The vascular endothelial growth factor mRNA contains an internal ribosome entry site

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Abstract Vascular endothelial growth factor (VEGF), an essential regulator of angiogenesis during early development as well as during the growth of solid tumours, bears an unusually large 5′ untranslated region (5′-UTR) in the mRNA of over 1000 nucleotides. We found that the VEGF 5′-UTR, despite being GC-rich and containing an upstream short open reading frame, promotes efficient translation of a luciferase reporter. The VEGF 5′-UTR also allowed translation of luciferase from a dicistronic mRNA when placed between the two cistrons, demonstrating that it contains an internal ribosome entry site. Deletion analysis indicated that the IRES resides towards the 3′ end of the 5′-UTR.

Key words: Vascular endothelial growth factor; Translation; 5′-Untranslated region; Ribosome

1. Introduction

All tissues, as they develop, must acquire a vasculature to deliver an adequate supply of blood, without which the tissue would be deprived of oxygen and nutrients. Vascular endothelial growth factor (VEGF) is an important initiator of vascular development both during normal development and, pathologically, during the growth of solid cancers. The effects of VEGF during early development appear to be highly dose-dependent because even in heterozygous form the VEGF knockout is lethal in the embryo due to inadequate development of blood vessels [1,2]. The crucial role of VEGF in tumour development is demonstrated by animal studies in which abrogation of VEGF signalling has been shown to abolish tumour establishment and progression [3,4].

VEGF is encoded by a single gene which gives rise to four isoforms (three isoforms in mouse) by alternative splicing [5,6]. All four isoforms share the same 5′ untranslated region (UTR), which is unusually long (1014 nucleotides), suggesting that elements regulating translation and/or stability of the mRNA may reside in this region. The VEGF 5′-UTR is not only very long, but it is also GC-rich and contains an AUG triplet upstream of the actual initiation codon. These features suggest translation of the VEGF mRNA would be inefficient if translation occurred by the usual mechanism, which involves binding of the small ribosomal subunit complex at the mRNA 5′ cap, followed by scanning for the initiation codon. Upstream AUGs tend to occur in mRNAs that encode key regulatory molecules and are postulated to limit production of proteins that would be deleterious if produced in excess [7]. However, an alternative mode of translation initiation has recently been described that avoids the requirement for the ribosome to commence scanning at the 5′ end of the mRNA. Some viral mRNAs and a few cellular mRNAs contain an RNA structure, called the internal ribosome entry site (IRES), that allows ribosomes to bind and initiate translation internally within the mRNA (reviewed in [8,9]). It has not been clear why some cellular mRNAs use internal ribosome entry to initiate translation, but two differences from cap-dependent translation are likely to be significant. Firstly, IRES-mediated translation does not involve the cap-binding translation initiation factor eIF4E, the availability of which is regulated by binding proteins and by direct phosphorylation in response to various physiological stimuli, including growth factors [10]. Consequently, IRES-containing mRNAs will be immune to changes in eIF4E availability. Secondly, an IRES can allow the 43S ribosome complex to bind at or near the initiation codon, thereby avoiding the influence of upstream structures or short open reading frames in the mRNA.

We report here that the VEGF 5′-UTR can mediate translation initiation via internal ribosome entry and that the IRES is located in the AUG-proximal region of the 5′-UTR. The major inducing stimulus for VEGF secretion by tumours is hypoxia and nutrient starvation in the undervascularised regions of the growing tumour [11–13]. Because overall protein synthesis is suppressed by hypoxia [14], the alternative mode of translation initiation of VEGF synthesis may allow VEGF production to escape this general suppression of translation.

2. Materials and methods

2.1. Plasmid construction

Plasmids used in these experiments were made by first cloning the required elements into pBluescript SK$^+$ (Stratagene), and then transferring the completed insert to expression vectors pRc/CMV or pCDNA3 (Invitrogen). The complete 5′-UTR of the murine VEGF gene [6] was PCR amplified from plasmid pV5NBgl63 [15] using *Pfu* polymerase (Stratagene) following manufacturer's specifications with oligonucleotide primers (forward primer: 5′-TCTAGAAGCTTAGCGCAGAGGCTTGGGGCAGCCGA-3′ and reverse primer: 5′-GAATTCCATGGTTTCGGAGGCCGTCCGGG-3′). The PCR fragment and pBluescript SK$^+$ were digested with *Hin*dIII and *Eco*RI (NEB) and ligated together to form plasmid pBSv5. Plasmid CMV-FF contains the firefly luciferase gene and was created by PCR using *Pfu* polymerase with oligonucleotides specific to the firefly luciferase (forward primer 5′-CCGGAAGCTTAAGCCATGGAAGACGCCAAAAACATAAAG-3′ and reverse primer 5′-AGAATTCTGCGGCCGCTGAATACAGTTACATTTTAC-3′) using plasmid pGL2prom

