and *Xho*I (position 312). This plasmid was named pRF12V.

The FGF-CAT fusion (Fig. 1A) was constructed by PCR amplification of the CAT coding sequence, using a 5′ DNA primer (5′-CCCCGGCGCCCAGGAGAAAAAAATCACT-3′) with a *Nar*I site in frame with the *Nar*I site located at position 539 of the bFGF sequence and a 3′ primer (5′-AATCTCGAGTCTAGAGTATTCGCCCCGCCCTGCCA-3′) introducing a *Sac*I site. The generated CAT fragment digested by *Nar*I and *Sac*I and the 5′ part of bFGF cDNA digested by *Xba*I and *Nar*I were introduced by tripartite ligation into the vector fragment pSCT-CAT digested by *Xba*I and *Sac*I.

The complete bFGF cDNA was introduced into vector pSCT by tripartite ligation of (i) the *Nar*I-*Bgl*II fragment of the FGF-CAT construct containing the 5′ part of the bFGF cDNA (upstream of nt 539) and one part of the vector, (ii) a *Bgl*II-*Xba*I fragment containing the other part of the vector, and (iii) a *Nar*I-*Xba*I fragment containing the 3′ part of bFGF cDNA (downstream of nt 539).

*Mlu*I sites were generated at positions 391 and 411 of bFGF cDNA by PCR amplification of ATR fragments. PCR fragments containing the sequence from nt 391 to 538 or 411 to 538 were generated by using *Mlu*I site-creating oligonucleotides (5′-GTGACGCGTTGCGGGTCGGAGGCC-3′ or 5′-GGGGGGACGCGTCCGGGGCCGTG-3′, respectively) as 5′ primers and oligonucleotide 5′-GCCGCCATCCTC GGGCAAGG-3′ as the 3′ primer. These ATR 3′ fragments were digested by *Nco*I (position 482) and reintroduced into pRF12 digested by *Sma*I (5′ end) and *Nco*I, generating constructs Δ1-391 and Δ1-411. Another PCR fragment containing the sequence from nt 312 to 385 (with a spontaneous deletion of nt 375 to 380, a CCGGGG motif in tandem repeat), followed by an *Mlu*I site, was generated by using construct Δ1-312 (see below) as the template. T3 primer and oligonucleotide 5′-GCTACGCGTCACGGCCCCGGCCCC-3′ were used as 5′ and 3′ primers, respectively. This ATR 5′ fragment digested by *Xho*I (position 312) and *Mlu*I as well as a 5′ UTR-containing fragment digested by *Xba*I (5′ end) and *Xho*I (position 312) were reintroduced by tripartite ligation into construct Δ1-391 digested by *Xba*I and *Mlu*I. This construct, named pRF12M1, containing a complete leader with an *Mlu*I site at positions 386 to 391, had the same pattern of translation as did the construct with wild-type leader pRF12V (data not shown). PCR amplification of ATR DNA fragments needed specific conditions because of the very high G+C percentage. Reactions were carried out in 2.5% formamide; the first cycle of 4 min at 94°C was followed by 30 cycles of 30 s at 50°C, 2 min at 74°C, and 1 min at 94°C and one cycle of 30 s at 50°C and 15 min at 74°C.

The CUG mutations were obtained by insertion into pRF12M1 of mutagenic double-stranded oligonucleotides, between the *Xho*I (position 312) and *Sac*II (position 330)

