**Translation of CUG- but Not AUG-initiated Forms of Human Fibroblast Growth Factor 2 Is Activated in Transformed and Stressed Cells**

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**Abstract.** Four isoforms of the human fibroblast growth factor 2 (FGF-2), with different intracellular localizations and distinct effects on cell phenotype, result from alternative initiations of translation at three CUG and one AUG start codons. We showed here by Western immunoblotting and immunoprecipitation that the CUG-initiated forms of FGF-2 were synthesized in transformed cells, whereas "normal" cells almost exclusively produced the AUG-initiated form. CUG-initiated FGF-2 was induced in primary skin fibroblasts in response to heat shock and oxidative stress. In transformed cells and in stressed fibroblasts, CUG expression was dependent on cis-elements within the 5' region of FGF-2 mRNA and was not correlated to mRNA level, indicating a translational regulation. UV cross-linking experiments revealed that CUG expression was linked to the binding of several cellular proteins to FGF-2 mRNA 5' region. Since translation of FGF-2 mRNA was previously shown to occur by internal ribosome entry, a nonclassical mechanism already described for picornaviruses, the cross-linking patterns of FGF-2 and picornavirus mRNAs were compared. Comigration of several proteins, including a p60, was observed. However, this p60 was shown to be different from the p57/PTB internal entry factor, suggesting a specificity towards FGF-2 mRNA. We report here a process of translational activation of the FGF-2 CUG-initiated forms in direct relation with trans-acting factors specific to transformed and stressed cells. These data favor a critical role of CUG-initiated FGF-2 in cell transformation and in the stress response.

**Fibroblast growth factor 2 (FGF-2),** also known as basic fibroblast growth factor, belongs to a family of nine genes encoding cytokines involved in the control of cell proliferation and differentiation. As a potent mitogen for a variety of cells including endothelial cells and fibroblasts, it displays multiple biological roles. FGF-2 is involved in the development of the nervous system (Wagner, 1991), displays angiogenic activities implicated in wound-healing and tumor-neovascularization processes (Rifkin and Moscatelli, 1989; Kandel et al., 1991; Yanagisawa-Miwa et al., 1992), and also has a potential oncogenic effect (Coudere et al., 1991; Quarto et al., 1991). This factor is synthesized by a large number of cell types and exists as four isoforms resulting from alternative initiations of translation at three CUG and one AUG start codons of the FGF-2 mRNA (Florkiewicz and Sommer, 1989; Prats et al., 1989).

The process of alternative initiation of translation has a crucial role in the localization and function of FGF-2. The CUG-initiated forms of 21, 22, and 24 kD contain a nuclear localization sequence (Bugler et al., 1991) and are mostly recovered in the nucleus (Renko et al., 1990). In contrast, the AUG-initiated form of 18 kD is mostly cytosolic (Brigstock et al., 1991; Bugler et al., 1991). In addition to their distinct localizations, the FGF-2 isoforms show radically different features; constitutive expression of the AUG-initiated form leads to transformation of adult bovine aortic endothelial and NIH 3T3 cells, whereas expression of the CUG-initiated forms leads to immortalization of adult bovine aortic endothial cells and confers a unique phenotype to NIH 3T3 cells (Coudere et al., 1991; Quarto et al., 1991). In contrast to the CUG-initiated isoforms, the AUG-initiated FGF-2 is able to stimulate cell migration (Mignatti et al., 1991) and to down-regulate its own receptor (Bikfalvi et al., 1995). The mechanism of action of these isoforms is different. The AUG-initiated...
form, which is secreted despite the absence of a signal sequence and via a pathway independent of the endoplasmic reticulum–Golgi complex, has an autocrine or paracrine effect mediated by interactions with the plasma FGF-2 membrane receptors (Bikfalvi et al., 1995; Mignatti et al., 1992). The nuclear CUG-initiated forms are not released from the cell and have an intracellular effect independent of the cell surface receptors (Bikfalvi et al., 1995).

The multiple roles carried out by FGF-2 require a strong and subtle control of its synthesis. Expression of the FGF-2 gene is transcriptionally regulated by interleukin 1, tumor growth factor β, and tumor suppressor p53 (Gay and Winkles, 1991; Pertovaara et al., 1993; Ueba et al., 1994). Transcriptional regulation cannot account for differential expression of FGF-2 isoforms, however. Furthermore, the structure of the FGF-2 mRNA suggests the existence of posttranslational controls; several species of FGF-2 messenger RNAs have been described that differ in the length of their 3′ untranslated regions (Bensaïd, 1989; Weich, 1990). 90% of the longest and most abundant FGF-2 mRNA, which is 6774-nucleotide (nt)-long, corresponds to untranslated regions (Prats et al., 1989). The 318-nt-long 5′ untranslated region (UTR), the 165-nt-long alternatively translated region (ATR, between the initiation codons), and the 5823-nt-long 3′ UTR can be expected to contain regulatory elements. FGF-2 expression is translationally controlled by five cis-acting elements of the RNA leader sequence (Prats et al., 1992). One of these regulatory elements, located in the 5′ UTR between nt 192 and 256 from the RNA 5′ end, acts as a strong inhibitor of global FGF-2 mRNA translation in wheat germ extract, but not in rabbit reticulocyte lysate or in transfected COS-7 cells. Such observations suggest the involvement of regulatory trans-acting factors specific to different cell systems.