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Abbreviations: IRES, internal ribosome entry site; UTR, untranslated region; VEGF, vascular endothelial growth factor

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(Promega) as the template. The 1660-bp PCR product was cut with *Hin*dIII and *Not*I and ligated into similarly cut pRc/CMV. Plasmid pBSv5FF was created by cutting CMV-FF with *Nco*I and *Not*I followed by isolation of a 1660-bp fragment (containing the firefly luciferase) and ligation into *Nco*I/*Not*I cut pBSv5, joining the VEGF 5′-UTR in frame with the firefly luciferase gene at the ATG. Plasmid v5FF was created by removing the insert from pBSv5FF with *Kpn*I and *Not*I and ligating into pCDNA3 also cut with *Kpn*I and *Not*I.

Stem loop constructs used in expression experiments were created using oligonucleotides designed to complement the pRc/CMV- and pCDNA3-derived sequence to form a 45-base pair stable stem loop, starting at the transcription start site. Forward oligo (5′-AGCTTGAGTCGTATTAATTTCGATAAGCCAGTAAGCAGTGGGTTCTCTGCGGCCGC-3′) and reverse oligo (5′-AGCTGCGGCCGCAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCA-3′) were kinased, annealed together, and ligated into *Hin*dIII-digested CMV-FF and v5FF to form plasmids SLFF and SLv5FF.

Plasmid pBSRLv5FF, containing the entire dicistronic insert, was created by isolating a 947-bp fragment (containing the Renilla luciferase gene) from *Nhe*I and *Xba*I digested pRL-TK (Promega), blunt ending the fragment with T4 DNA polymerase (NEB) according to manufacturer's specifications, and ligating into pBSv5FF cut with *Hin*dIII and similarly blunt ended. Plasmid RLv5FF was created by removing the insert from pBSRLv5FF with *Kpn*I and *Not*I, and ligating into similarly cut pCDNA3.

Deletion constructs were assembled in pBluescript, and then the entire insert was transferred into pCDNA3 in the same manner as described for RLv5FF. RLv5B/E-FF was made by cutting pBSRLv5FF with *Bsa*I and *Eag*I, blunt ending, and isolating a 496-bp fragment. This fragment was cloned into pBSRLv5FF cut with *Pst*I and *Nco*I (to remove the v5), followed by blunt ending. RLv5A/N-FF was made by cutting pBSRLv5FF with *Ava*II, blunt ending, cutting with *Nco*I, and isolating a 550-bp fragment, which was cloned into pBSRLv5FF cut with *Pst*I, blunt ended, and cut with *Nco*I.

2.2. Sequencing

Sequencing was performed using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer), following manufacturer's specifications. Inserts were sequenced in pCDNA3 using T7 and SP6 primers. Deletions were sequenced to confirm identity using primers specific to the 3′ end of RL (5′-GATGCACCTGATGAAATGGG-3′) and the 5′ end of FF (5′-TTATGCAGTTGCTCTCCAGC-3′).

2.3. Cell culture and transfection

BALB/c 3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10 mM HEPES, 7.5% heat inactivated fetal calf serum, 1.2 μg/ml penicillin, 1.6 μg/ml gentamycin, and 200 nM additional L-glutamine. Transient transfections were performed using lipofectin (Gibco-BRL) as recommended by the manufacturer. Stable polyclonal lines of dual luciferase constructs were made by $CaPO_4$ transfection of 1×10^6 cells with 20 mg *Sca*I linearised plasmid, followed by selection in 0.5 mg/ml G418 (geneticin, Gibco-BRL) for 14 days. The resultant polyclonal lines were maintained in 0.25 mg/ml G418.

2.4. Luciferase assays

Firefly luciferase from monocistronic constructs was assayed as described [16], but using 40 μg protein in an assay volume of 200 μl. The reactions were initiated by addition of 40 μl of 1 mM D-luciferin. The light signal was measured using a Top-Count (Packard). The dual luciferase assays for dicistronic constructs were performed following specifications by Promega. The light signal was measured using a scintillation counter with the coincidence circuit switched off.

