Control of the Vascular Endothelial Growth Factor Internal Ribosome Entry Site (IRES) Activity and Translation Initiation by Alternatively Spliced Coding Sequences*

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The vascular endothelial growth factor-A (VEGF) gene locus contains eight exons that span 14 kb. Alternative splicing generates multiple, different mRNAs that in turn translate into at least five protein isoforms. While the canonical AUG start codon is located at position 1039 in exon 1, there also exists an upstream, in-frame CUG initiation codon that drives expression of L-VEGF, containing an additional 180 amino acids. Two separate internal ribosome entry sites (IRES) regulate the activity of each initiation codon. Thus the 5′-UTR of VEGF, which comprises the majority of exon 1, consists of IRES B, the CUG, IRES A, and the AUG, from 5′ to 3′. Previously, it has been shown that IRES B regulates initiation at the CUG and IRES A regulates AUG usage. In this study, we have found evidence that the exon content of the VEGF mRNA, determined through alternative splicing, controls IRES A activity. While the CUG is most efficient at initiating translation, transcripts that lack both exons 6 and 7 and therefore contain an exon 5/8 junction lack AUG-initiated translation. The process of splicing is not responsible for this start codon selection since transfection of genomic and cDNA VEGF sequences give the same expression pattern. We hypothesize that long range tertiary interactions in the VEGF mRNA regulate IRES activity and thus control start codon selection. This is the first report describing the influence of alternatively spliced coding sequences on codon selection by modulating IRES activity.

Diverse roles have been found for VEGF1 in both physiological and pathological processes. These include roles in embryonic development, differentiation of the vascular system, wound healing, and reproductive functions. VEGF is a potent mitogen for endothelial cells and controls the initiation of blood vessel formation. In addition, VEGF has been shown to be involved in pathological angiogenic processes such as proliferative retinopathies, tumor growth, arthritis, psoriasis, and ischemia (for review, see Refs. 1–3).

Expression of VEGF is subjected to regulatory mechanisms involving transcriptional and post-transcriptional controls. Alternative splicing of human VEGF generates six polypeptides with 121, 145, 165, 183, 189, and 206 amino acids (4–8). The precise functions of all the different VEGF isoforms have not been defined. Most VEGF producing cells preferentially express VEGF121, 165, or 189 (120, 164, or 188 in mouse), which are considered the three major isoforms and exist as glycosylated dimers (9). In contrast, expression of VEGF145 and 206 is restricted to cells of placental origin (10).

The three major isoforms of VEGF show different properties with respect to receptor binding and extra-cellular localization. While VEGF121 is fully diffusible, VEGF165 and 189 are anchored to the cell surface and in the extracellular matrix (ECM) through interactions with heparan sulfate containing molecules (11). While VEGF isoforms are functionally redundant in the processes leading to the initial formation of arch arteries (12), they are expressed in distinct spatio-temporal patterns during embryonic development and in adult tissues (13). Each isoform of VEGF contributes differentially to the process of tumor neo-vascularization, with the more soluble isoforms acting at more distal sites to promote vascular recruitment, and the more extracellular membrane-associated isoforms acting to promote local expansion of capillary beds (14). The fact that the same VEGF isoform can have distinct activities at different anatomical sites suggests that the microenvironment of different tissues can dictate VEGF function (15). Different transgenic mice have been generated to express a single VEGF isoform. Mouse embryos expressing only VEGF120 (120/120) develop to term but die shortly after birth. 120/120 mice display vascular defects, including impaired post-natal cardiac angiogenesis, resulting in severe myocardial ischemia and early postnatal lethality. These mice also have impaired vascular development in their lungs (16). These studies show an essential role for the heparin sulfate binding VEGF isoforms in lung vascular development (17). Half of the 120/120 embryos die in the perinatal period due to congenital birth defects and the other half succumb within 2 weeks after birth, in part due to myocardial ischemia (18). While mice expressing only VEGF164 are normal (19), half of the 188/188 mice die between E9.5 and E13.5. Taken together, these data suggest a requirement for the expression of multiple different VEGF isoforms for normal development.

