**A Single Internal Ribosome Entry Site Containing a G Quartet RNA Structure Drives Fibroblast Growth Factor 2 Gene Expression at Four Alternative Translation Initiation Codons**

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The 484-nucleotide (nt) alternatively translated region (ATR) of the human fibroblast growth factor 2 (FGF-2) mRNA contains four CUG and one AUG translation initiation codons. Although the 5′-end proximal CUG codon is initiated by a cap-dependent translation process, the other four initiation codons are initiated by a mechanism of internal entry of ribosomes. We undertook here a detailed analysis of the cis-acting elements defining the FGF-2 internal ribosome entry site (IRES). A thorough deletion analysis study within the 5′-ATR led us to define a 176-nt region as being necessary and sufficient for IRES function at four codons present in a downstream 308-nt RNA segment. Unexpectedly, a single IRES module is therefore responsible for translation initiation at four distantly localized codons. The determination of the FGF-2 5′-ATR RNA secondary structure by enzymatic and chemical probing experiments showed that the FGF-2 IRES contained two stem-loop regions and a G quartet motif that constitute novel structural determinants of IRES function.

The human FGF-2 gene belongs to a gene family of 23 fibroblast growth factors and is involved in various fundamental cellular processes, such as cell proliferation, differentiation, and angiogenesis (1). The contribution of translational control in FGF-2 gene expression has been particularly well documented. First, a process of alternative initiation of translation occurs on the unique FGF-2 mRNA and leads to the production of five FGF-2 protein isoforms with extended NH2-terminal ends (2–4). Second, translation of four of these isoforms is initiated at non-canonical CUG codons (2–3). Third, the use of alternative initiation codons is controlled in transformed and stressed cells (5). Fourth, protein synthesis on the FGF-2 mRNA can occur by internal ribosome entry (6).

Whereas the usual mechanism of translation in eukaryotes involves the recruitment of the 40 S ribosomal subunit to the 5′-cap structure of the mRNA, a restricted but growing number of viral and cellular mRNA initiate their translation through the recruitment of the 40 S ribosomal subunit to internal sequences of the mRNA called IRES (7–9). As regards the FGF-2 mRNA, this process is active *in vivo* in transgenic mice (10). Interestingly, the FGF-2 IRES is able to contribute to the choice of initiation codons because translation at the cap-proximal CUG codon occurs by a cap-dependent process, whereas translation at the other four codons occurs by an IRES-based mechanism (4). To understand translation initiation codon selection in the FGF-2 mRNA, it is therefore important to define the cis-elements required for FGF-2 IRES function.

The RNA sequences and structural features of cellular IRESs remain largely unknown. Various attempts to define the cis-acting elements required for the function of cellular IRES have failed to find a common RNA sequence. Cellular IRES seem to be very diverse in nature, without stringent sequence similarity. Because IRES elements in viral RNA genomes contain higher order structures whose integrities are essential for IRES activity, it was proposed that eukaryotic IRESs could also share some common RNA structural determinants. A Y-shaped double hairpin RNA secondary structure was found in various cellular IRESs (11), although its functional role has never been clearly demonstrated. The only experimentally determined RNA secondary structure model of a cellular IRES revealed that the c-myc IRES contains a number of structural motifs, including a pseudoknot helix, but not the predicted Y-shaped structure (12).

In this study, our goal was to determine the secondary structure of the FGF-2 5′-ATR with chemical and enzymatic probing experiments and to precisely define the RNA sequences and structural determinants required for internal entry of ribosomes at each of the four IRES-dependent translation initiation codons. Our initial expectation, because of the reported modular nature of IRES, was that each of the initiation codons would be controlled by a distinct IRES element. A mutation and deletion study led us to show that a single IRES was able to control translation initiations at four downstream initiation codons in the FGF-2 mRNA. The FGF-2 IRES is 176 nucleotides long, is highly structured, and contains two RNA stem-loops and a G quartet motif, each of which contributes to IRES activity.

**EXPERIMENTAL PROCEDURES**

*Plasmid Construction*—Detailed information about DNA cloning procedures can be obtained from the authors upon request.