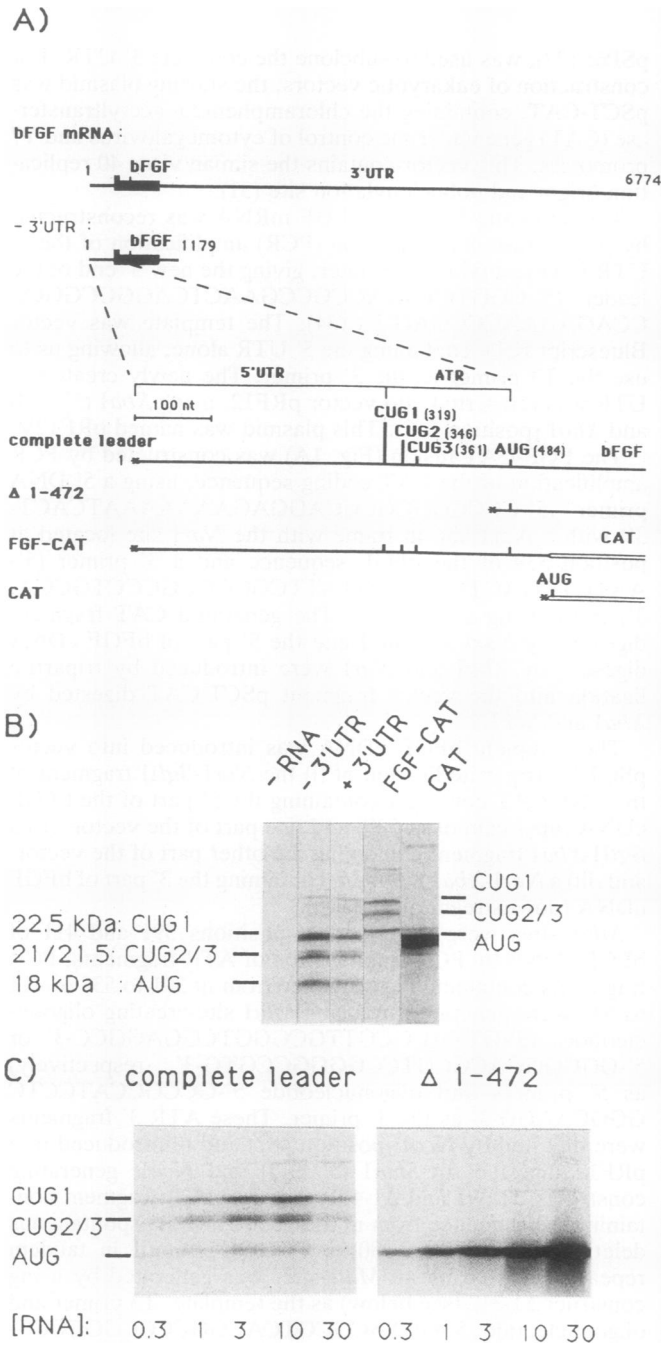

FIG. 1. (A) Schema of the bFGF mRNA with or without the 3′ UTR. Under the dotted lines, the 5′ portions of the different RNAs translated in the assays shown in panels B and C are represented. For RNA with complete leader, the different initiation codons and their positions from the 5′ end are indicated. Δ1-472 corresponds to deletion of the 472 nt 5′ of bFGF mRNA (5′ UTR plus ATR). FGF-CAT is a protein fusion of CAT with bFGF at position 539 from the bFGF mRNA 5′ end, preserving 66 nt of bFGF sequence downstream of the AUG codon (see Materials and Methods). (B) RNAs depicted in panel A were translated in rabbit reticulocyte lysate (see Materials and Methods) at a concentration of 10 μg/ml. Translation of the long RNA with the 3′ UTR did not increase at higher concentrations. For each band, the corresponding initiation codon and the apparent molecular masses of the bFGF forms are indicated. The name of the corresponding RNA is indicated above each lane. (C) RNAs with complete leader and RNA Δ1-472, both without the 3′ UTR, were translated as described above at five concentrations between 0.3 and 30 μg/ml.

sites for mutation of CUG1 to CCU (oligonucleotides 5′-AGCTCGAGGCCTGGGGACCGCGGAA-3′ and 5′-TTC CGCGGTCCCCAGGCCTCGAGTC-3′) and between the *Not*I (position 336) and *Mlu*I (position 391) sites for mutations of CUG2 and CUG3 to CUC and CCU, respectively (oligonucleotides 5′-TACGCGTCACGGCCCCGGCCCCG GCCCCCAGGCCTCCCGCCCGGGAGCGCGCGGCCGC TT-3′ and 5′-AAGCGGCCGCGCGCTGCCGGGCGGGAG GCTGGGGGGCCGGGGCCGGGGCCGTGACGCGTA-3′). These oligonucleotides also restored nt 375 to 380 deleted in pRF12M1. For mutation of CUG1 to AUG, we used a previously described construct (27) after having restored the complete 5′ UTR.

The deletions shown in Fig. 3 were obtained by digestion of plasmids having the complete leader with restriction enzymes cutting at appropriate positions in the leader and/or in the 5′ polylinker, followed by ligation, as follows: Δ1-192, *Pst*I deletion in pRF12; Δ1-256, *Sma*I deletion in pRF12; Δ1-312, *Xho*I deletion in pRF11; Δ1-391 and Δ1-411, site-directed mutagenesis by *Mlu*I site insertion, described above; Δ1-472, *Apa*I deletion in pRF11; Δ182-256, *Sma*I deletion in pRF12V; Δ264-312, *Nae*I-*Xho*I deletion in pRF12V (Klenow treatment); Δ312-341, *Xho*I-*Bss*HII deletion in pRF12V (Klenow treatment); Δ341-391, *Bss*HII-*Mlu*I deletion in pRF12M1; Δ385-411, *Mlu*I digestion of pRF12M1 (site at positions 385 to 391) and of construct Δ1-411 (site at positions 405 to 411), followed by Klenow and *Hin*dIII treatment (cut at position 1179) and then religation of the two appropriate fragments from each plasmid; Δ385-472, *Mlu*I-*Apa*I digestion of pRF12M1, followed by ligation with an *Mlu*I-*Apa*I sticky octanucleotide (5′-CCGGCGCG-3′); Δ385-472 with three mutated CUGs, same strategy as described above, using the CUG1/2/3-mutated plasmid.

For some of the plasmids with internal deletions (Δ341-391, Δ385-411, and Δ385-472; see Fig. 4D), the 5′ UTR was also removed by recloning the *Xho*I-*Hin*dIII fragments (nt 312 to 1179) of these constructs into vector Bluescript KS+.

To obtain the deleted or mutated FGF-CAT plasmids (see Fig. 6), DNA fragments 5′ of the *Nar*I site (position 539) were excised from constructs Δ1-256, Δ1-312, and Δ1-472, the CUG1-to-AUG mutant, and the three-CUG mutant by digestion with an appropriate enzyme cutting at the 5′ end (Klenow treatment if necessary) and with *Nar*I; then they were introduced into the FGF-CAT plasmid in place of the wild-type sequence.

Vector pSCT-CATt encoding a truncated CAT protein was synthesized by PCR, using pSCT-CAT as the template. We used T7 primer as the 5′ primer and two overlapping oligonucleotides (5′-AAAGTCGACATCGATTCATTCGAA TATGTGTAGAAACTGCCG-3′ and 5′-AAAGACGTCG ATATCGGTACCTCTAGAGTCGACATCGATTCAT TC-3′) as 3′ primers. This strategy allowed us to introduce several restriction sites 3′ of the truncated CAT coding sequence. The truncated CAT had no CAT activity but was recognized by anti-CAT antibodies (5 Prime-3 Prime, Inc.) (see Fig. 6).

Mutations were checked by DNA sequencing using the dideoxy method (32).