In addition to the AUG 1039 initiated isoforms resulting from alternative splicing, we and other laboratories demonstrated the existence both in vitro and in vivo, of the L-VEGF isoform initiated at the CUG 499, located 539 nucleotides upstream of the canonical AUG codon (20–22). Start codons se-
lecion is controlled by two independent internal ribosome entry sites (23). The first identified IRES (IRES A) is located within the 300 nucleotides upstream of the AUG. IRES B located at the 5'-end of the mRNA controls translation initiation of L-VEGF at the CUG (22, 23). The 180 additional amino acids present in L-VEGF are highly conserved between mammals. L-VEGF is detectable in different mouse tissues and in COS-7 cells transfected with a full-length cDNA. L-VEGF is proteolytically processed leading to a 23-kDa N-terminal fragment of 206 amino acids, named N-VEGF, and a protein with a similar size to that of the canonical AUG-initiated VEGF (22). While the C-terminal cleavage product of L-VEGF and the AUG-initiated VEGF are secreted, both the N-terminal fragment and intact L-VEGF, which contain a hydrophobic sequence, are retained in the Golgi apparatus (22).

In this study, we reveal a new level in the regulation of VEGF expression. VEGF can be synthesized by translation initiation at the AUG, CUG, and rare codons, which is dependent on the exon content of the coding region. While translation initiation at the CUG is always efficient regardless of which splice variant is expressed, usage of the AUG depends on which exons are present in the mRNA. These data suggest for the first time that the structure of the VEGF mRNA, generated through alternative splicing, controls the selection of the translation initiation codon by modulating the activity of IRES A.

MATERIALS AND METHODS

Plasmid Constructions—The human VEGF189 coding sequence and the DNA fragment corresponding to the 5’-UTR of the messenger were kindly provided by J. Abraham (4). The constructs p5'121, p5'165, p5'189, and pVc55Sp have already been described (22). The hemagglutinin (HA) tag was added at the signal peptide was performed using the hybridizing PCR method (23). Each cell type was transfected with 5 μg of DNA, kindly provided by J. Abraham (4). The constructs p5'121, p5'165, and p5'189 (ClaI and AagI sites) were digested with pEX4 forward and EX6 forward/EX8HA reverse, using genomic DNA from HeLa cells. The products named EX4-7 and EX6-8 were directly cloned in the TOPO cloning vector PCR2.1 (Invitrogen) leading to the pEX4-7 and pEX6-8 and sequenced. pEX4-7 was digested with BsiWI and BamHI, pEX6-8 with BamHI and ClaI for insertion into BsmI and ClaI p121mSPHA vector to produce pmminiVEGF (Fig. 4). The plasmids p121mSPHA, p165mSPHA and p189mSPHA were digested with NgoMI and XhoI and cloned into NgoMI- and XhoI-digested pCRVL2 (23) to create plasmids p121mSPHA, p165mSPHA, and p189mSPHA. The vector pVEGF13 (pGL2-Basic vector containing the VEGF promoter sequence from -1176 to +54, kindly provided by G. Pages) was digested with EcoRI and NheI. Plasmids p121mSPHmAUG, p165mSPHmAUG, and p189mSPHmAUG were digested with AatII, blunted with Klenow, digested with BamHI and the 4-kb pair fragments were gel-purified. The vector p121mSPHA was digested with NheI/BamHI and the 740-bp fragment was ligated with the XmaI-DraIII-digested fragment of pVEGF13, respectively (Fig. 3).

We substituted the CMV promoter in vectors p121mSPHA, p165mSPHA, and p189mSPHA with the VEGF promoter to create pV121mSPHA, pV165mSPHA and pV189mSPHA. The vector pVEGF13 (pGL2-Basic vector containing the VEGF promoter sequence) was digested with HindIII and NheI. Plasmids p121mSPHmAUG1123HA, p165mSPHmAUG1123HA, and p189mSPHmAUG1123HA containing mutated AUG1123 were digested with EcoRI and NheI and cloned into EcoRI-NheI-digested pVEGF13 to create plasmids pCA121mSPHmAUG1123HA, pCA165mSPHmAUG1123HA, and pCA189mSPHmAUG1123HA containing mutated AUG1123 (1, 2, and 3, respectively), were created using the QuikChange method (Stratagene) using plasmids pCA121mSPHA, pCA165mSPHA, and pCA189mSPHA and primers mATG1123foward and mATG1123reverse.

Cell Culture and DNA Transfection—Cell lines were obtained from the American Type Culture Collection (ATCC), COS-7 (ATCC no. CRL 1654) is a monkey kidney cell line transformed by the simian virus 40 large T antigen. HeLa (ATCC no. CCL2) is a human cervical carcinoma cell line of epithelial origin. These cell lines were grown in Dulbecco’s modified Eagle’s medium supplemented with gentamicin, amphotericin, glutamine, and 5 or 10% fetal calf serum, respectively.