*DNA Transfection and Luciferase Activities*—The SK-Hep 1 human
hepatoma cell line was transfected with 1 μg of plasmid DNA per 60-mm tissue culture dish with the Fugene-6 transfection reagent (Roche Applied Science). Cell extracts were prepared 24 to 36 h after transfection and analyzed for luciferase activities by using the Dual-Luciferase reporter assay system (Promega) as described in Ref. 13.

Structural-probing Reactions on in Vitro-synthesized RNA—In vitro-transcribed RNA (4 pmol) was denatured for 1 min at 95 °C and chilled on ice for 5 min. Incubation was continued for 10 min at 25 °C after addition of the various reaction buffers (10 mM HEPES-NaOH, pH 7.5, 50 mM KCl, 2.5 mM MgCl₂ for RNases T1 (Invitrogen) and V1 (Kemotex); 50 mM sodium borate, pH 8.0, 50 mM KCl, 2.5 mM MgCl₂ for CMCT; 25 mM cacodylate-NaOH, pH 7.5, 50 mM KCl, 2.5 mM MgCl₂ for DMS and kethoxal). Chemical modifications with DMS, CMCT, and kethoxal were done during 5, 20, and 15 min, respectively, in a 25-μl final volume as described previously (14). Enzymatic digestions were done with 0.05 units of RNase T1 or 0.0002 units of RNase V1 for 10 min at 25 °C.

For primer extensions, the five different primers (RT75, 5'-CCCAC-CAGCCTCCCGCGC-3'; RT205, 5'-CGCCGCTGGGGAGAGCC-3'; RT285, 5'-CGCTCTTCTGTCCGCCGGC-3'; RT365, 5'-CCCGGCCCCGGCCC-3'; and RT445, 5'-GGATCCCCGGACGCGTGGA-3') were labeled with [γ-32P]ATP and combined with 2 pmol of synthetic RNA. Hybridization and reverse transcription with the murine leukemia virus reverse transcriptase were carried out as described previously (14). The samples were analyzed on 8% polyacrylamide sequencing gels containing 8 M urea.

RESULTS

Secondary Structure of the FGF-2 5'-ATR—The secondary structure of the FGF-2 5'-ATR RNA was determined by analyzing its enzymatic cleavage and chemical modification pattern obtained in native conditions in solution. The probing experiments were performed using an unlabeled in vitro-transcribed RNA corresponding to the first 539 nucleotides of the human FGF-2 mRNA. We subjected this RNA to partial RNase digestions with RNases T1 or V1. RNase T1 is known to cut in the 3'-direction from G residues present in single-stranded regions, whereas RNase V1 cuts indicate doubled-stranded or stacked bases. We also used limited chemical modifications with three chemical probing agents, DMS, CMCT, and kethoxal, as described under "Experimental Procedures." The sites of cleavage or modification were then identified by primer extension with reverse transcriptase, using various radiolabeled oligonucleotides complementary to five FGF-2 ATR sequences. Analysis of the resulting cDNAs was performed on sequencing polyacrylamide gels that were run together with the corresponding RNA sequencing ladder to allow identification of the modified residues. A typical example of the results is shown in Fig. 1.

A secondary structural model, presented in Fig. 2, was further derived by combining experimental data and free energy data calculated using the mFOLD program (15). The overall secondary structure of FGF-2 5'-ATR appears as a succession of six independent stem-loop structures (labeled I to VI) (Fig. 2). The stem-loops are separated by short regions, most of...
which are single stranded (I–IV), or are directly adjacent (IV–VI). Because of the length and high GC content of their stems, these stem-loops are probably very stable, which is also indicated by the absence of reactivity toward the single strand-specific probes and the numerous V1 cleavages within the stems. Some irregularities are present in the stems because of some bulged residues and internal loops. Some of these internal loops do possess some degree of order, as indicated by the presence of RNase V1 cuts concomitant with single strand-specific probes. This reflects the possible dynamic equilibrium between open and closed conformation in these regions (e.g. regions 194/216 and 305/348).