In vitro translation. Plasmids were linearized downstream from the 3′ end of the bFGF coding sequence, and capped RNAs were in vitro generated by T7 or T3 RNA polymerase according to the manufacturer's instructions in the presence of m^7GpppG (0.5 mM). Incorporation of m^7GpppG was verified by competition with [γ-^{32}P]GTP. RNA transcripts were quantitated by A_{260} and ethidium bromide staining on agarose gels, which also allowed us to verify their integrity.

In vitro translation in rabbit reticulocute lysate and in wheat germ extract was performed as specified by the manufacturer (Promega) in the presence of [^{35}S]methionine (Amersham). However, in the reticulocyte lysate, some modifications allowed optimization of the results; i.e., translation assays (20 μl) were incubated for 1 h at 37°C (instead of 30°C) and in a 50% (instead of 70%) diluted lysate, as described previously (27). In the wheat germ extract, the best conditions were those given by the manufacturer. Translation products were analyzed by electrophoresis in sodium dodecyl sulfate–12.5% polyacrylamide gels (32) and autoradiography of dried gels.

DNA transfection, Western immunoblotting, and CAT assay. COS-7 monkey cells were transfected by the DEAE-dextran method (3). Then, 0.5 μg of each plasmid and 2.5 μg of plasmid pSCT-CATt (truncated CAT), used as an internal standard in Western blots, were incubated with the cells for 20 min at 37°C in the presence of 1 mg of DEAE-dextran per ml; chloroquine was added at 40 μg/ml, and incubation was continued for 4 h. DNA was removed, and cells were incubated in 10% dimethyl sulfoxide for 2 min. Cell lysates were prepared 48 h later by scraping cell monolayers. Cell pellets were frozen-thawed, resuspended in 0.1 M Tris (pH 7.8), and sonicated. Total proteins were quantitated by the Bio-Rad assay (A_{595}), and the normalized cell lysates were used for Western blots and CAT assays. For Western blots, lysates were heated for 2 min at 95°C in sodium dodecyl sulfate- and dithiothreitol-containing sample buffer, separated in a 12.5% polyacrylamide gel, and transferred onto a nitrocellulose membrane (32). CAT proteins were immunodetected with anti-CAT antibodies (5 Prime-3 Prime) and an enhanced chemiluminescence kit (Amersham). CAT assays were performed by using the diffusion-based CAT assay kit provided by NEN. The method is based on the differential organic solubilities of acetyl coenzyme A and the enzymatic reaction product, acetylchloramphenicol. The enzymatic reaction components, including [^{14}C]acetyl coenzyme A, chloramphenicol, and cell lysate in 0.1 M Tris (pH 7.8), were combined directly in a mini-scintillation vial and then immediately overlaid with the water-immiscible counting cocktail Econofluor and counted. The [^{14}C]acetylchloramphenicol diffuses linearly over time into the organic Econofluor phase.

RESULTS

Translational inhibition of AUG-initiated bFGF expression by the mRNA leader sequence. The AUG codon initiating the small form of bFGF (18 kDa) is located in the canonical context ACCAUGG (17). However, its translational efficiency is very weak (27), suggesting translational inhibition. To test this hypothesis, several DNA constructs were made. First, the original 5′ end of bFGF mRNA (34) was reconstructed to rule out an artifactual inhibition of translation due to the poly(G) resulting from the cDNA tailing (data not shown). Then, to evaluate the contributions of the different parts of bFGF mRNA in translational regulation, several modifications were generated in bFGF cDNA (Fig. 1A). (i) The long 3′ UTR was removed, (ii) the 5′ 539 nt of bFGF cDNA (leader plus 66 nt from the CTR) were fused with the CAT coding sequence (FGF-CAT) so as to conserve the alternative translation context, and (iii) the leader sequence was removed (Δ1-472). The corresponding RNAs were synthesized in vitro and translated in rabbit reticulocyte lysate.

As shown in Fig. 1B, the entire bFGF mRNA gave rise to four products migrating at 22.5, 21.5, 21, and 18 kDa, corresponding to alternative initiations of translation at CUG1, CUG2, CUG3, and AUG, respectively. However, these initiations were very inefficient in comparison with translation of CAT mRNA. Such a weak level of translation could be expected for noncanonical codons like CUGs but not for the AUG codon located in a favorable context. Removal of the 3′ UTR resulted in only a slight increase of all initiations, whereas translation of chimeric RNA FGF-CAT was similar to that of its counterpart encoding bFGF (Fig. 1B).

Unexpectedly, translation efficiency could not be improved by increasing the RNA concentration; the translation level of bFGF mRNA with a complete leader reached a plateau at RNA concentrations of 5 to 10 μg/ml and decreased at higher concentrations (Fig. 1C), whereas CAT mRNA translation was still increasing under the same conditions (data not shown). In contrast, when the leader was removed (RNA Δ1-472), AUG initiation was 15-fold more efficient than in the presence of the leader at an RNA concentration of 10 μg/ml, and it was still increasing at an RNA concentration of 30 μg/ml.

The results showed that under these conditions, the CTR and the 3′ UTR had little effect on translation initiation of bFGF mRNA, whereas the leader sequence or sequences surrounding the AUG codon strongly down-regulated synthesis of the 18-kDa AUG-initiated bFGF. Both the weak effect of the 3′ UTR and the strong influence of the leader sequence on translation initiation prompted us to use RNAs devoid of the 3′ UTR in the following experiments.

Lack of competition between CUG and AUG codons. To determine whether the weak translation initiation at the AUG codon was the result of a competition with the CUG codons located upstream, mutagenesis of one or all CUGs was performed (Fig. 2A) and the corresponding RNAs were translated as described above (Fig. 2B). AUG initiation was not enhanced by mutation of one or all CUGs.