Each cell type was transfected with 5 μg of plasmid using FuGENE-6 transfection reagent (Roche Applied Science) according to the manufacturer’s instructions, in 60-mm Petri dishes. Cells lysates were prepared for Western blot experiments 24 to 48 h after transfection. The 8 h cobalt chloride treatment (100 μM (Sigma) and the cycloheximide treatment (100 μM (Sigma) were initiated 24 h after transfection.

Western Immunoblotting—Transfected cells were scraped in phosphate-buffered saline, resuspended in dithiothreitol and β-mercapto-
ethanol-containing sample buffer (1×) and subjected to sonication. Total proteins were quantified by the Bio-Rad assay (absorbance at 595 nm). The 30-μg protein samples were heated for 2 min at 95°C and separated by 12.5% PAGE before being transferred onto a nitrocellulose membrane. HA-tagged VEGF proteins were detected using the HA antibody (monoclonal Ab HA.11, BabCO) (dilution 1:1000). HIF1α, Rb, and β-actin proteins were each detected using specific antibodies (anti-HIF1α and anti-pRb from BD Biosciences and anti-β-actin from Sigma). Antibodies were detected using a chemiluminescence ECL kit (Amer- sham Biosciences).

UV Cross-linking Experiments—S10 cytoplasmic extracts were prepared from COS-7 cells as already described (24). $1 \times 10^6$ cpm of $^{32}$P-labeled in vitro transcribed RNA were incubated with 10 μg of S10 extract and UV-irradiated with an energy of 400,000 J/cm² at 254 nm. The samples were then treated with 10 units of RNase One (Promega) at 37°C for 30 min, before 12.5% PAGE analysis and autoradiography.

**Fig. 1.** Alternative initiation of translation of the different VEGF mRNAs. A, VEGF mRNA is schematized with the two initiation sites and the two IRESs. Beneath the mRNA, the two protein isoforms, L-VEGF and VEGF, are represented together with their maturation products (N-VEGF and VEGF) and their cellular localization. B, schematic representation of the constructs used for transfection experiments (see “Materials and Methods”). These constructs encode the three VEGF isoforms with an HA tag in exon 8 (p121mSPHA, p165mSPHA, and p189mSPHA numbered 1, 2, and 3) or exon 2 (p121mSPHA2, p165mSPHA2, and p189mSPHA2 numbered 7, 8, and 9). The constructs pAUG121mSPHA, pAUG165mSPHA, and pAUG189mSPHA (numbered 4, 5, and 6) correspond to the three VEGF alternatively spliced cDNAs fused to the HA tag in exon 8, beginning 24 nucleotides upstream the AUG1039. The constructs 1–3 and 7–9 give rise to the VEGF and the L-VEGF proteins and constructs 4–6 give rise to the VEGF proteins only. C, constructs 1–6 were transiently transfected into COS-7 cells and 1–3 into HeLa cells. Their expression was analyzed by Western immunoblotting using an anti-HA antibody. Control (Ct) lanes correspond to untransfected COS-7 or HeLa cells. The positions of L-VEGF proteins are indicated by dots (from top to bottom, L-VEGF189, L-VEGF165, L-VEGF121) and VEGF proteins by arrows (from top to bottom, VEGF189, VEGF165, VEGF121). D, constructs 7, 8, and 9 were transiently transfected into COS-7 cells and their expression was analyzed by Western immunoblotting using an anti-HA antibody. Positions of L-VEGF and VEGF proteins are described as in Fig. 1C.
VEGF Expression, Splicing/Translation Cross-talk

**RESULTS**

Differences in Protein Expression Pattern between the Three Major VEGF mRNA Splice Variants—Previously, we demonstrated that VEGF can be generated through both AUG initiated translation and cleavage of the L-VEGF precursor protein (22), depicted in Fig 1A. In order to detect differences between translational initiation at the AUG and CUG codons for each VEGF mRNA splice variant, we constructed expression plasmids containing VEGF121, -165, or -189 with mutated signal peptide sequences (22). This mutation abrogates the cleavage of CUG initiated L-VEGF into a 23-kDa N-terminal fragment (N-VEGF) and a peptide with a molecular weight indistinguishable from the AUG-initiated VEGF (Fig. 1A). Each mutant VEGF isoform is discernable from endogenous VEGF due to a C-terminal HA tag and is under the transcriptional control of a CMV promoter (Fig. 1B).