We have recently reported that the mechanism of translation initiation of the FGF-2 mRNA differs from the classical model (Vagner et al., 1995a). The cap-dependent scanning model predicts that the ribosome binds at the capped mRNA 5′ end and scans the RNA molecule until it recognizes a start codon (Kozak, 1978; Sonenberg, 1988). 90% of the longest and most abundant FGF-2 mRNA, which is 6774-nucleotide (nt)-long, corresponds to untranslated regions (Prats et al., 1989). The 318-nt-long 5′ untranslated region (UTR), the 165-nt-long alternatively translated region (ATR, between the initiation codons), and the 5823-nt-long 3′ UTR can be expected to contain regulatory elements. FGF-2 expression is translationally controlled by five cis-acting elements of the RNA leader sequence (Prats et al., 1992). One of these regulatory elements, located in the 5′ UTR between nt 192 and 256 from the RNA 5′ end, acts as a strong inhibitor of global FGF-2 mRNA translation in wheat germ extract, but not in rabbit reticulocyte lysate or in transfected COS-7 cells. Such observations suggest the involvement of regulatory trans-acting factors specific to different cell systems.

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These different observations suggest the existence of cellular trans-acting factors implicated in the translational control of FGF-2 expression. In this report, we showed that FGF-2 expression in different cell types displayed very distinct profiles of the four isoforms, supporting the hypothesis of a trans-regulation. Translation of CUG-, but not of AUG-initiated isoforms, was activated in transformed and stressed cells. This process, dependent on the FGF-2 mRNA 5′ region, was directly related to the RNA binding of cellular factors. Comparison with picornavirus IRES-binding proteins suggested that some of these factors could be internal entry factors. However, we provide evidence that this translational control does not involve the p57/pyrimidine tract–binding protein (PTB) known as a picornavirus internal entry factor (Hellen et al., 1993), suggesting the implication of trans-acting factors specific to FGF-2 mRNA.

Materials and Methods

Cell Types

Normal cells: Human skin fibroblasts were obtained from the Laboratory of Human Skin Cultivation (C.H.U. Rangueil, Toulouse, France and from the Laboratory of Pediatric Endocrinology, C.H.U. Purpan, Toulouse, France). They were between the first and fourth passage. Human retinal pigmentary epithelial cells (RPEH) were a gift of M. Guérin and J. Plouet (CNRS Toulouse, France). Human aortic endothelial cells, sampled during renal transplantation in a comatose patient, in accordance with the French legislation, were a gift of B. Malavaux (Department of Urology, C.H.U. Purpan, Toulouse, France). Adult bovine aortic endothelial cells were already described (Coudere et al., 1991).

Cell lines were obtained from American Type Culture Collection (Rockville, MD): SK-Hep-1 is a human liver adenocarcinoma (American Type Culture Collection: No. HTB 32), HeLa epithelial cells from a human uterine carcinoma (No. CCL2), A-431 human epithmoid carcinoma (No. CRL 1555), MIA PaCa-2 human pancreas carcinoma (No. CRL 1420), CAPAN-1 and BxPC-3 human pancreas adenocarcinoma (Nos. HTB 79 and CRL 1687), HT-29 colon adenocarcinoma (No. HTB 38), MCF-7 human breast adenocarcinoma (No. HTB 22), and COS-7 simian kidney cells transformed by SV-40 large T antigen (No. CRL 1654).

Plasmid Construction

The plasmid pPS used to synthesize the internal standard RNA was constructed as follows: a DNA fragment was synthesized using the template pFC1 linearized by SmaI site (GGG) and the 5′ primer 5′-AATAAGCTTGGCATTCAAAGGACCCAGGCTAAAGGAAGCTAAA-3′ and the 3′ primer 5′-GGGTACCTCTAGAGCACTGGTAAAGGGCGTAAATA-3′. The 5′ primer hybridizes to nt 1 to 15 preceding the CAT AUG start codon (bold) and has a tail corresponding to nt 554–573 of FGF-2 cDNA with a 5′ HindIII site. The 3′ primer hybridizes to nt 119–134 downstream of the CAT AUG (bold), and its tail is complementary to nt 931–951 of FGF-2 cDNA, with a 5′ half Smal site (GGG). The 200-nt-long PCR fragment was cloned into the HindIII-Smal sites of the vector pBluescript KS (Stratagene, La Jolla, CA)), and the 5′ primer 5′-AATAAGCTTGGCATTCAAAGGACCCAGGCTAAAGGAAGCTAAA-3′ and the 3′ primer 5′-GGGTACCTCTAGAGCACTGGTAAAGGGCGTAAATA-3′. The 5′ primer hybridizes to nt 1 to 15 preceding the CAT AUG (bold) and has a tail corresponding to nt 554–573 of FGF-2 cDNA with a 5′ HindIII site. The 3′ primer hybridizes to nt 119–134 downstream of the CAT AUG (bold), and its tail is complementary to nt 931–951 of FGF-2 cDNA, with a 5′ half Smal site (GGG). The 200-nt-long PCR fragment was cloned into the HindIII-Smal sites of the vector pBluescript KS, resulting in the plasmid pPS (for FGF standard).