Within the FGF-2 5'-ATR reactivity pattern, one region (involving nt 57–108) was particularly remarkable. Thus, this region was poorly reactive to probing and prediction structure programs failed to predict any structure involving it. The presence of strong pauses of reverse transcriptase progression immediately before this purine-rich region led us to test for the presence of an intramolecular purine-quartet. In a G quartet, the guanines are hydrogen-bonded via Hoogsteen base pairs in a square-planar symmetric array, and the quadruplex is stabilized by coordination of a monovalent K⁺ ion within the planes of the guanine tetrads (16). The formation and stability of G quartets is cation-dependent, with a preference for K⁺ over Na⁺ or Li⁺ (17). We therefore performed reverse transcriptase reactions with either KCl, NaCl, or LiCl in the reaction buffer and found that stops of reverse transcriptase progression at positions U94, G101–102, U103, G104, and A106 in KCl-containing buffer disappeared in NaCl- or LiCl-containing buffers (Fig. 3A). Because the guanines in between positions 57 to 102 are accessible to neither RNase T1 digestion nor DMS modification (position N1), we propose the presence of a G quartet in this region (Fig. 3B).

Stem-loop II Is Critical for IRES Activity at the CUG1 Codon—To analyze the elements of the FGF-2 5'-ATR required for IRES activity, we used the bicistronic vector assay in which translation of the first cistron is cap-dependent and translation of the second cistron is IRES-dependent (7, 8, 18). Bicistronic mRNAs containing an upstream *Renilla reniformis* luciferase open reading frame and a downstream firefly luciferase (LucF) open reading frame were expressed from plasmid vectors transfected into the SK-Hep1 human liver adenocarcinoma cell line, which was previously shown to harbor FGF-2 IRES activity (10).

To determine the elements required for IRES activity at each of the initiation codons, we used constructs in which the downstream LucF open reading frame was inserted either at the CUG1/320 (pCRFL1), CUG2/347 (pCRFL2), CUG3/362 (pCRFL3), or AUG/485 (pCRFL4) positions (Fig. 4). Moreover, to avoid LucF activity coming from upstream in-frame trans-
lution initiations, an in-frame UAG termination codon was inserted just upstream of AUG485 in the pCRFL4 plasmid and the CUG1 and/or CUG2 codons were mutated to non-initiating UUA codons in the pCRFL2 and pCRFL3 plasmids. Because in these vectors the R. reniformis luciferase activity reflects capdependent translation and is expected to be proportional to the amount of RNA produced, whereas LucF activity measures the IRES activity, the firefly/R. reniformis luciferase ratio gives the IRES activity normalized to the amount of RNA produced (10). Compared with the entire FGF-2 5’-ATR, in which the relative luciferase activity was set to an arbitrary value of 100% (pCRFL1/100%), a control bicistronic vector that contains a short hairpin structure between the two cistrons functioned more than 10 times less efficiently in mediating second cistron translation (pCRHL/8%) (Fig. 4). The luciferase activity for plasmids pCRFL1 to pCRFL4 was between 59 and 69%, showing that an IRES activity can be measured at each of these four initiation codons. We therefore confirmed our previous observations (6) that these vectors the R. reniformis luciferase ratio in transiently transfected SK-Hep1 cells. The average relative luciferase activity was calculated from at least six experiments. An arbitrary value of 100 was set for the pCRFL1 background (Fig. 5A). We therefore concluded that various elements in domains I, G4, and II, but not in domain III, contribute to IRES activity and that a 17-nucleotide region located within domain II, at positions 128–144 of the FGF-2 5’-ATR, is the main element required for IRES activity at CUG1.

The Same 17-Nucleotide Region in Domain II Is Required for IRES-mediated Translation at Four Downstream Translation Initiation Codons—We next sought to determine whether nucleotides 128–144 of domain II would also be responsible for IRES activity at the CUG2 and CUG3 codons. To address this point, we deleted nucleotides 128 to 144 in the pCRFL2 and pCRFL3 background (Fig. 5, B and C). The results showed that this deletion affected IRES activity at the CUG2 and CUG3 positions (Fig. 5, B and C).