Fig. 1C shows that these three VEGF splice variants have distinct properties in terms of translation start site codon selection. In both HeLa and COS-7 cells, Western blots with the HA-antibody show that VEGF121 is exclusively expressed from AUG initiation events utilizing the CUG start codon, which, due to the absent signal peptide, only generates the high molecular weight VEGF. In contrast, the VEGF189 splice variant leads to the production of more AUG-initiated protein and the VEGF165 splice variant produces equivalent amounts of each isoform.

Another group has demonstrated that the large 47-kDa isoform of VEGF165 was predominantly produced after transient transfection in COS-1 cells as well as in an ovarian cancer cell line (20).

Two possibilities exist to explain the lack of the AUG-initiated VEGF121 isoform: inefficient protein synthesis and protein instability. To rule out these possibilities, the 1015 nucleotides upstream of the AUG were deleted from each plasmid, forcing the translation of the tagged protein in a cap-dependent manner. As shown in lanes 4, 5, and 6 of Fig. 1C, these three VEGF isoforms are capable of being synthesized and accumulate to equivalent amounts in COS-7 cells. In order to check if the position of the HA tag influences the choice of the initiation codon, we inserted the HA sequence in the exon 2 of the VEGF coding sequence, as seen previously, the plasmid p121mSPHA2 leads to the exclusive expression of the 42 kDa CUG initiated isoform whereas the constructs p165mSPHA2 and p189mSPHA2 express both isoforms. The change of the HA tag position in the VEGF sequence does not lead to any modification of the protein expression pattern. Despite the mutation of the signal peptide sequence, it may in fact be possible that the low molecular weight isoforms detected by Western blotting are cleavage products and not actually AUG-initiated translation products. Therefore, we mutated both AUG start codons, at positions 1039 and 1123 and repeated the above described expression analyses. The constructs shown in Fig. 2A were transfected into COS-7 cells for Western blotting with HA antibody (Fig. 2B). The complete disappearance of low molecular weight VEGF protein demonstrates that the previously detected proteins of these sizes were AUG initiated translation products and not the results of L-VEGF proteolysis. To further investigate if the lack of the VEGF121 AUG-initiated form (Fig. 1C, lane 1) is not due to a higher instability of this isoform, cells...
were transfected with VEGF cDNAs that begin just upstream the AUG1039 codon. Then, cells were treated by cycloheximide for different times. To control the efficiency of the translational blockade, and provide evidence of the reliability of this experiment, we have analyzed pRB stability. Results shown in Fig. 2B are in good agreement with pRb half-life described in the literature (about 5 hr in this cell type) (29, 30). In these conditions, the half-lives of the VEGF121, 165, and 189 AUG-initiated proteins are very similar (Fig. 2C), indicating that the difference in the VEGF expression pattern is not due to a differential stability of the three isoforms.

Initiation Codon Selection Is Not Dictated by Hypoxia, the 3'-UTR, or the VEGF Promoter—VEGF is transcriptionally and post-transcriptionally up-regulated by hypoxia (31–34). Therefore we were interested in whether or not selection between CUG and AUG translation initiation also is influenced by hypoxic conditions. By treating cells transfected with the constructs shown in Fig. 3A, with cobalt chloride for 8 hr to mimic hypoxia, Western blotting revealed that such treatment has no effect on initiation codon selection (Fig. 3B). Since hypoxia increases the stability of VEGF mRNA through AU-rich sequences in its 3'-UTR, we investigated initiation codon choice using constructs containing the VEGF 3'-UTR sequence (Fig. 3A). Western blots of transfected COS-7 cells probed with the HA antibody revealed the same expression pattern as seen in experiments without the VEGF 3'-UTR sequence (Fig. 3C). Finally, we replaced the CMV promoter with the VEGF promoter and repeated these experiments. Again, as shown in Fig. 3D, constructs containing the VEGF promoter, did not respond differently to hypoxic stimuli. These data confirm that the selection of initiation codon is not influenced by hypoxia, by the presence of the 3'-untranslated region, or the VEGF promoter.