In Vitro Transcription

The transcription templates corresponded to various linearized DNAs. pFS linearized by XbaI allowed synthesis of the internal standard RNA for reverse transcriptase–polymerase chain reaction (RT-PCR). pFC1 linearized by NarI (position 539) allowed synthesis of the 5′ region (UTR + ATR) of FGF-2 mRNA (Prats et al., 1992), pTM1 linearized by EcoRI allowed synthesis of the 5′ region (UTR + ATR) of FGF-2 mRNA (Prats et al., 1992), pTM1 linearized by EcoRI allowed synthesis of the 5′ region (UTR + ATR) of FGF-2 mRNA (Prats et al., 1992), pTM1 linearized by EcoRI allowed synthesis of the 5′ region (UTR + ATR) of FGF-2 mRNA (Prats et al., 1992), pTM1 linearized by EcoRI allowed synthesis of the 5′ region (UTR + ATR) of FGF-2 mRNA (Prats et al., 1992), pTM1 linearized by EcoRI allowed synthesis of the 5′ region (UTR + ATR) of FGF-2 mRNA (Prats et al., 1992).
lowed synthesis of the complete untranslated region of human rhinovirus (HRV-2; see Borman et al., 1993), and pSCT CAT linearized by BglII allowed synthesis of CAT RNA (Vagner et al., 1995a). These RNAs, labeled or unlabeled, were used either as probes or as competitors, respectively. RNAs were generated in vitro by T7 or T3 RNA polymerase and quantitated as previously (Vagner et al., 1995b). RNA labeling was performed in 50 μl with 100 μCi of [35S]CTP (without unlabeled CTP).

### Cell Transfection

The different cell types were transfected by using either DEAE dextran as described previously (Vagner et al., 1995a), Lipofectin according to manufacturer’s instructions (Life Technologies, Inc., Grand Island, NY), or electroporation. For the lipofectin method, 5 μg of plasmid pFC1 was incubated with lipofectin (50 μl) in a final volume of 0.5 ml of DME without serum for 15 min at room temperature (rt) and then added to serum-depleted medium in a 9-cm dish containing exponentially growing cells. Electroporation was performed with a gene pulsar (960 μF capacitance, 260V, BioRad Labs, Hercules, CA) using 2 × 10⁶ cells mixed in 0.4 ml with 20 μg of plasmid. With the three transfection methods, cell lysates were prepared 48 h later for Western immunoblotting analysis.

### Western Immunoblotting

The cell monolayers (1-5 × 10⁶ cells) were scraped in a subconfluent state. Total proteins were prepared, quantitated, and analyzed by Western immunoblotting (20 μg of proteins from each cell lysate) as previously described (Vagner et al., 1995a). CAT and FGF-2 proteins were immunodetected using rabbit polyclonal anti-CAT antibodies prepared in our laboratory (1/200,000 dilution) and anti–FGF-2 antibodies (Oncogene Science, Inc., Manhasset, NY; 1/2000 dilution), respectively.

### 35S Labeling and Immunoprecipitation

Cell cultures were incubated with 100 μCi/ml of [35S]methionine and [35S]cysteine (Trans3SS-label; ICN Biomedicals, Inc., Costa Mesa, CA) for 60 min. Cells were then harvested by scraping. Cell extracts were prepared by lysis in a PBS/NP-40 buffer containing PBS, pH 7.4, 50 mM NaF, 2 mM EDTA, 2 mM EGTA, 0.05% NP-40, 1 mM PMSF, 2 μg/ml aprotinin (Sigma Chemical Co.), followed by sonification for 20 s. Immunoprecipitation of the 35S-labeled proteins was performed with magnetic beads (DYNAL France s.a., Compiegne, France), according to manufacturer’s instructions. Briefly, 100 μg of total proteins (400 μl) was precleared for 45 min at rt with 50 μl of magnetic beads coupled with anti–mouse IgG (Dynabeads M-450; DYNAL, Inc.) and then incubated for 45 min at rt with 50 μl of Dynabeads M-450 coupled with anti–FGF-2 antibody (Ab-3; Oncogene Science, Inc.). The beads were washed six times in a Heps/NP-40 buffer (15 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40). The samples were then recovered in SDS–sample buffer, denatured at 95°C for 5 min, and analyzed by 12.5% PAGE after bead removal. The gel was fixed, dried, and autoradiographed.

The cross-linked proteins were immunoprecipitated with pansorbin: 10 μl of the cross-linked 3P-labeled sample (see below) was diluted to 150 μl in the PBS/NP-40 buffer described above and precleared by incubation with 50 μl of pansorbin for 10 min at rt. The supernatant was incubated for 30 min at rt with 5 μl of anti–PTB antibody (kindly provided by J.G. Patton, Vanderbilt University, Nashville, TN; see Patton et al., 1991) and then for 30 min at rt with 50 μl of pansorbin. After five washes in the Heps/NP-40 buffer, the samples were analyzed by 10% PAGE as above.

### Cell RNA Purification

The cell monolayers (5 × 10⁶ cells) were scraped in a subconfluent state. Total cellular RNA was prepared from the cell pellets by the Trizol method (Life Technologies, Inc.) as previously described (Vagner et al., 1995a). RNA was quantitated by measuring the absorbance at 260 nm and checked for integrity by electrophoresis on agarose gel and ethidium bromide staining.

### RNA Quantification by RT-PCR

The cDNAs were synthesized using the Superscript™ premplification system from Life Technologies, Inc., according to the manufacturer’s instructions. The reverse transcription reaction was carried out using 1 μg of total RNA and 50 ng of random hexamers in a final volume of 20 μl. For FGF-2 mRNA quantification, variable amounts of internal standard RNA synthesized from the FC plasmid were added to the reactions (see Fig. 3).

