Fibroblast Growth Factor 2 Internal Ribosome Entry Site (IRES) Activity Ex Vivo and in Transgenic Mice Reveals a Stringent Tissue-specific Regulation

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Abstract. Fibroblast growth factor 2 (FGF-2) is a powerful mitogen involved in proliferation, differentiation, and survival of various cells including neurons. FGF-2 expression is translationally regulated; in particular, the FGF-2 mRNA contains an internal ribosome entry site (IRES) allowing cap-independent translation. Here, we have analyzed FGF-2 IRES tissue specificity ex vivo and in vivo by using a dual luciferase bicistronic vector. This IRES was active in most transiently transfected human and nonhuman cell types, with a higher activity in p53−/− osteosarcoma and neuroblastoma cell lines. Transgenic mice were generated using bicistronic transgenes with FGF-2 IRES or encephalomyocarditis virus (EMCV) IRES. Measurements of luciferase activity revealed high FGF-2 IRES activity in 11-d-old embryos (E 11) but not in the placenta; activity was high in the heart and brain of E 16. FGF-2 IRES activity was low in most organs of the adult, but exceptionally high in the brain. Such spatiotemporal variations were not observed with the EMCV IRES. These data, demonstrating the strong tissue specificity of a mammalian IRES in vivo, suggest a pivotal role of translational IRES-dependent activation of FGF-2 expression during embryogenesis and in adult brain. FGF-2 IRES could constitute, thus, a powerful tool for gene transfer in the central nervous system.

Key words: central nervous system • development • FGF • in vivo • translational control

Introduction

FGF-2 or bFGF is a prototype member of the ever increasing FGF family that comprises, to date, 22 genes involved in the control of cell proliferation and differentiation (Nishimura et al., 1999). FGF-2 is a pleiotropic factor that induces the proliferation of most mesoderm- and neuroectoderm-derived cells (Slack and Isaacs, 1994; Bikfalvi et al., 1997). In vitro, it stimulates endothelial cell growth and migration; in vivo, it promotes angiogenesis and is a major tumor angiogenic factor (Kandel et al., 1991). FGF-2 is also implicated in wound-healing processes (Bikfalvi et al., 1997; Ortega et al., 1998). It regulates gap junction communications in astroglial cells (Reuss et al., 1998). Furthermore, FGF-2 is a neurotrophic factor able to promote nerve survival and regeneration after central nervous system (CNS) injury (Eckenstein, 1994; Houle and Ye, 1999). Finally, invalidation of the FGF-2 gene in mice has established its role in controlling the fate, migration, and differentiation of neuronal cells (Dono et al., 1998; Ortega et al., 1998; Zhou et al., 1998). The homeostatic process of vascular tone control by the autonomous nervous system is impaired in these knockout mice (Dono et al., 1998; Zhou et al., 1998).

Five FGF-2 isoforms are expressed by the FGF-2 mRNA, resulting from a process of alternative initiation of translation from five different initiation codons, including four noncanonical CUGs (Florkiewicz and Sommer, 1989; Prats et al., 1989; Arnaud et al., 1999). These isoforms have different cellular localizations and functions (Bugler et al., 1991; Coularcq et al., 1991; Quarto et al., 1991; Bikfalvi et al., 1995; Arnaud et al., 1999). Their relative abundance varies according to cells and tissues (Coffin et al.,

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† Abbreviations used in this paper: CMV, cytomegalovirus; CNS, central nervous system; EMCV, encephalomyocarditis virus; IRES, internal ribosome entry site; RT, reverse transcriptase.
The two plasmids pCRHL and pCREL have been described already (Huez et al., 1998). The two luciferase genes, Renilla luciferase (LucR) and firefly luciferase (LucF), are controlled by the cytomegalovirus promoter (CMV) and separated by either a hairpin or the encephalomyocarditis virus (EMCV) IRES, for pCRHL and pCREL, respectively (see Fig. 1; Huez et al., 1998). The plasmid pCRFL was constructed by replacing the EMCV IRES with the FGFR-IRES functions in different species and in all the tested cell types, with greater efficiency in neuroblastoma and p53 minus osteosarcoma cells. IRES activity was analyzed in the embryo of transgenic mice, particularly in the heart and brain. In contrast, IRES activity in the adult was low in most organs but remained very high in brain. The in vivo specificity of this activity contrasted strikingly with the relatively ubiquitous expression of viral EMCV IRES.