When CUG1 was mutated to AUG, initiations at CUG2 and CUG3 were extinguished, as predicted by the scanning model. However, initiation at the new AUG was not stronger than when it was a CUG, and initiation at the downstream AUG decreased but was still detectable.

These results showed a lack of competition between CUG and AUG codons, indicating that the inhibition of translation initiation at AUG is not significantly influenced by the presence of upstream initiation codons.

RNA elements involved in the regulation of alternative translation in reticulocyte lysate. The involvement of RNA *cis*-acting elements was further investigated by deletion mutagenesis. Either progressive deletions from the 5′ end or small internal deletions were generated in the leader (Fig. 3).

The results obtained with progressive deletions (Fig. 4A) indicated that deletion of part or all of the 5′ UTR (Δ1-192, Δ1-256, or Δ1-312) did not affect the global translation rate. However, deletion of nt 1 to 312 modulated alternative translation by specifically decreasing CUG1 initiation. Such a behavior was not the result of a too short 5′ end; in this construct, the CUG1 was located 36 nt downstream from the T3 transcription start. Furthermore, the same results were obtained with a similar deletion in which CUG1 was preceded by a completely different leader of 16 nt (data not shown), allowing us to exclude a possible effect of the small heterologous leader. Removal of nt 1 to 391 and 1 to 411 in the ATR did not increase AUG initiation. In contrast, translation was strongly derepressed by removal of nt 1 to 472.

Translation of the internally deleted RNAs (Fig. 4B) again indicated no major effect of deletions in the 5′ UTR (Δ182-

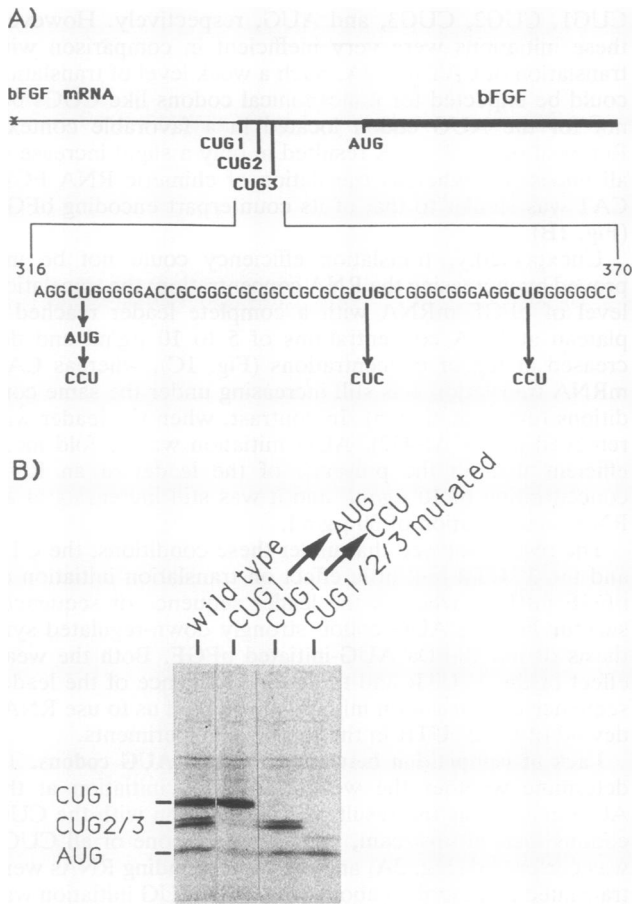

FIG. 2. (A) Schema of the 5′ end of bFGF mRNA and sequence from nt 316 to 370, including the three CUGs. Mutations of the different CUGs (see Materials and Methods) are indicated by arrows. (B) Translation in reticulocyte lysate of the CUG-mutated RNAs (devoid of the 3′ UTR). RNAs were used at 10 μg/ml.

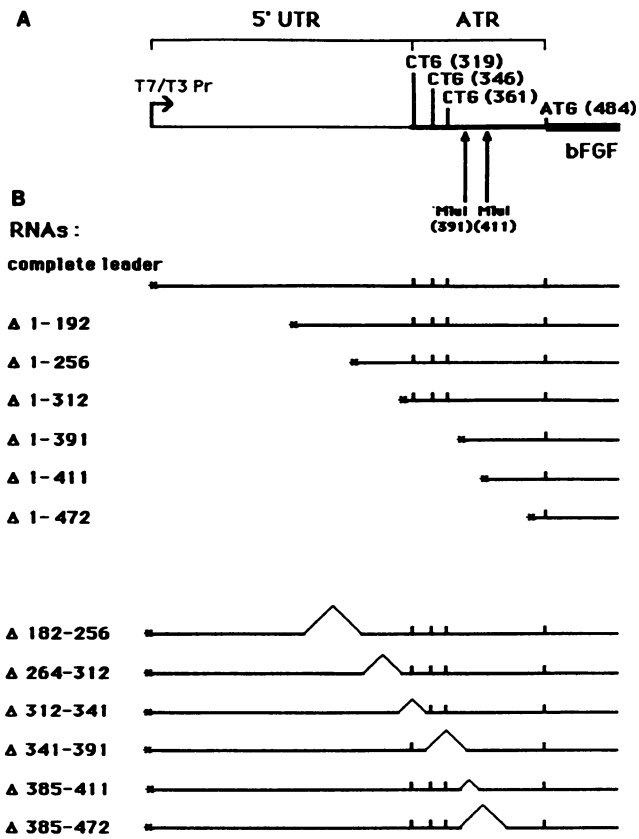

FIG. 3. (A) Schema of the 5′ end of bFGF cDNA; (B) 5′ portions of the different deleted RNAs used for Fig. 4. All of these RNAs were devoid of the 3′ UTR (see Fig. 1A). They were obtained from T7 or T3 transcription, depending on the orientation of each construct in vector Bluescript KS+ (see Materials and Methods). The name of each deletion, indicated on the left, corresponds to the nucleotides that were removed, counting from the RNA 5′ end. The two *Mlu*I sites, indicated by arrows, were generated by site-directed mutagenesis (see Materials and Methods).