The PCR was performed with the 5′ primer 5′-GCCACCTTCAGAGAGCACGG-3' and the 3′ primer 5′-TCGCTCTTACAAGAGCATTTGG-3', hybridizing to regions 554-573 and 931-951 of FGF-2 cDNA, respectively. The 5′ primer degeneration corresponds to a divergence between the human and bovine sequences. The two primers hybridize in two different exons, ruling out contamination of the PCR reactions by the genomic sequence. The PCR reactions were carried out using 0.5 U of Goldstar Taq DNA polymerase (Eurogentec France s.a., Angers, France), in a final volume of 50 μl, using variable amounts of cDNA (1 μl or less). The reaction was done on a TrioThermoblock apparatus (Eurogentec) in the following conditions: 94°C for 3 min and then 30 cycles of 94°C for 30 s, 65°C for 1 min, 72°C for 1 min, and finally 72°C for 5 min. Amplification results (1/5 of the reactions) were analyzed on 6% polyacylamide gels (Tris Borate/EDTA), followed by ethidium bromide staining. The intensity of the ethidium bromide luminescence was measured by image acquisition on a UV max apparatus (Fisher Scientific OSL, Elancourt, France) followed by an image treatment with NIH Image software, in conditions where the intensity of fluorescence was linear (Nakayama et al., 1992).

### UV Cross-linking Assays

S10 cytoplasmic extracts from the various cell types were prepared as already described after a PBS wash (Vagner et al., 1995b). HeLa S100 extracts were prepared by centrifugation of the HeLa S10 at 100,000 g for 1 h. HeLa ribosome salt wash was prepared from the S100 as previously described (Meerovitch et al., 1989). 1 × 10⁶ cpm (UV cross-linking) of 3P-labeled RNA was incubated with 10 μg of S10 extract and UV irradiated as previously (Vagner et al., 1995b). For competition experiments, cold competitor RNAs or homoribopolymers (Boehringer Mannheim France s.a., Meylan, France) were preincubated with the S10 extract for 15 min at 30°C. The 3P-labeled RNA was then added and the mixture was further incubated at 30°C for 15 min to allow complex formation before UV irradiation. The samples were then treated with 2.5 U of RNase A and 10 U of RNase T1 (Sigma Chemical Co.) at 37°C for 30 min and, when indicated, with Proteinase K (Sigma Chemical Co.) at 37°C for 20 min at a final concentration of 1 mg/ml, before 10% PAGE analysis and autoradiography.

### Results

#### Specific Expression of FGF-2 Isoforms in Various Cell Types

Expression of the four FGF-2 isoforms in different human cell types was analyzed by Western immunoblotting. As shown in Fig. 1, A and B, three distinct patterns of expression were detected. In a first group of cells including normal cells like skin fibroblasts, RPEH grown in primary culture, or aortic endothelial cells picked up in situ, large amounts of the 18-kD AUG-initiated form were present, whereas the CUG-initiated forms were barely detectable (lanes 1–3). In a second group comprising transformed cell lines such as uterus carcinoma HeLa cells, liver adenocarcinoma SK-Hep-1, epidermoid carcinoma A-431, and pancreas carcinoma Mia PaCa-2, the four isoforms were visible, with a major band corresponding to the CUG2-initiated form (lanes 4–7). The third group, another set of transformed cell lines including breast adenocarcinoma MCF-7, colon adenocarcinoma HT-29, or pancreas adenocarcinoma CAPAN-1, showed only one band, migrating as the CUG3-initiated FGF-2 (lanes 8–10).

These data clearly indicated that the four FGF-2 isoforms were differentially expressed in different cell types. Interestingly, the CUG-initiated forms could only be detected in transformed cell lines, whereas three normal cell types expressed large amounts of 18-kD AUG-initiated FGF-2. To check these different expression patterns at the level
Absence of Correlation between FGF-2 mRNA Level and CUG-initiated Form Expression

FGF-2 expression in different cell types was analyzed at the RNA level using the method of RT-PCR. RNA was quantitated from purified total RNA by using an internal RNA standard synthesized in vitro (Fig. 3). This procedure included several steps shown in Fig. 3, A and B, for skin fibroblast RNA (Wang et al., 1989). First, an approximate quantitation was performed by adding growing amounts of competitor internal RNA standard (not shown). Then the RT was performed using an RNA standard amount corresponding to the approximate number of FGF-2 mRNA molecules determined in the first experiment, and PCR amplification was carried out with serial 1:3 dilutions of the RT product containing both the FGF-2 and internal standard cDNAs (Fig. 3 A). A straight line was obtained from these different points, giving a precise quantitation of the FGF-2 mRNA (Fig. 3 B). This experiment was performed for most cell types shown in Fig. 1, and also for monkey COS-7 cells, whose FGF-2 isoforms expression profile corresponded to the second group (Vagner et al., 1995a; Fig. 2). The final values are represented by histogram (Fig. 3 C).

These results showed that two normal cell types, RPEH and skin fibroblasts, contained the highest levels of FGF-2 mRNA, with respective values of $9.1 \times 10^4$ and $4.8 \times 10^4$ molecules/ng of total RNA. SK-Hep-1 was the only transformed cell line presenting a high level of this mRNA (3.8 $\times 10^4$ molecules/ng), whereas the other transformed cell lines expressed it very poorly ($1.3 \times 10^4$-3.0 $\times 10^2$ molecules/ng). This study showed that the AUG-initiated FGF-2 expression was approximately related to the mRNA levels in the different cell types. However, we found no correlation between FGF-2 mRNA levels and expression of the CUG-initiated FGF-2 isoforms. These data favor a mechanism of translational activation or derepression of CUGs expression.