Materials and Methods

Plasmid Construction

The two plasmids pCRHL and pCREL have been described already (Huez et al., 1998). The two luciferase genes, Renilla luciferase (LucR) and firefly luciferase (LucF), are controlled by the cytomegalovirus promoter (CMV) and separated by either a hairpin or the encephalomyocarditis virus (EMCV) IRES, for pCRHL and pCREL, respectively (see Fig. 1; Huez et al., 1998). The plasmid pCRFL was constructed by replacing the EMCV IRES with the FGFR-IRES functions in different species and in all the tested cell types, with greater efficiency in neuroblastoma and p53 minus osteosarcoma cells. IRES activity was analyzed in the embryo of transgenic mice, particularly in the heart and brain. In contrast, IRES activity in the adult was low in most organs but remained very high in brain. The in vivo specificity of this activity contrasted strikingly with the relatively ubiquitous expression of viral EMCV IRES.

Cell Transfection

Cell lines were obtained from American Type Culture Collection (ATCC, CRL-1573) is a human kidney epithelial cell, transformed with adenovirus 5. SK-Hep-1 (accession number for ATCC, HTB 52) is a human liver adenocarcinoma of epithelial origin; SK-N-AS (accession number for ECACC, 94092302) and SK-N-NE (accession number for ECACC, 9001815) are human neuroblastoma. Saos2 (accession number for ATCC, HTB-85) is a p53-/- human osteosarcoma of epithelial origin. Cells were cultivated according to ATCC or ECACC instructions.

Generation of Transgenic Mice

Transgenic mice were obtained by injecting fragments containing either the CMV-LucR-EMCV IRES-LucF or the CMV-LucR-FGF2IRES-LucF sequences into one of the pronuclei of (C57BL/6×CBA)F1 embryos (Birnstiel et al., 1985). Transgenic embryos and adult mice were identified by PCR and Southern blot analysis of placenta or tail DNA using a LucF 500-bp-long probe amplified with the LucF-specific oligonucleotides 5'-CAATGAGACATTTGCCAG-3' and 5'-CTGAGGACTCTGAAC-3'. Stable lines were maintained by successive crosses with transgenic mice. The transgene copy number was determined by FluorImager scanning of Southern blots.

Reverse Transcription and PCR Analysis

The cDNAs were synthesized as previously described, using 5 μg of poly(dT)-treated total RNA (Touriol et al., 1999). The PCR was performed with the primer couples, 5'-GATCAGGATTCCAGTGCG-3' and 5'-CTGAGGACTCTGAAC-3', for the FGF-2 IRES and firefly luciferase (LucF), respectively (see Fig. 1; Huez et al., 1998). The plasmid pCRFL was constructed by replacing the EMCV IRES with the FGFR-IRES functions in different species and in all the tested cell types, with greater efficiency in neuroblastoma and p53 minus osteosarcoma cells. IRES activity was analyzed in the embryo of transgenic mice, particularly in the heart and brain. In contrast, IRES activity in the adult was low in most organs but remained very high in brain. The in vivo specificity of this activity contrasted strikingly with the relatively ubiquitous expression of viral EMCV IRES.