256 and Δ264-312) on the global rate of translation. However, alternative translation was modulated by deletion of nt 264 to 312. In contrast to the deletion of nt 1 to 312, which led to a decrease of CUG1 initiation, the internal deletion of nt 264 to 312 resulted in an increase of CUG1 initiation. Regarding the deletions in the ATR, CUG2/3 and AUG initiation was enhanced by removal of nt 312 to 341 (which also removed CUG1), whereas CUG2/3 initiation was inhibited by removal of nt 385 to 411 relative to the other codons. Deletion of nt 385 to 472 led to an increase of translation, particularly of the smaller band resulting from comigration of CUG2/3 and AUG products. The removal of the three CUGs in that construct confirmed that the derepression primarily affected the AUG codon (Fig. 4B).

These results revealed four mRNA elements, A, B, C, and D (Fig. 5). Each of these elements, having a specific effect on the efficiency of one or more initiation codons, had a specific role in the modulation of alternative translation of bFGF mRNA. Unexpectedly, two elements showing an inhibiting effect (B and D) were located within the ATR (a translated region), whereas the G+C-rich 5′ UTR did not inhibit bFGF mRNA translation in the reticulocyte lysate.

Translational regulation of bFGF expression in the wheat germ extract. To evaluate whether the translational regulation of bFGF expression in reticulocyte lysate could be generalized to other translation systems, translation of the different deleted RNAs was carried out in wheat germ extract.

In this translation system, the bFGF mRNA with complete leader was still more weakly translated than in the reticulocyte lysate; the translation products were barely detectable (Fig. 4C and D). However, in contrast to the results obtained with reticulocyte lysate, deletions from nt 1 to 256 and 1 to 312 resulted in a significant increase of global translation, whereas no decrease of CUG1 initiation was observed for the deletion from nt 1 to 312 (Fig. 4).

As in the reticulocyte lysate, AUG initiation was strongly derepressed only with deletion from nt 1 to 472. Internal deletions in the ATR confirmed the modulation of global translation observed in the reticulocyte lysate; deletion from nt 385 to 411 inhibited, whereas deletion from nt 385 to 472 derepressed, bFGF translation. Nevertheless, modulation of alternative translation initiation could not be detected.

In summary, these results showed very different behaviors of the 5′ UTR in the reticulocyte lysate and the wheat germ extract. They revealed a fifth regulatory element, W, with a strong inhibiting effect specific to the wheat germ extract (Fig. 5). The *cis* elements located in the ATR seemed to regulate bFGF translation in both reticulocyte and wheat