The 5' Region of FGF-2 mRNA Is Sufficient for the Cell-specific Control of Alternative Translation Initiation

To see whether the different patterns of FGF-2 expression shown in Fig. 2 resulted from a translational control, we transfected skin fibroblasts, HeLa, and SK-Hep-1 cells with a chimeric DNA in which the 5' region of FGF-2 mRNA sequence has been fused to the CAT-coding sequence and which encodes four FGF-CAT isoforms from the four FGF-2 initiation codons (Prats et al., 1992, Fig. 4 A). The proteins expressed by the three cell types were detected by Western immunoblotting using an anti-CAT serum (Fig. 4 B).
Figure 3. Quantitative RT-PCR analysis of FGF-2 mRNA in different cell types. (A) Ethidium bromide staining of RT-PCR products from human skin fibroblast total RNA. Reverse transcription and polymerase chain reaction were performed in the conditions described in Materials and Methods. Before carrying out the quantitative RT-PCR (Wang et al., 1989), we determined the amount of internal standard RNA to add in the reaction that must be close to the amount of FGF-2 mRNA. An approximate quantification was done by competitive RT-PCR using 1 µg of total cell RNA and 1:5 serial dilutions of the internal standard RNA (6.2 x 10^9-6.2 x 10^4 molecules, not shown), giving the approximate value of 3.1 x 10^7 molecules (equal intensity of the two bands). Then, for the quantitative RT-PCR experiment, reverse transcription was done in 20 µl with 1 µg of total RNA and 3.1 × 10^7 molecules of internal standard RNA. 1:3 serial dilutions of the obtained cDNA (starting from 3 µl of the RT reaction, lane 4) were amplified by PCR and analyzed on 6% TBE polyacrylamide gels (lanes 4-10). Lanes 1 and 2 correspond to reverse transcription with 1 µg of fibroblast RNA alone, or with 3.1 × 10^7 molecules of internal standard alone, respectively, followed by PCR with 1 µl/20 of each cDNA sample. Lane 3 corresponds to the PCR negative control without cDNA, lane M to the DNA ladder (Life Technologies, Inc.). The bands corresponding to endogenous FGF-2 and to internal standard are indicated by arrows. (B) The fluorescence of the bands shown in A was scanned (see Materials and Methods). The variable template concentrations of internal standard FC RNA and skin fibroblast total RNA were plotted against the fluorescence intensity of the PCR products. The dotted line with an arrow at the end indicates the number of molecules contained in 1 ng of total RNA. (C) The procedures described in A and B were used to quantitate the FGF-2 mRNA in different human cell types, as well as in simian COS-7 cells. For each cell type, 1 µg of total RNA was used, in presence of internal standard amounts determined by competitive RT-PCR (see A). The numbers of internal standard molecules added were: 12.4 × 10^7 for RPEH, 3.1 × 10^7 for skin fibroblasts, 6.2 × 10^7 for HeLa, 6.2 × 10^7 for SK-Hep, 6.2 × 10^6 for MIA PaCa-2, 6.2 × 10^6 for MCF-7, 6.2 × 10^5 for CAPAN-1, and 5.0 × 10^6 for COS-7. The quantitative RT-PCR was reproduced four to seven times for each cell type. The average values obtained from fluorescence intensity measurements detailed in B are represented by histograms.

The same expression profile was observed for the FGF-CAT proteins as for the endogenous FGF-2. We could only detect AUG-initiated FGF-CAT in transfected skin fibroblasts (Fig. 4 B, lane 2), whereas all four isoforms were synthesized in transfected HeLa or SK-Hepl cells (lanes 4 and 6). These data clearly indicate that the 5′ region of FGF-2 mRNA contains the cis-acting elements necessary for the cell-type specific control of alternative translation initiation.

CUG-initiated FGF-2 Is Translationally Induced by Stress in Human Skin Fibroblasts

We have previously shown that the 5′ untranslated region of FGF-2 mRNA contains an IRES localized upstream from the CUG codons (Vagner et al., 1995a). The results obtained in Fig. 4 suggested that the selection of the initiation codons could involve this IRES and thus occur by internal ribosome entry. To address this question, we subjected human skin fibroblasts to a heat shock treatment previously shown to abolish the cap-dependent translation (Panniers et al., 1985).

Skin fibroblasts were heat shock treated at 45°C for increasing periods of 15-60 min. The endogenous FGF-2 present in the cells was then analyzed by Western immunoblotting (Fig. 5 A), and the level of FGF-2 mRNA checked by quantitative RT-PCR (Fig. 5 B). The results clearly showed that heat shock induced the synthesis of the CUG-initiated forms. This effect was time dependent, being detected after 15 min and more pronounced after 60
Figure 4. Transfection of different cell types by an FGF-CAT chimeric construct. (A) Schema of the FGF-CAT chimeric RNA expressed by the plasmid pFC1 used for transfection. This construct contains the entire 5' leader of FGF-2 mRNA and is able to express the four FGF-CAT isoforms from the four FGF-2 start codons, under the control of the complete FGF-2 mRNA 5' UTR (Prats et al., 1992; Vagner et al., 1995a). (B) Human skin fibroblasts, HeLa, and SK-Hep-1 cells were transiently transfected by the chimeric plasmid pFC1 using lipofectin. 48 h after transfection, the cell proteins were analyzed by PAGE and Western immunoblotting using anti-CAT antibody. The migration of the different FGF-CAT isoforms is indicated (CUG1, CUG2/3, and AUG), as well as the size standards. The name of the cell type is indicated on the top of each block, mock, the negative control transfected without DNA. FCI, the transfection with 5 μg of pFC1 DNA.

min of stress (Fig. 5 A, lanes 2–5), whereas the FGF-2 mRNA level did not change (Fig. 5 B, histograms 2–5).