2.5. RNA isolation and RNase protection assay

RNA was isolated by the method of Chomczynski and Sacchi [17]. Luciferase and GAPDH transcripts were quantitated by RNase protection assay using complementary RNA probes synthesised with SP6 RNA polymerase from *Cla*I-restricted CMV-FF and with T7 RNA polymerase from *Dde*I-restricted pGAPM, respectively, essentially as described [18]. Signals were quantitated by phosphorimaging (Molecular Dynamics).

3. Results

3.1. The VEGF 5′-UTR allows efficient translation

To investigate the influence the long VEGF 5′-UTR has on translation we compared the translational efficiency of luciferase mRNA with and without insertion of the VEGF 5′-UTR. The complete VEGF 5′-UTR was inserted into CMV-FF (Fig. 1) so that the luciferase initiation codon replaced the VEGF initiation codon, thereby creating v5FF. The CMV-FF and v5FF transcripts have 60 nucleotides in common at the 5′ end that are derived from the Rc/CMV expression vector. The luciferase reporter genes were transiently transfected into BALB/c 3T3 fibroblasts and two days later the levels of reporter mRNA and enzyme activity were measured. Contrary to expectation, insertion of the VEGF 5′-UTR had no inhibitory effect on the ratio of luciferase activity to mRNA (Fig. 1), indicating that the long VEGF 5′-UTR allows translation to proceed efficiently.

3.2. The VEGF 5′-UTR contains an internal ribosome entry site

To test whether the VEGF 5′-UTR might contain an IRES we created a large stem-loop at the very 5′ end of the v5FF mRNA by inserting 45 nucleotides of complementary sequence, generating SLv5FF (Fig. 1). This 5′ stem-loop should inhibit cap-dependent translation by preventing the binding of eIF4E to the 5′ cap [19]. Inhibition of cap-dependent trans-

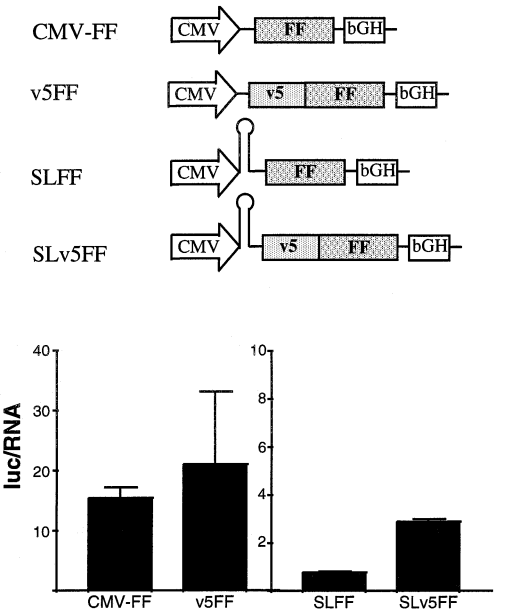

Fig. 1. Effect of the VEGF 5′-UTR on the translational efficiency of firefly luciferase. The reporter genes are shown schematically above. All genes use the CMV promoter (CMV) and the 3′-UTR and polyadenylation signals from the bovine growth hormone gene (bGH). The regions coding for Renilla luciferase (RL) and firefly luciferase (FF), and the VEGF 5′-UTR (v5) are indicated and the 45-bp stem-loop at the very 5′ end of SLFF and SLv5FF is shown pictorially. Plasmids bearing the indicated genes were transiently transfected into Balb/c 3T3 fibroblasts. Cells were lysed 48 h after transfection and separate aliquots used to measure firefly luciferase mRNA by RNase protection assay and luciferase enzymatic activity. The means and standard errors of the ratio of enzyme activity to mRNA from 2 independent experiments are shown; note the 4-fold change in scale for SLFF and SLv5FF. The standard errors for SLFF and SLv5FF are too small to be evident in the figure.