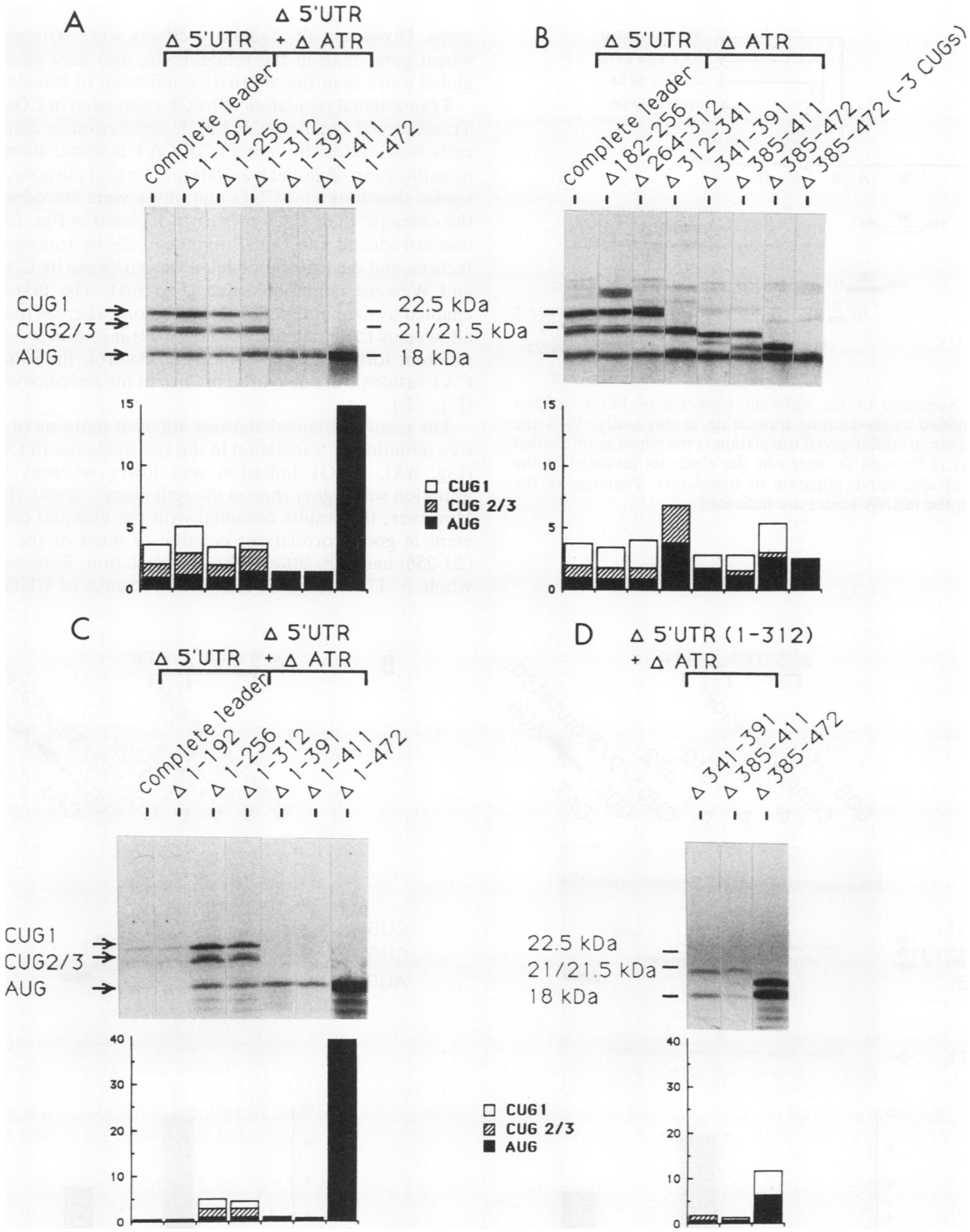

FIG. 4. In vitro translation of the RNAs depicted in Fig. 3 in rabbit reticulocyte lysate (A and B) or wheat germ extract (C and D). RNAs were used at the optimal concentrations of 10 and 30 μg/ml in reticulocyte lysate and in wheat germ, respectively. The deletions are indicated above the lanes. The positions of the four bFGF forms and their apparent molecular masses are indicated. The deletions in the ATR generated smaller CUG-initiated products and sometimes abolished one of them. Δ312-341, no CUG1 product; Δ341-391, CUG1 product of 20.5 kDa (apparent molecular mass), no CUG2/3 product; Δ385-411, CUG1 product of 20.5 kDa, CUG2/3 product of 19 kDa; Δ385-472, CUG1 product of 19.5 kDa, CUG2/3 products comigrating with AUG product at 18 kDa. For Δ182-256, an additional band of 25 kDa, probably resulting from an initiation at an upstream codon (CUG or ACG?) set in frame by the deletion, is visible. In wheat germ extract (D), RNAs with internal deletions were also devoid of the 5′ UTR (Δ1-312) so as to obtain measurable signals. The radioactivity corresponding to each band was counted. The values (disintegrations per minute) were normalized to the value for AUG initiation either of RNA with complete leader (reticulocyte lysate) or of RNA with 5′ UTR deletion Δ1-256 (wheat germ). The relative values are represented by histograms under the corresponding lanes. The results shown correspond to representative experiments that were repeated more than five times in reticulocyte lysate and three times in wheat germ extract.

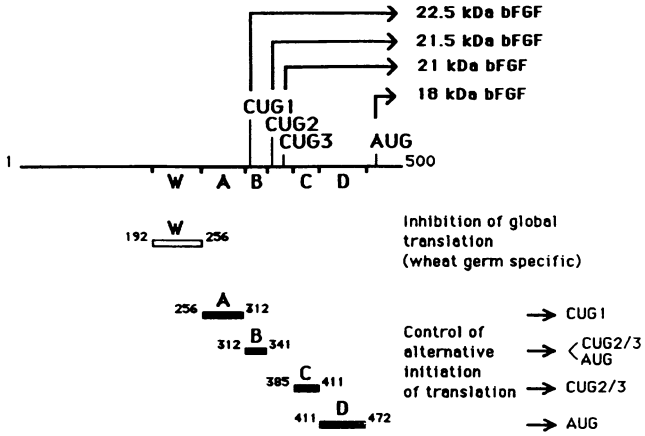

FIG. 5. Summary of the different elements of bFGF mRNA leader identified as modulating translation in this study. W is the element shown to inhibit global translation in the wheat germ extract specifically; A through D represent the elements involved in the regulation of alternative initiation of translation. Positions of the elements in the mRNA leader are indicated.

germ. However, all regulatory effects were stronger in the wheat germ than in the reticulocyte, and they affected the global more than the alternative initiation of translation.

Translational regulation of bFGF expression in COS-7 cells. Translational regulation of bFGF expression in mammalian cells was studied by using FGF-CAT fusions, allowing the quantification of global translation. For that purpose, several leader deletions and CUG mutations were introduced into the chimeric FGF-CAT construct depicted in Fig. 1A. DNA was introduced into COS-7 monkey cells by transient transfection, and the translation level was analyzed by CAT assay and Western immunoblotting (Fig. 6A). The first method enabled us to quantify global translation, whereas the second allowed us to distinguish the relative translation levels of the different forms of FGF-CAT. As a control, the same FGF-CAT fusions were also used in vitro in the reticulocyte lysate (Fig. 6B).

The results obtained showed different patterns of alternative initiation of translation in the two systems. In COS cells (Fig. 6A), CUG1 initiation was lower whereas CUG2/3 initiation was higher than in the reticulocyte lysate (Fig. 6B). However, the results obtained with the mutated constructs were in good correlation; deletion of most of the 5′ UTR (Δ1-256) had very little effect on translation. Removal of the whole 5′ UTR (Δ1-312) led to an increase of CUG2/3 and