To find out whether this process was dependent on the 5' region of the FGF-2 mRNA, skin fibroblasts were transfected by the construct used above (Fig. 4 A) and able to express the four FGF-CAT fusion proteins (Fig. 6). The transfected fibroblasts were heat shocked and expression of the FGF-CAT proteins then analyzed by Western immunoblotting with anti-CAT serum: the CUG-initiated fusion FGF-CAT proteins (Fig. 6 A, lanes 2–4) were induced simultaneously with endogenous CUG-initiated FGF-2, although with a lower efficiency (Fig. 6 B, lanes 2–4). This indicates that the 5' of FGF-2 mRNA comprises the cis-acting elements necessary and sufficient for heat shock response.

Figure 5. Analysis of FGF-2 expression in heat shock–treated skin fibroblasts. (A) Subconfluent human skin fibroblasts were heat shocked by rapid and complete immersion of the flasks in a 45°C bath for variable times. Cells were harvested directly after the heat treatment and FGF-2 expression analyzed as in Fig. 1, by Western immunoblotting with anti-FGF-2 antibody. The heat shock durations (0–60 min) are indicated at the top of the lanes. SK-Hep-1 cell extract was used as a control of FGF-2 migration. The expected positions of FGF-2 isoforms are indicated (CUG1, CUG2/3, and AUG), as well as the size standards. (B) FGF-2 mRNA present in each cell extract used in A was quantitated by RT-PCR as in Fig. 3. The heat shock duration is indicated on the top of each block.

The question of CUG-initiated forms induction as a general response to other stress conditions was addressed by subjecting skin fibroblasts to oxidative stress using xanthine oxidase. The analysis of FGF-2 expression by Western immunoblotting showed that the CUG-initiated forms were induced in response to oxidative stress (Fig. 7, lane 3). Taken together, these data point out a process of translational regulation modulating the expression of the FGF-2 CUG-initiated forms in normal human cells as a response to stress. The appearance of such a process in conditions of inhibited cap-dependent translation and the involvement
Figure 6. Transfection of heat-shocked skin fibroblasts by an FGF-CAT chimeric construct. Skin fibroblasts were transfected by electroporation (see Materials and Methods) with the plasmid pFC1 used in Fig. 4, expressing a chimeric FGF-CAT RNA (see the schema). After 48 h, the cells were subjected to heat shock treatment (0, 15, 30, or 45 min; lanes 1 to 4) and harvested as in Fig. 5. Western immunoblotting was performed either with anti-CAT antibody or with anti-FGF antibody to detect FGF-CAT (A) or endogenous FGF-2 (B) isoform expression, respectively. Size standards are indicated.

of cis-acting elements located in the 5' part of FGF-2 mRNA (containing the IRES) favor a process of internal ribosome entry (Panniers et al., 1985; Vagner et al., 1995a).

Specific Binding of HeLa Cell Proteins to FGF-2 mRNA 5' Region

The involvement of the 5' region of FGF-2 mRNA in the translational control of its expression prompted us to look for cell proteins that could interact with this 5' region. The proteins interacting with the FGF-2 mRNA 5' region (UTR + ATR) were characterized by UV cross-linking experiments. A 32P-labeled RNA probe corresponding to this 5' region (nt 1–539) was UV irradiated in the presence of HeLa S10 extracts. Then the assay was RNase treated before analysis on PAGE. Several cross-linked bands were detected, the most abundant one migrating around 110 kDa (Fig. 8, lane 3). Proteinase K treatment confirmed that these bands corresponded to proteins (lane 11). The specificity of RNA–protein interactions was tested by addition of an excess of different competitor RNAs; the cross-linked proteins were displaced from the labeled probe by addition of a 100-fold excess of homologous FGF-2 RNA (lane 6), but not by heterologous CAT RNA added in the same proportions (lane 10), confirming the specificity of the interactions between the FGF-2 mRNA and the HeLa proteins. Interestingly, the EMCV IRES RNA was able to displace several proteins, except for p110 (lane 8), suggesting that FGF-2 and EMCV RNAs could share the binding of common factors.

The Binding of Cell Proteins to FGF-2 mRNA Is Related to CUG-initiated FGF-2 Expression

To find out whether the cross-linked proteins had something to do with translation regulation, we prepared a HeLa ribosome salt wash retaining the ribosome-associated proteins expected to be involved in translation (see Materials and Methods and Meerovitch et al., 1989). The cross-linking reaction performed with the ribosome salt wash showed the proteins already detected with the S10 extract (Fig. 9, lanes 2–5), thus favoring a role of these proteins in translation regulation.

The question of the cell specificity of the interacting proteins and of a possible relation between the profiles of protein binding and FGF-2 expression (Fig. 1) was studied by cross-linking experiments using cell-free extracts from various cell types. Different profiles of cross-linked proteins were obtained according to the cell types (Fig. 9, lanes 6–16). Interestingly, most of the factors visible in HeLa (lane 3) could be detected in the transformed cell
was also visible in HeLa, but in low amounts (Figs. 9 and 10 A). The binding of these proteins and the expression of the FGF-2 CUG-initiated form is regulated at the translational level by a cell state-specific process; indeed, the expression of the CUG-initiated forms of FGF-2 occurs in human transformed cell lines of several origins, almost exclusively expressed by CUG-initiated forms in skin fibroblasts. We show that the positive regulation of CUG initiation depends on cis-

The p60 bound to FGF-2 mRNA is different from the PTB internal entry factor

The apparent molecular weight of the p60 observed in transformed cell lines and in the stressed skin fibroblasts suggested that it could correspond to the PTB shown to be a splicing factor and an internal entry factor involved in picornavirus IRES function (Patton et al., 1991; Hellen et al., 1993).