Luciferase Activity Analysis

The two luciferase activities were measured in cell or tissue extracts and the IRES activity was determined by calculating the LucR/LucFL ratios. The IRES efficiency between different cell types was compared ex vivo by calibrating the pCRFL ratio (FGF IRES activity) to pCRHL (background activity without an IRES). For the in vivo analysis, E11 embryos, heart, limb bud, tail, brain, liver, and placenta of the E16 embryos, and tissue fragments of the adults were frozen in liquid nitrogen and stored at −80°C. They were homogenized in 200 μl of passive lysis buffer (Promega) using thurax and centrifuged for 20 min at 4,500 rpm at 4°C. The supernatant was centrifuged for 15 min at 13,000 rpm at 4°C, and the last supernatant was used for luminescence dosage (30 μl). LucR and LucF activities were measured using the Dual Luciferase kit from Promega.

Results

FGF-2 IRES Activity Is Optimal in Neuroblastoma and Osteosarcoma Cell Lines

The bicistronic vector strategy, which was previously used to demonstrate the existence of the two vascular endothelial growth factor IRESes (Huez et al., 1998), was used here to test the activity of the FGF-2 IRES in different cell types. This bicistronic vector expresses two highly sensitive luciferases, LucR and LucF, which are encoded by the same mRNA under the control of the cytomegalovirus promoter (Fig. 1). This promoter was chosen because it had been shown previously to control the strong expression of reporter genes in tissue-cultured cells and the
FGF-2 IRES Activity In Vivo

Luciferase activities in the transgenic lines were measured in the whole embryo and placenta for E11 and in various tissues of the E16 embryo. A study shown in Fig. 3 (left), the activity of FGF-2 IRES was high in the E11 embryo, whereas it was low in the placenta of the two RFL transgenic lines. Such a difference was also observed in the E16 embryo, where the placenta showed no significant FGF-2 IRES activity, whereas IRES was active in several tissues exhibiting the same CMV transcriptional efficiency, such as the heart and brain (Fig. 3 and Table I). The level of IRES activity varied according to the organ: it was hardly detectable in the liver; and low in limb bud and tail, regardless of the high activity of the CMV promoter in these tissues; and was high in the heart and brain. In contrast, EM CV IRES activity was high in all the tissues of the two REL transgenic lines embryos tested, except in the liver of E16 embryos (Fig. 3, right). These data show that FGF-2 IRES is active in the early embryo, but is strongly regulated in a tissue-specific manner.

In Adult Mice, the FGF-2 IRES Shows Very High Brain-specific Activity

The activity of FGF-2 IRES was also measured in different adult organs. As shown in Fig. 4 and Table I, the IRES activity variations were exacerbated in comparison with the data obtained from cell cultures and embryos. The
variation in FGF-2 IRES activity between the different organs attained 400-fold, as illustrated by the activities found in the stomach (0.4 and 1.2 for the lines RFLD and RFL12, respectively) and in the brain (193 and 454, respectively; Fig. 4, left). FGF-2 IRES was weakly expressed in most organs, except the testis (up to 40.7) and the brain, where it presented exceptionally high activity (up to 454). In contrast, except for the liver where neither LucR nor LucF activity was significantly detected (this could result from proteolytic activities known to be present in this organ), the EMCV IRES activity was high in most adult organs (Fig. 4, right, and Table I). The high activity of FGF2 IRES observed in the brain strongly suggests that this cellular IRES is upregulated in this tissue. To check that the strong signal observed in the brain was due to IRES activity and not to any aberrant event, comparative reverse transcriptase (RT)–PCR was used to measure the relative levels of LucF and LucR cistrons. We show that the ratio of LucF RNA/LucR RNA cistrons was similar in all the organs tested, including the brain, as expected for such bicistronic mRNAs (Fig. 5). In summary, these data indicate that the FGF-2 IRES is subjected to stringent regulation in the adult, and that its activity is strongly upregulated in the brain.

### Discussion

IRESes, which allow cap-independent translation initiation, have been discovered in several cellular capped mRNAs during the last few years. The presence of such elements in capped mRNAs has raised the question of their function in the regulation of gene expression. This report shows for the first time that in contrast to the broad-range activity of a picornavirus IRES, a mammalian IRES, (i.e., human FGF-2) is highly tissue-specific in vivo. The tissue specificity that we observed is strongly reminiscent of the proposed role of FGF-2 in embryogenesis and the adult CNS (Ekendief, 1994; Slack and Isaacs, 1994; Houle and Ye, 1999). These data constitute a new step in our understanding of the physiological relevance of cellular mRNA IRESes.