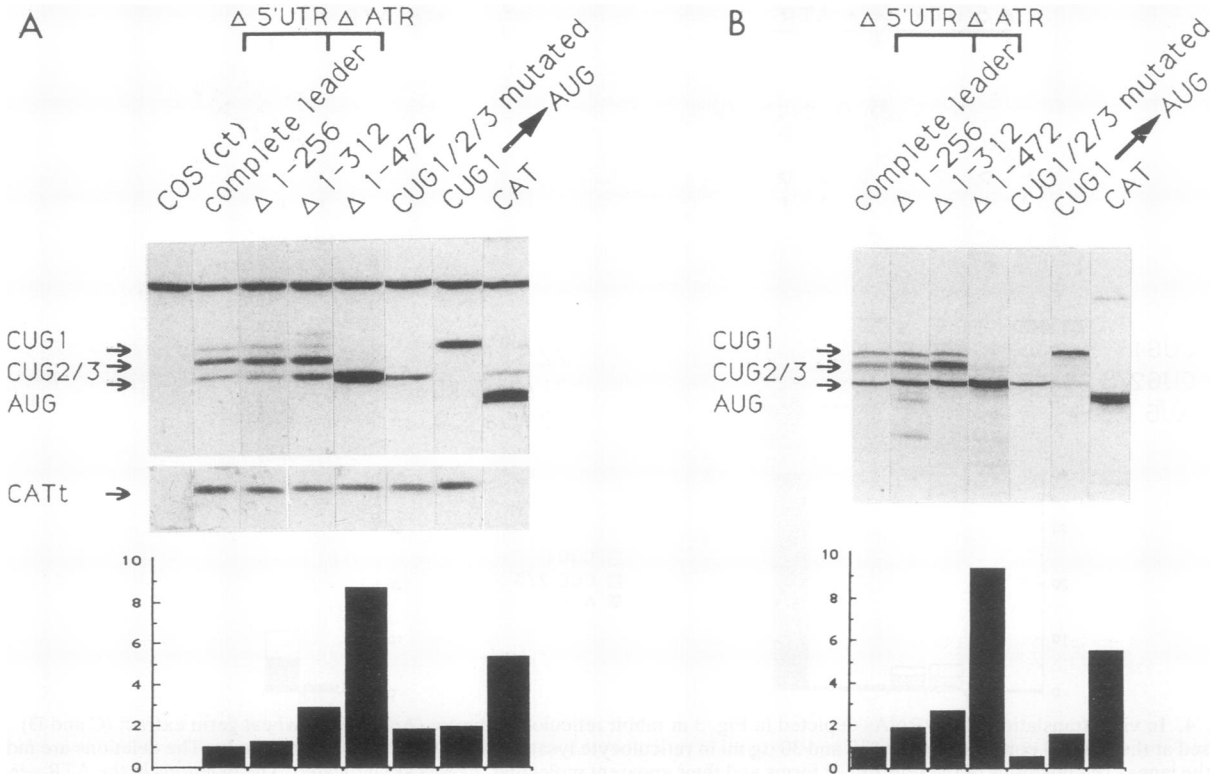

FIG. 6. Western blots and translation assays. Constructs Δ1-256, Δ1-312 (5′ UTR), and Δ1-472 (ATR), as well as mutations of the CUGs, were introduced into fusion plasmid FGF-CAT (see Fig. 1A and Materials and Methods). The plasmids were used either for transfection of COS cells or for RNA synthesis in vitro and translation in reticulocyte lysate. For a transfection internal standard, the different plasmids (except the CAT control) were cotransfected with pSCT-CATt DNA, encoding an inactive truncated CAT (CATt) product of 13 kDa (see Materials and Methods). (A) Western blots from transfected cell lysates with anti-CAT antibody (see Materials and Methods). ct, control. (B) Translation in reticulocyte lysate of the RNAs corresponding to the transfected plasmids. The constructs used for transfection and translation are indicated above the lanes. For each band, the corresponding initiation codon is indicated. Under each lane, the relative values corresponding to global CAT activity (A) (see Materials and Methods) or to global ^{35}S incorporation (B) are represented by histograms (normalized to the value for plasmid FGF-CAT with complete leader).

AUG but not of CUG-1, resulting in modulation of alternative translation. A strong derepression of AUG initiation was observed with deletion of the 5′ UTR plus ATR (Δ1-472), whereas FGF-CAT expression remained weak when the three CUGs were mutated. Only the mutation of CUG1 to AUG gave different translation patterns in the two systems; initiation at the internal AUG could be detected in the reticulocyte lysate but not in COS cells.

Slight differences were also observed between CAT fusions and bFGF RNAs in the reticulocyte lysate. In particular, a small inhibiting effect of the 5′ UTR was observed with FGF-CAT RNAs but not with bFGF RNAs (Fig. 4A and B and 6B), indicating a possible influence of the CTR on the translation level and suggesting that the global structure of mRNA could be involved in translation.

In conclusion, these experiments with FGF-CAT constructs showed, despite slightly different patterns of alternative translation between COS cells and reticulocyte lysate, that the *cis*-acting elements involved in translational regulation of bFGF mRNA had similar effects in the two systems.

DISCUSSION

This study was designed to identify *cis*-acting elements in bFGF mRNA that are able to modulate the global or alternative use of the four initiation codons implicated in translation of bFGF mRNA. We identified five RNA elements (Fig. 5), each having a specific effect on the modulation of translation initiation at one or all of the start codons. Moreover, three different translation systems were studied, allowing us to correlate translation regulation in vivo and in vitro but also clearly indicating that two of the *cis*-acting elements, located in the 5′ UTR, had specific effects, depending on the translation system used.