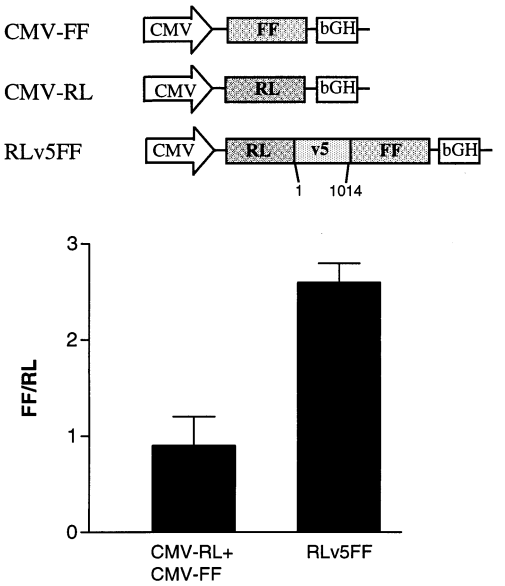

Fig. 2. Ratios of activities of firefly luciferase to Renilla luciferase in extracts from cells expressing the two luciferase enzymes either from separate genes or from a single dicistronic gene with the VEGF 5′-UTR inserted between the reporters. The reporter genes are shown schematically above. All genes use the CMV promoter (CMV) and the 3′-UTR and polyadenylation signals from the bovine growth hormone gene (bGH). The regions coding for Renilla luciferase (RL) and firefly luciferase (FF), and the VEGF 5′-UTR (v5) are indicated. Extracts were prepared from polyclonal cell lines stably transfected with equal amounts of plasmids bearing the monocistronic genes (CMV-RL+CMV-FF) or the dicistronic gene RLv5FF and the relative amounts of each luciferase measured using the Promega dual luciferase assay. The data shown are means and standard errors of assays in triplicate of duplicate extracts and are representative of two cultures from the transfection.

lation was verified by creating the same stem-loop in the CMV-FF mRNA, creating SLFF (Fig. 1). IRES-mediated translation should be insensitive to such a stem-loop so long as the stem-loop did not interfere with formation of the IRES structure or impede access to the IRES. The presence of the VEGF 5′-UTR allowed only partial relief of the inhibition resulting from the stem-loop, producing a 3.2-fold increase in translation compared to SLFF (Fig. 1). This result suggests that the VEGF mRNA may contain an IRES but the stem-loop structure at the 5′ end of SLv5FF may interfere with its function. Alternatively, translation of VEGF may result from a combination of cap-mediated and IRES-mediated translation, with cap-mediated initiation predominating under the culture conditions used in this experiment.

To further investigate the possibility that VEGF translation can be IRES-mediated we constructed a dicistronic gene encoding two independently assayable enzymes, with transcription driven by the CMV promoter (RLv5FF, Fig. 2). Normally only the upstream (5′-most) protein would be produced from such a gene in eukaryotes because ribosomes are released at the completion of translation of the first encoded protein. Thus, when the reporter gene is introduced into cells, the upstream reporter (Renilla luciferase) would be synthesised by normal cap-dependent translation, whereas the downstream reporter (firefly luciferase) would not be translated unless an IRES is present in the region between the two reporters. The ratio of firefly luciferase activity to Renilla luciferase activity provides a measure of the ability of the region inserted between the two luciferases to function as an IRES. We inserted the entire 5′-UTR of VEGF between the two luciferase cistrons, creating RLv5FF (Fig. 2). The dicistronic gene was transfected into BALB/c 3T3 fibroblasts, polyclonal pools of stable transformants were selected and assayed for Renilla and firefly luciferase activities. For comparison we selected a polyclonal line that had been cotransfected with equal amounts of monocistronic luciferase reporter genes (CMV-RL and CMV-FF, Fig. 2). The ratio of firefly luciferase to Renilla luciferase was almost 3-fold greater in extracts from cells transfected with the dicistronic gene than in extracts from cells cotransfected with the monocistronic genes (Fig. 2), demonstrating that the VEGF 5′-UTR can promote internal initiation of translation. It is interesting that the VEGF IRES appeared to function efficiently in the dicistronic context (Fig. 2) but inefficiently in a monocistronic context when a stable stem-loop was inserted upstream of the VEGF 5′-UTR (Fig. 1, compare SLv5FF to v5FF). This suggests that the stem-loop structure in SLv5FF adversely affects the structure or function of the IRES.

3.3. *The IRES is located primarily in the 3′ half of the VEGF 5′-UTR*

IRESs were originally identified in a number of viral mRNAs and the viral IRESs remain the best characterised. One class of viral IRES, typified by the EMCV IRES, includes the initiation codon at their 3′ boundary, whereas the other class can be located upstream of the initiation codon [8]. To test whether the VEGF IRES is located near the initiation codon we inserted into the dicistronic reporter gene either