Minigene Construct Transfection Gives Results Similar to cDNA Transfection—The coding region of the VEGF gene spans 14 kb and contains 8 exons (4, 8). Through alternative mRNA splicing, the VEGF isoforms differ by the presence or

![Diagram](https://example.com/diagram.png)

**Fig. 3. Hypoxia does not influence VEGF alternative translation initiation.** A, schematic representation of constructs used in transfections (see "Materials and Methods"). These constructs encode the three VEGF isoforms fused to HA without 3'-UTR (p121mSPHA, p165mSPHA, and p189mSPHA numbered 1, 2, and 3) or with 3'-UTR (p121mSPHA3', p165mSPHA3', and p189mSPHA3' numbered 4, 5, and 6). The constructs 1, 2, and 3 (B) and 4, 5, and 6 (C) depicted in panel A were transiently transfected in COS-7 cells. The cells were cultured under normoxic (−) or hypoxic (+) conditions using an 8 h cobalt chloride (100 μM) treatment before harvest (see "Materials and Methods"). The expression of the different isoforms was analyzed by Western immunoblotting using an anti-HA antibody. The positions of VEGF and L-VEGF proteins are indicated. The HIF-1α protein level was detected with anti-HIF-1α monoclonal antibody as a control for hypoxic stimulation and β-actin was measured as a control for equal loading. D, constructs 1', 2', and 3' are identical to constructs 1, 2, and 3 but their transcription is under the control of the VEGF promoter (see "Materials and Methods") instead of the CMV promoter. For testing VEGF expression under hypoxia, transiently transfected COS-7 cells were cultured and analyzed as described above.
absence of sequences encoded by exons 6 and 7. Previously, we have determined protein expression patterns using cDNA constructs that represent the end products of VEGF splicing pathways. By replacing the cDNA sequence between exons 4 and 8 with the genomic sequence, we were able to examine these protein expression patterns in a more natural context (Fig. 4A). While VEGF 189 was undetectable in both HeLa and COS-7 cells, results obtained with VEGF121 and -165 paralleled those seen with the cDNA constructs. This result is in agreement with the literature which states that most cell types predominantly produce VEGF121 and -165 isoforms (35, 36). Again, VEGF121 is predominantly produced as the CUG-initiated L-VEGF isoform and VEGF165 is found to be expressed as both AUG- and CUG-initiated translation products (Fig. 4B).  

In conclusion, the alternative initiation of translation at both AUG and CUG codons undergoes specific regulations correlated to the isoform synthesized (121, 165, or 189 amino acids), suggesting a direct linkage between the splicing of VEGF mRNA and the choice of the translation initiation codon.  

VEGFAlternativelySplicedRegionModulatesIRESActivity—Previous studies have demonstrated that IRES A, located within the 238-nt upstream of AUG 1039, controls AUG initiated translation (23). Here we show that the alternative translation initiation of the three VEGF isoforms implies mainly a modulation of the initiation at the AUG codon. We hypothesize that the differential expression of AUG initiated isoforms is exerted through a modulation of IRES A activity.  

To determine whether or not the differential protein expression pattern we have observed is exerted though a modulation of IRES A activity, we generated bicistronic constructs for each splice variants. The Renilla luciferase reporter gene is used as the first cistron and VEGF121, 165, or 189 cDNAs are the second cistrons. In between the two coding sequences, we placed the IRES A sequence from nucleotides 745 to 1038 of the VEGF mRNA (Fig. 5A). In each of these constructs VEGF translation can be initiated exclusively at the AUG codon through an internal ribosome entry mechanism utilizing the IRES A sequence.  

We have first verified that the luciferase activity is dependent on the bicistronic mRNA level (data not shown). In these experiments, equal loading of Western blots was normalized by the measure of Renilla luciferase activity in each lysate. Cells transfected with the VEGF121 construct showed poor IRES A activity and in turn no VEGF protein expression. On the other hand, both VEGF165 and -189 containing constructs produced IRES A-dependent AUG initiated translation. Two proteins, potentially corresponding to translation initiation at both AUG 1039 and AUG 1123, were detected in each lysate (Fig. 5B, lanes 1, 2, and 3). To verify the usage of these two start codons, we repeated the experiments with constructs in which these AUG codons were mutated. The lower molecular weight isoform corresponds to translation product initiated at AUG 1123, since the mutation of this codon leads to the disappearance of this protein (Fig. 5B, lanes 1′, 2′, and 3′). Fig. 5B, lanes 1′′, 2′′, and 3′′ show that no VEGF proteins are expressed when both AUGs are mutated, demonstrating that no more downstream initiation codons could be used.  