The analysis of our transgenic mice showed that the human FGF-2 IRES is particularly active in the embryo proper at midgestation. At a later stage, its activity is par-
particularly high in the brain and persists in the adult. In contrast, as observed from transfection experiments, FGF-2 IRES functions in most tissue culture cell types ex vivo. All this suggests that the IRES-dependent translation of FGF-2 might be correlated with cell status, as it is functional in actively proliferating cells, embryonic or in vitro cultured, and extinguished in fully differentiated and quiescent cells, except for the CNS. Interestingly, among the different cell lines tested, the strongest IRES activity is observed in Saos2, a p53−/− osteosarcoma cell line. A s p53 has been shown to have an inhibitory effect on FGF-2 translation (B. Galy, L. Créancier, C. Zanibellato, A.C. Prats, and H. Prats, unpublished results), this could indicate that the FGF-2 IRES is not only subjected to activation, but also to silencing by trans-acting factors. This silencing could be temporarily removed in response to specific stimuli, such as stress or tissue lesion (Vagner et al., 1996). An aberrant activation of the FGF-2 IRES could participate in acquisition and/or maintenance of the transformed cell status, in concordance with our previous observations that the HMW FGF-2 isoforms are constitutively expressed in transformed cells while they are regulated as a function of cell density in subprimary skin fibroblasts (Galy et al., 1999).

These data also show that the human FGF-2 IRES activity is more tissue-specific than species-specific. Indeed, it is not only active in human cultured cells, but also in hamster, monkey, and mouse cells or tissues, suggesting that specific cellular trans-acting factors are able to recognize FGF-2 IRES independently of their origin. This corroborates previous observations in transgenic mice that expressed a human FGF-2 cDNA transgene encoding the different FGF-2 isoforms (Coffin et al., 1995). In those transgenic animals, the pattern of expression of the different human isoforms was highly tissue-specific and corresponded to that of the endogenous FGF-2 isoforms. Interestingly, FGF-2 proteins were observed in tissues such as the brain where the FGF-2 mRNA could not be detected, even by RT-PCR (Coffin et al., 1995). This suggests that the level of translation of the endogenous FGF-2 mRNA could be upregulated in a few tissues, a hypothesis which is consistent with our observations of very high IRES activity in the brain of the IRES FGF-2 transgenic mice.

While the transgenic mice that overexpress FGF-2 (Coffin et al., 1995) provide important information concerning the pattern of expression of the various FGF-2 isoforms and its conservation between human and mouse, they cannot be used to distinguish between cap-dependent and IRES-dependent translation. The bicistronic vector ap-

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**Table 1:**

| Organ   | Human FGF-2 IRES Activity (LucF / LucR) x100 | Viral EMCV IRES Activity (LucF / LucR) x100 |
|---------|---------------------------------------------|---------------------------------------------|
| Lung    | 4.2                                         | nd                                         |
| Liver   | 0.9                                         | nd                                         |
| Skin    | 0.9                                         | nd                                         |
| Muscle  | 2.0                                         | nd                                         |
| Brain   | 454.0                                      | 173.5                                      |
| Tongue  | 4.0                                         | 81.2                                      |
| Thymus  | 19.5                                       | 83.8                                      |
| Heart   | 1.8                                         | 8.3                                       |
| Stomach | 3.5                                         | 7.5                                       |
| Kidney  | 3.8                                         | 3.8                                       |
| Intestine | 1.2                                     | 1.2                                       |
| Ovary   | nd                                         | nd                                         |
| Testis  | 40.7                                       | 21.1                                      |

**Figure 4:** FGF-2 and EMCV IRESes activities in transgenic adult mice. Different organs (indicated on the left) were prepared from adult transgenic mice and luciferase activities were measured as in Fig. 3. IRES activities are represented by histograms corresponding to the LucF/LucR activities (see Table I). The results are shown for each of the two independent transgenic lines, expressing one or other bicistronic construct pCRFL (FGF-2) or pCREL (EMCV), as means ± SEM of experiments that were performed 3–10 times. The lines expressing pCRFL (left) are RFLD (gray boxes) and RFL12 (black boxes). The lines expressing pCREL (on the right) are RELB (gray boxes) and RELA (black boxes). nd corresponds to experiments giving no detectable LucR activity.