Our analysis of the translational regulation of bFGF expression has shown an important role of RNA *cis*-acting elements present in both the 5′ UTR and the ATR. In fact, the regulation of bFGF expression seems to result from the cooperation of at least five elements of the RNA leader. Such a conclusion is not really surprising, as it has been shown previously that the translation level of mRNAs with long G+C-rich leaders depends on the action of several elements within the 5′ UTR. However, in the cases of ornithine decarboxylase (14, 23) and TGF-β3 (2), the 5′ part of the 5′ UTR has strong inhibiting effects due mostly to a very stable secondary structure, whereas in the 3′ part of the 5′ UTR, the regulation seems to depend on small ORFs. The regulatory features of bFGF mRNA leader appear to be somewhat different; the regulatory elements have been identified in the 3′ part of the 5′ UTR, as reported for platelet-derived growth factor 2 (28), and in the ATR, surrounding the initiation codons. Furthermore, the lack of AUGs in these regions rules out the possible involvement of small (AUG-initiated) ORFs, in contrast with what has been reported in the case of FGF-5 (4).

In fact, the bFGF mRNA is very different from other mRNAs whose translational regulation has been studied; the essential difference is the existence of four start codons, including three CUGs, used for translation initiation. Our results show that each initiation site can be specifically regulated, making the regulation mechanisms still more complex. In contrast with previous reports indicating that translation regulation involves only UTRs, we show that not only the 5′ UTR but also the ATR, a translated region, modulates the alternative initiation of translation. Even the CTR, the coding sequence downstream of the AUG, might have some effect, as shown by the use of CAT fusions. As regards the ATR, an important question remains unanswered: are all initiation codons used on the same RNA molecule, or are there several populations of RNA, for instance, molecules initiating only at the CUGs and molecules initiating only at the AUG? In the latter case, the ATR would be an untranslated region for all molecules of the second category. The absence of competition between CUGs and AUG, suggested in a previous report (11) and shown in Fig. 2, would argue in favor of this hypothesis.

At first sight, the translational regulation described here could correspond to the predictions of the ribosome scanning model. The results can be interpreted in terms of very stable RNA structures, due to the G+C-rich composition of 5′ UTR and ATR, that would prevent unwinding by the initiation factors eIF-4F and eIF-4A. In particular, we show that RNA sequences surrounding each codon modulate the level of initiation at that codon; deletions of nucleotides upstream of the start codons increase translation initiation at the codon located downstream (Fig. 5, elements B and D), suggesting that these RNA elements hinder codon accessibility. In contrast, removal of nucleotides located downstream of CUG2/3 results in a specific inhibition of CUG2/3 initiation (Fig. 5, element C). This observation is in agreement with previous studies (19) showing that stable hairpins located downstream of a non-AUG codon increase translation initiation at this codon. Such RNA structures could generate a pausing of ribosomes at the level of the initiation codon and facilitate its recognition. The behavior of element A is more difficult to interpret; whereas the internal deletion of this element located upstream of CUG1 increases the level of CUG1 initiation (Fig. 4B, Δ264-312), removal of the entire 5′ UTR, including element A, decreases the level of CUG1 initiation. This observation suggests that RNA structures surrounding a start codon can act positively or negatively on its accessibility.

In contrast, several features of bFGF mRNA translation emerging from our study could not be predicted by the scanning mechanism. Particularly, the lack of competition between the AUG and the CUGs (Fig. 2) suggests that the mechanisms governing bFGF mRNA translation are probably more complex than predicted by the classical scanning model. Moreover, the absence of a significant inhibition by the 5′ UTR in reticulocyte lysate and COS cells is surprising (Fig. 4A and B and 6). Indeed, the 318-nt-long 5′ UTR is very G+C rich and therefore presumably highly structured. One can predict that such a leader must hinder the ribosome progression along the RNA molecule, and this is the case in the wheat germ extract (Fig. 4C and D). Our results suggest the existence in the two mammalian systems of a specific factor able to unwind the secondary structure of the 5′ UTR or of a different mechanism of translation initiation, for instance, an internal binding of ribosomes, which would render unnecessary the 5′ UTR unwinding.

Another question remains unanswered: does the 3′ UTR have a role in translational regulation of bFGF expression? The existence of several potential polyadenylation sites (27) giving rise to at least three bFGF mRNAs species, depending on the cell type (5, 35), is in favor of this hypothesis. Furthermore, other reports have shown that mRNA 3′ UTRs are involved in the modulation of translation initiation, either negatively in the case of human beta interferon mRNA (22) or positively in the case of ornithine decarboxylase mRNA (14). Here we show that the 3′ UTR of bFGF mRNA has little effect on translation initiation in reticulocyte lysate (Fig. 1B). However, this result does not allow us to rule out

an involvement of this 3′ UTR in translation initiation. Indeed, it has recently been shown in the case of tumor necrosis factor that the mRNA 3′ UTR has a suppressive effect that is abolished in the presence of the 5′ UTR and of a cell-specific *trans*-dominant factor (21). In that context, the role of the 3′ UTR of bFGF mRNA will be further investigated.

Our results clearly indicate that the mRNA *cis*-acting elements regulating bFGF translation have different effects, depending on the translation system studied. This difference has been shown especially for elements W (specific inhibition in wheat germ extract) and A (modulation of alternative translation only in reticulocyte lysate and COS cells). Such observations suggest that the *cis*-acting elements are regulated by *trans*-acting factors. This hypothesis has already been suggested for the translation of c-*myc* and TGF-β3 expression (2, 25). Regarding TGF-β3, it has been reported that the inhibiting effect of the 5′ UTR is inversely proportional to RNA concentration, arguing in favor of a control by limiting *trans*-acting factors. In the case of bFGF mRNA, *trans*-acting factors, by acting on the different *cis* elements, would be able to regulate not only the global translation but also the relative expression of the different forms of bFGF.

As the CUG- and AUG-initiated forms of bFGF have immortalizing and transforming effects, respectively (8), control of the alternative initiation of translation of bFGF mRNA will have an important impact on cell behavior. The *cis*-acting elements in mRNA could be the targets for specific *trans*-acting factors involved in cell growth and differentiation.

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