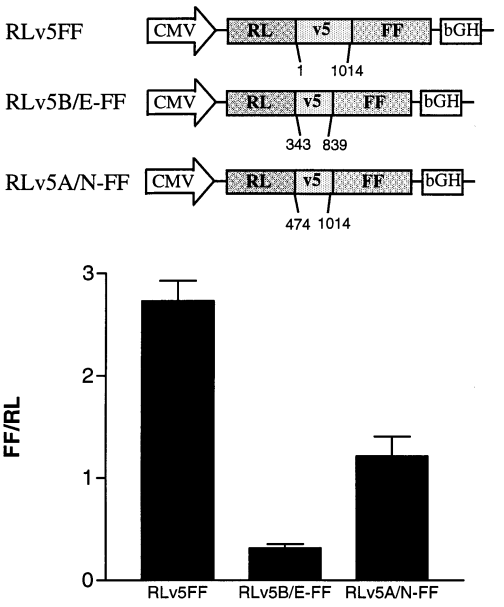

Fig. 3. Ratios of firefly luciferase to Renilla luciferase in extracts from cells expressing dicistronic reporters with various regions of the VEGF 5′-UTR inserted between the luciferase cistrons. The reporter genes are shown schematically above. The various regions of VEGF 5′-UTR (v5) inserted in the dicistronic genes are shown with numbering from the first base of the VEGF 5′-UTR. Extracts were prepared from polyclonal cell lines stably transfected with the indicated dicistronic genes and the relative amounts of each luciferase measured using the Promega dual luciferase assay. The data shown are means and standard errors of assays in triplicate of duplicate extracts and are representative of three cultures from the transfection.

the 3′ half of the VEGF 5′-UTR (nucleotides 474–1014, creating RLv5A/N-FF), or a central region of the 5′-UTR (nucleotides 343–839, RLv5B/E-FF) (Fig. 3). In RLv5A/N-FF the fusion of VEGF and luciferase sequences occurs precisely at the initiation codon. The RLv5B/E-FF produced little firefly luciferase relative to Renilla luciferase, whereas RLv5A/N-FF produced significant levels of firefly relative to Renilla luciferase, although not as high as produced by the dicistronic construct containing the entire VEGF 5′-UTR (Fig. 3). Thus sequences between nucleotides 839 and 1014 are essential for IRES function. Partial IRES function is conferred by the region downstream of nucleotide 474, but for optimal IRES activity sequences upstream of nucleotide 474 appear to be necessary.

4. Discussion

Initiation of translation of most mRNAs involves binding of the mRNA 5′ cap structure by the initiation factor eIF4E. The concentration of eIF4E in the cytoplasm is rate-limiting, forcing mRNAs to compete for eIF4E to initiate translation, which places limits on the production of cellular proteins. This is particularly important for certain key regulators of cell growth, especially those with secondary structure in the 5′-UTR, which are translated inefficiently [20–22].

Viruses such as poliovirus have developed an effective method to circumvent this competitive environment, allowing translation of viral mRNAs despite limitations in the level of eIF4E [8,9]. These viruses turn off translation of host mRNAs by cleaving eIF4G, which uncouples the cap-binding complex from the rest of the translation initiation machinery. The viral mRNAs continue to be translated because they contain internal ribosome entry sites, which allow ribosome binding, and therefore translation, without involving the 5′ cap.

To date only a few cellular mRNAs, encoding PDGF-B [23], FGF-2 [24], IGF-II [25], immunoglobulin heavy chain binding protein (BiP) [26], *c*-myc [27,28], eIF4G [29], *Drosophila antennapedia* [30], and VEGF (reported here, and in agreement with [31]) have been found to contain an IRES. The physiological role of these IRESs is not yet clear, but it is striking that most are important regulatory genes, involved in growth control. The presence of an IRES in these mRNAs may reflect the need to maintain translation of key survival proteins under conditions of stress, when normal cap-dependent translation is compromised. Furthermore, three of these (bFGF, PDGF-B and VEGF) are involved in formation or maintenance of blood vessels [11,32,33]. Since VEGF synthesis is induced both by hypoxia and by nutrient deprivation, and one function of VEGF is to stimulate angiogenesis to restore the blood supply to ischaemic tissues, the presence of the IRES in the 5′-UTR may be important to allow VEGF secretion by cells that are severely starved.

The VEGF IRES may also have an important role during embryonic development. VEGF is an essential regulator of vascular development in the embryo, where its effects appear to be exquisitely dose-dependent, since mice with only one functional allele of the VEGF gene die in utero from failure of adequate vascular development [1,2]. Thus it seems likely that VEGF must be produced at a precise rate in the developing embryo. The IRES-mediated translation of VEGF may act as a buffer against changes in the cellular concentration of eIF4E, allowing an appropriate rate of synthesis of VEGF despite modulation of eIF4E levels in response to changes in the cellular environment.

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