The proteins bound to FGF-2 mRNA 5' (comprising the FGF IRES) were compared to those bound to the IRES's of two picornaviruses, EMCV and human rhinovirus (HRV) by carrying out cross-linking experiments using FGF, EMCV, and HRV 32p-labeled probes incubated with HeLa S10 extracts (lanes 4 and 6), as previously described (Jang and Wimmer, 1990; Borman et al., 1993). The FGF-2 p60 comigrated with the upper band of the PTB doublet (lane 2).

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Figure 8. UV cross-linking of HeLa cellular factors with FGF-2 RNA leader. HeLa cells S10 extract was incubated with RNA probe (106 cpm) corresponding to the FGF-2 leader RNA (nt 1-529) containing the 5' UTR and the ATR. Competition experiments were carried out by addition of unlabeled RNA in 20-100-fold molar excess. The competitors were FGF 5' (nt 1-539), EMCV (nt 261-837), or CAT (coding sequence of 0.7 kb). UV irradiation was performed as described in Materials and Methods with an energy of 400,000 μJ/cm² at 254 nm. Samples were treated with RNases A and T1 before analysis by SDS-PAGE. Addition of HeLa extract, competitors, protease K, and UV irradiation is indicated at the top of the lanes. Migration of the size standards is shown. Several cross-linked products are indicated by arrows.

Discussion

The results reported here demonstrate that FGF-2 expression is regulated at the translational level by a cell state-specific process; indeed, the expression of the CUG-initiated forms of FGF-2 occurs in human transformed cell lines but not in primary cell types. Furthermore, this expression is completely independent of the FGF-2 mRNA levels in the cells. The three studied "normal" cell types, of endothelial, epithelial, and fibroblastic origin, almost exclusively express the AUG-initiated 18-kD FGF-2. Stress conditions, however, are able to induce the synthesis of the CUG-initiated forms in skin fibroblasts. We show that the positive regulation of CUG initiation depends on cis-

Cross-linking experiments were also performed with extracts from fibroblasts treated by oxidative stress (Fig. 10 B). The same change in the cross-linked proteins profile as after heat shock was observed, with a stress-induced binding of several proteins mostly including a p60. These data strongly suggest that one or several of these bound proteins, including the p60, could be involved in translational activation of FGF-2 CUG-initiated forms expression.

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elements present in the 5' leader of the FGF-2 mRNA and is concomitant with the binding of several cell proteins to this leader region. This suggests that the CUG-initiated forms are translationally induced by trans-acting factors that would be active in transformed cells and activated as a response to stress in normal cells. The rapidity of the stress response favors a posttranscriptional or -translational activation of the trans-acting factors.

Although a large variety of cells have been described as producing FGF-2, little data about the regulation of FGF-2 isoforms expression from one cell type to another has been reported. The CUG-initiated forms are slightly induced after cAMP or protein kinase C signal pathways stimulation, suggesting a possible regulation of the relative synthesis of the four isoforms (Stachowiak et al., 1994). More recently, a study using FGF-2 transgenic mice has suggested that the expression of FGF-2 isoforms is translationally regulated in a tissue-specific manner (Coffin et al., 1995). Our data show that the balance between FGF-2 isoforms not only varies with the cell type but also with stress conditions in one same cell type, demonstrating the existence of this translational regulation, in relation to cell transformation or stress. There is an apparent contradiction between our study, describing that normal cell types selectively express the AUG-initiated form, and the report of Coffin et al. showing that some normal cells (contained in tissue extracts) express the CUG-initiated forms. However these data are not incompatible; indeed, we have observed that skin fibroblasts are able to express the CUG-initiated forms in certain cell physiological conditions. The most important difference between normal and transformed cells, regarding the CUG-initiated forms of FGF-2, would be that the expression of these isoforms is regulated in normal cells, whereas it is constitutive in transformed cells (Galy, B., unpublished results).

Most previously described examples of translational control correspond to negative regulations (Standart and Jackson, 1994). In contrast, CUG-initiated FGF-2 expression is induced by stress conditions (Figs. 5, 6, and 7) and is related to the binding of trans-acting factors to FGF-2 mRNA (Figs. 9 and 10), demonstrating a mechanism of positive regulation. Several mechanisms could account for CUG activation. Firstly, the presence of a hairpin downstream of non-AUG codons could increase translation by generating a ribosome pausing (Kozak, 1990). We have identified such an element downstream of CUG3 (position 361), between nt 385 and 411 (Prats et al., 1992). Trans-acting factors targeting this element could mediate CUG activation by stabilizing the RNA structure. A second possible mechanism would be dependent on the cap-binding protein, eIF-4E; previous reports show that overexpression of this factor not only affects cell growth and induces cell transformation (DeBenedetti and Rhoads, 1990; Lazaris-Karatzas et al., 1990) but is responsible for translational activation of messengers possessing structured leader sequences, such as ornithine decarboxylase mRNA (Rousseau et al., 1996). It has also been shown in a recent report that rat FGF-2 is translationally induced in