**Figure 5:** Analysis of the bicistronic mRNA in transgenic tissues by RT-PCR. Total RNA was extracted from RFL12 or RELA transgenic mice brain (B), tongue (To), or testis (Te), and the level of LucF and LucR cistrons was analyzed by RT-PCR (as described in Materials and Methods). The negative control (C−) corresponds to a PCR experiment performed using RNA from the brain of a nontransgenic mouse. The positive control (C+) was performed with an in vitro-transcribed bicistronic RFL mRNA. The bands corresponding to amplification of LucR (465 nucleotide) or LucF (366 nucleotide) cDNAs are indicated by arrows. This experiment enabled us to check the LucF/LucR ratios at the RNA level in the different organs.
proach that we describe in the present study allows us to enlighten the involvement of the FGF-2 IRES in vivo. Strikingly, this mechanism of cap-independent translation is more generally active during embryogenesis than in the adult, where it is restricted to a few organs. Indeed, we find high IRES activity in the brain and weaker activity in the testes, ovaries and lung, whereas all the other tested organs show no significant IRES activity, even those in which the CMV promoter is active. In particular, the IRES is silent in the heart where HMW murine and human FGF-2 proteins have been detected in FGF-2 transgenic mice (Coffin et al., 1995), suggesting that a majority of these isoforms could result from cap-dependent translation of FGF-2 mRNA s in this organ. The physiological activation of FGF-2 IRES observed in the brain, correlated with the high IRES efficiency in two neuroblastoma cell lines, suggests the existence of a specific CNS internal entry activating factor. The location of the IRES just upstream from the CUG1 codon and the observation of higher HMW FGF-2 synthesis in nervous system cells, in particular after nerve injury, strongly suggest that IRES could have an essential role in the brain (Vagner et al., 1995; Huber et al., 1997; Meisinger and Grothe, 1997).

IRESes constitute powerful tools as they allow the expression of several genes from the same transcription unit. Two groups have previously reported that a bicistronic retroviral vector with the EMCV IRES works stably in chicken and mouse embryos (Ghattas et al., 1991; Kim et al., 1992). We show in this study that EMCV IRES functions in a ubiquitous manner not only in the embryos, but also in most organs of adult transgenic mice. This is in agreement with a recent report that demonstrates a quasi-ubiquitous activity of the IRES of another picornavirus, Theiler’s murine encephalomyelitis virus, in young and adult transgenic mice (Shaw-Jackson and Michiels, 1999). In contrast, we demonstrate that the FGF-2 IRES, a cellular mRNA IRES, shows strong tissue-specific activity. These observations may be related to another study performed in transgenic flies indicating that the IRES contained in the 5’ untranslated regions of antennapedia and ultrabithorax exhibited a high degree of developmental regulation and were not functionally equivalent (Y e et al., 1997). Furthermore, the IRES of an avian retrovirus shows considerable activity in multipotent neural cells and in their glial and neuronal progeny (Derrington et al., 1999). We hypothesize from these results that retroviral IRESes and cellular IRESes, which both belong to capped and polyadenylated transcripts, would present stringent tissue specificity, whereas the activity spectrum of picornavirus IRESes, which in most cases belong to uncapped mRNA, would be broader.

Finally, the stringent tissue specificity of a given cellular IRES might be very useful as a biotechnological tool to drive specific gene expression in a particular tissue. A illustrated in this study, the FGF-2 IRES could be powerfully used to express neurotrophic factors from multicistronic vectors in CNS regeneration programs.

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