The same strategy was used to localize the element(s) required for IRES activity at the AUG485 codon, and various deletion constructs were made in the pCRFL4 background. As shown in Fig. 5D, deletions of the first 314, 338, or 377 nucleotides of the FGF-2 ATR strongly affected the IRES activity at the AUG, showing that the elements required for IRES activity at this codon were not located in its close vicinity and might be present in the first 314 nucleotides of the FGF-2 ATR. Indeed, deletion of part of domain II (Δ128/144) strongly decreased IRES activity at the AUG (Fig. 5D). Because the overall structure of the FGF-2 ATR, as determined by structural probing experiments, is not affected by deletion of stem-loop II (data not shown), we concluded that stem-loop II, located 308 nucleotides upstream of the AUG, is required for IRES activity at the AUG. Moreover, these data demonstrate that the same features are necessary for IRES-mediated translation at four downstream initiation codons. The ability of the FGF-2 IRES to control translation initiation at four downstream codons was also observed in the Saos-2 osteosarcoma and SK-N-BE neuroblastoma cell lines that harbor strong FGF-2 IRES activity (10). Indeed, deletion of nucleotides 128–144 led to a 3–4-fold reduction in IRES activity in these cell lines (data not shown).

The Integrity of Stem-loop II Is Required for IRES Activ-
ity—To test the structure of stem-loop II more thoroughly, we generated deletions and mutations in this region in the context of bicistronic luciferase construct pCRFL1. We first mutagenized the loop structure of stem-loop II. Deletion (ΔH9004141/152) or mutation (M142/150) of loop II led to a strong reduction in IRES activity (Fig. 6). We next mutagenized a nine-nucleotide sequence (position 132–140) that was identical to a region present in the Gtx 5'-untranslated region shown to mediate an interaction with the 18 S ribosomal RNA (19) and internal entry of ribosomes (20). However, mutation of this sequence in the FGF-2 context (M132/140) did not affect IRES activity (Fig. 5, A and B). Mutation of the other side of the stem (M156/160) had no consequences on IRES activity (Fig. 6). Overall, these data show that the loop sequence in stem-loop II is required for IRES activity.

A 176-Nucleotide RNA Module Is Sufficient to Confer IRES Activity—To determine whether stem-loop II was sufficient to mediate internal entry of ribosomes, we tested the IRES activity of a construct that contained sequences normally located downstream of stem-loop II (pCRFL1 Δ1/126) was still very weak, showing that sequences downstream of residue 176 are dispensable for IRES function. However, we found that constructs containing sequences normally located upstream of stem-loop II (pCRFL1 1/176 or pCRFL4 1/176) gave strong IRES activities that were very close to those of the parental constructs (pCRFL1 or pCRFL4) (Fig. 5, A and B). Deletion of stem-loop I alone (pCRFL1 50/176), stem-loop I and half of the G4 domain together (pCRFL1 76/176 or pCRFL4 76/176), or stem-loop II alone (pCRFL1 1/126 or pCRFL4 1/126) in this 176-nt fragment reduced IRES activity. We therefore concluded that a 176-nt sequence is necessary and sufficient for IRES activity.

**DISCUSSION**

We report here the RNA sequence and structural determinants of the FGF-2 IRES. Compared with other cellular IRES, in which the IRES has been localized and shown to function in a modular fashion, with each module able to contribute singly to IRES activity (12, 21, 22), the FGF-2 IRES contains a single module. This module is 176 nt long and is composed of two stem-loops and a G quartet motif.

Studying the process of internal entry of ribosomes on the alternatively translated FGF-2 mRNA therefore allowed us to...
define novel structural determinants of IRES function. Strikingly, none of the known RNA features involved in IRES function are present in the FGF-2 IRES module. For instance, the FGF-2 IRES lacks the polypyrimidine tract required for IRES function of several cellular IRESs (23–25). The FGF-2 IRES contains a nine-nt sequence that is also present in the Gtx IRES and that functions on its own as an IRES (20). However, mutation of these nine nucleotides in the FGF-2 context did not impair internal entry of ribosomes. Several higher-order structures are also involved in IRES function. For instance, pseudoknot structures were shown to be required in various IRES (12, 26–28). Furthermore, stem-loop structures containing a GNRA motif are shared by several picornavirus IRESs (29, 30) and could be involved in RNA-RNA tertiary interactions. We could find no evidence of the existence of such structures in loops I and II of the FGF-2 IRES. The lack of similarities between the FGF-2 IRES and other cellular or viral IRES could therefore reflect its unique property of being regulated at given physiological conditions by specific IRES trans-acting factors. Indeed, we have recently shown that the FGF-2 IRES does not require some of the classic IRES trans-acting factors, such as hnRNP I/PTB and La autoantigen; instead, we have demonstrated the role of hnRNP A1 as a novel IRES trans-acting factor for FGF-2-mediated translation.2 More generally, because a wide variety of nucleotide sequences seem to function as IRES and several proteins were identified as IRES trans-acting factor, the mechanisms by which translation preinitiation complexes are recruited to IRES-containing mRNA are likely to be different and should provide several targets for translational regulation of gene expression.