![Figure 9. UV cross-linking of FGF-2 RNA leader with extracts from different cell types. S10 extracts from different cell types were prepared as in Fig. 8. Hela S10 and ribosome salt wash were also prepared as described in Materials and Methods. Rabbit reticulocyte lysate and wheat germ extract were provided by Promega Corp. (Madison, WI). All these extracts were used as in Fig. 8 in a cross-linking experiment with 10^5 cpm of RNA probe corresponding to the FGF-2 RNA 5' region (nt 1-529). The origin of the cell extract is indicated on the top of the lanes. Migration of the size standards is shown. Cross-linked products are indicated by arrows.](image-url)
CHO cells overexpressing eIF-4E (Kevil et al., 1995). A third possible regulatory mechanism would involve an internal ribosome entry, a translation-activating process that requires a cis-element, the IRES (Jackson, 1991), and trans-acting factors constituting the so-called IRESome (Witherell and Wimmer, 1994). Indeed, the FGF-2 mRNA contains an IRES, located upstream of the CUGs, which could be responsible for their activation (Vagner et al., 1995a). The internal entry hypothesis is supported by an important phenomenon, at least regarding the stress-induced CUG activation: heat shock has been shown to inhibit the cap-dependent translation by inactivation of eIF-4E (Panniers et al., 1985). We did in fact observe a strong shut off of cell global translation in our heat shock experiments (not shown). Consequently, it seems probable that the translational induction of FGF-2 CUG-initiated forms occurs by a cap-independent mechanism. This would be the first case of translational activation of a cellular mRNA using an internal entry process.

An interesting question to address is the importance of the CUGs as initiating codons in the FGF-2 regulation process described here. Indeed it has been shown in the case of hepatitis C virus IRES that translation initiation efficiency is remarkably unperturbed when the AUG codon is substituted by non-AUG codons (Reynolds et al., 1995). This suggests that translation by ribosome internal entry is less stringent towards the start codon than the classical scanning mechanism and that initiation by internal entry could specifically regulate the use of CUGs or other non-AUG codons. This point will be investigated by using mutants of the FGF-2 5' UTR without the IRES or having AUG for CUG substitution.

The present report shows that the binding of several proteins to the mRNA 5' is related to CUG-initiated forms expression. This suggests that one or several of these factors are involved in FGF-2 mRNA translational activation. The presence of an IRES in the FGF-2 mRNA, located upstream of the CUG codons, also suggests that these factors may be internal entry factors (Vagner et al., 1995a). Several observations favor this hypothesis. (a) The CUG-initiated forms and factor binding are induced by heat shock (see above); (b) the binding of several proteins is displaced by EMCV IRES (Fig. 8); and (c) the only translational specific trans-activators described up to now says (see Materials and Methods). RNA probe, UV irradiation (left), homoribopolymer nature and amount are indicated on the top of each lane (right). Size standards are indicated (in the middle), as well as the size of FGF RNA cross-linked proteins on the left). The arrow indicates the migration of the PTB (left) and of the FGF specific p60 (right).
are the internal entry factors constituting the picornavirus IRESomes (Standart and Jackson, 1994). Indeed, based on their apparent molecular weights, we can notice that some proteins among the FGF-2 mRNA cross-linked factors might correspond to viral IRES binding proteins. For instance, the p110 and the p75 described here (Fig. 11) can correspond to the p100 and p70 bound to HRV and EMCV IRES’s (Borman et al., 1993; Witherell and Wimmer, 1994); the p50 bound to FGF mRNA may be related to the p52/La protein involved in poliovirus IRES function (Meerovitch et al., 1989; Svitkin et al., 1994). Finally, the protein migrating around 40 kD in our experiments might be similar to the p43-44 cross-linked to picornavirus and murine leukemia virus IRES’s (Fig. 11; Hellen et al., 1994; Vagner et al., 1995b). These factors can also be specific to FGF-2 mRNA, as illustrated by the p60 shown here, which is clearly different from the p57/PTB splicing factor involved in viral IRES functions (Borovjagin et al., 1994; Toyoda et al., 1994). A recent report showing that PTB, necessary for internal entry only in the case of EMCV IRES, is not a universal internal entry factor (Kaminski et al., 1995) let us hypothesize that the IRESome composition could be specific to each IRES and involve common factors together with specific factors. In particular, cellular mRNA IRESomes could involve proteins different from viral mRNAs IRESomes, allowing specific regulations such as that of FGF-2 expression.

We would like to point out that the cross-linked factors we have characterized are not necessarily implicated in internal ribosome entry; further investigation is required to identify them and demonstrate their translational regulatory function. As far as the p60 protein is concerned, it has been suggested in the literature that c-src (pp60) may regulate the trafficking or translation of RNAs in a cell cycle-dependent manner (Taylor and Shalloway, 1994). However, the proteins of the src family have never been described as RNA-binding proteins, and furthermore, the src proteins are anchored in the cell membrane by their acylated end. As the p60 is clearly an RNA-binding protein and the cell extracts used in the cross-linking experiments are cytoplasmic, we can rule out the possibility that the p60 interacting with FGF-2 mRNA is c-src or an src family member. On the other hand, an src protein could indirectly regulate RNA translation through its interaction with other proteins involved in its intracellular transduction cascade. Indeed two src-associated proteins, p68 and p62, have been described as RNA-binding proteins (Wong et al., 1992; Fumagalli et al., 1994; Taylor and Shalloway, 1994). These proteins could correspond to the p70 and p60 shown here. Interestingly, the ability of p62 to bind RNA is altered by tyrosine phosphorylation (Wang et al., 1995), and this is compatible with the rapid induction of CUG-initiated expression in heat shock conditions.

The stress induction reported here suggests a novel role for the CUG-initiated forms of FGF-2; their rapid synthesis in response to stress suggests that they could behave as survival factors. The stress induction could be representative of physiological situations such as tissue lesion requiring FGF-2 for wound healing. The expression of CUG-initiated isoforms in transformed cells indicates that they might be related to a critical state of the cell.

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