Interestingly, we have also found in this study that the FGF-2 IRES contains an intramolecular G quartet motif. Such a motif has never been described in IRES sequences. It was found in several mRNAs that are bound by the fragile X mental retardation protein (31–33). The FGF-2 G quartet motif contributes to IRES activity and may therefore behave as a positive translational element. This is surprising given the reported role of purine-quartet motifs in translation repression (34). Interestingly, the inefficient translation initiation at the cap-proximal CUG0 codon (4) may be explained by its localization within the G quartet. The binding of the fragile X mental retardation protein or other proteins to the FGF-2 G quartet motif and the role of these interactions in IRES-mediated translation await investigation.

Finally, we report here the relative position of an IRES and of alternative translation start sites. Although IRESs have been shown to be present in many mRNAs that contain a single translation initiation codon, their existence has been demonstrated on several mRNAs containing alternative initiation codons. The logical expectation is that IRESs present in alternative translation initiation systems contribute to the selection of the cap-distal internal codon, whereas the cap-proximal codon is selected by a cap-dependent mechanism. This is indeed the case for the FGF-2 mRNA, in which translation initiation at the 5′-end proximal CUG codon occurs by a cap-dependent mechanism, whereas translation at the four downstream initiation codons occurs by an internal ribosome entry process (4). We provide evidence in this study that the position of the IRES (i.e. overlapping the cap-proximal codon but upstream of the internal codons) may explain this fact. This situation has also been found in the Moloney murine leukemia virus gag mRNA, the human vascular endothelial growth factor mRNA, or the human PITSLRE protein kinases mRNA, in which an IRES is

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FIG. 6. Analyzing the requirements for FGF-2 IRES-mediated translation in stem-loop II. Schematic representation of the wild-type stem-loop II and of the different mutants. Wild-type nucleotides in the loop are in gray, whereas mutated nucleotides are in bold. The mutants were constructed in the pCRFL1 plasmid. The values for the average relative luciferase activities in transiently transfected SK-Hep1 cells are shown at the bottom and were calculated from at least four experiments performed in duplicate. An arbitrary value of 100 was set for the parental pCRFL1 plasmid.

FIG. 7. Mapping the domains sufficient for IRES-mediated translation. The various constructs were transfected in SK-Hep1 cells and the average relative luciferase activities were calculated from three independent experiments performed in duplicate.
present between two translation initiation codons and leads to the selection of the internal codon, thereby contributing to the control of alternative initiation of translation (13, 35, 36).

Conversely, IRESs can also be found upstream from several translation initiation codons without an apparent contribution to the selection of the alternative initiation codon. This situation is encountered in foot-and-mouth disease virus (37), c-myc (38, 39), Bag-1 (40), vascular endothelial growth factor (13), and FGF-2 mRNAs. Regarding the FGF-2 mRNA, a single IRES controls initiation at four downstream translation initiation codons located in a 308-nt RNA segment. After the first step of internal entry of ribosomes, which is determined by sequences present in the first 176 nt of the FGF-2 mRNA, the molecular events that will contribute to the selection of the four downstream initiation codons remain to be determined. Because the FGF-2 5′-leader is highly structured, a “leaky scanning” mechanism is very unlikely (41). Although a ribosomal shunt mechanism (42) may account for selection of the four IRES-dependent translation initiation codons present in the human FGF-2 mRNA, we favor a model in which the choice of alternative initiation codons depending on a single IRES will result from alternative RNA conformations, controlled by trans-acting factors, that will in turn favor ribosome recruitment at each of the alternative initiation codon.

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