Examination of Potential Mechanisms—Taken together, these results imply that AUG initiated translation is dependent on the presence of exon 7 (VEGF165) or exons 6 and 7 (VEGF189). Conversely, it is also conceivable that AUG-initiated translation is inhibited by the presence of an exon 5/8 junction (VEGF121). In addition, we have demonstrated that this effect of exon composition on start codon selection is exerted through the IRES A sequence of VEGF (Fig. 6A).  

There exist at least two possibilities with respect to the mechanism whereby alternatively spliced mRNA sequences can exert an effect on IRES activity and in turn regulate start codon selection. Either distinct protein complexes can preferentially form on a particular mRNA or one or more of the mRNA species can adopt particular structures that favor or inhibit the observed protein expression patterns.  

To test the first possibility, we analyzed cellular proteins that bind to the different splice junctions that occur in the VEGF121, -165, and -189 mRNAs. Results from UV crosslinking experiments using 32P-labeled RNA probes and cellular COS-7 extracts are shown in Fig. 6B. While a set of proteins were found to interact with these RNA sequences (two major 70 and 40 kDa as well as three minor bands of 25, 29, and 45 kDa) no discernable differences were detectable between them.
CUG initiating codon. Constructs 1/H11032 contains the VEGF IRES A sequence (23) that does not contain any intercistronic region, responsible for translation of the second cistron, cistrons encode one of the three VEGF isoform (121, 165, and 189). The constructs 1/pCRA165mATG1123mSPHA, and pCRA189 mATG1123mSPHA). Constructs 1, 2, and 3 except that AUG1123 is mutated to a pCRA189mSPHA (numbered 1, 2, and 3). In these constructs the first sequences. Fig. 6 computational search of VEGF121 and 165/189 mRNA junctions and IRES A, we obtained much more promising re-

VEGF IRES A activity. Consequently, when we investigated these experiments did not enable us to identify any specific scanning factor potentially implicated in the modulation of VEGF IRES A activity. Consequently, when we investigated potential tertiary interactions between the different exon/exon junctions and IRES A, we obtained much more promising results that allow us to propose working hypothetical models.

Several complementary regions were discovered during a computational search of VEGF121 and 165/189 mRNA sequences. Fig. 6C shows a hypothetical structural model of tertiary interactions formed between the exon 5/8 junction and a region located 8 nucleotides downstream of AUG 1039 in VEGF121 mRNA ($\Delta G = -47.88$ kCal/mol). Such an intramolecular tertiary interaction could be part of the mechanism by which the exon composition of VEGF121 mRNA negatively influences translation initiation at AUG 1039. In addition, we found complementarity between the exon 7 and a region upstream of AUG 1039 (Fig. 6D). This base pairing is stabilized, in the VEGF189 mRNA, by the presence of exon 6 ($\Delta G$ of $-77.24$ kCal/mol, Fig 6D).

We then performed in silico structural analyses of the VEGF IRES A sequence to investigate what types of local secondary structures would consume bases required for such long range tertiary interactions. We first characterized potential stable helices (I and II), that are common to the three alternative VEGF messengers (Fig. 6, C and D). We found a helical region (Helix I in Fig. 6, C and D) with a loop that has a complementary counterpart downstream of where the exon 5/8 junction interacts (Helix II in Fig. 6, C and D). These data present a working hypothetical model by which interactions between the exon 5/8 junction could stabilize Helix II formation and affect ribosome entry at either AUG 1039 or AUG 1123. In contrast, the exon 6/7 junction, when bound to its complementary region upstream of the AUG could disrupt local IRES A structure and make the AUG more accessible. Since the VEGF IRES A functions in bicistronic constructs containing a reporter gene in place of the VEGF coding sequence (17), it is perhaps more likely that there exists a negative regulatory function in the VEGF121 sequence over a positive regulation by the VEGF165 and 189 mRNA sequences.

**DISCUSSION**

VEGF has served as an exemplar for the discovery of complex methods of genetic regulation. We report here an additional layer of that complexity and have found a novel mechanism whereby a regulatory network can exist within a single mRNA molecule. The VEGF pre-mRNA transcriptional product undergoes alternative exon splicing in order to generate a distinct set of mature mRNAs, which encode different VEGF protein isoforms. In this report, we demonstrate an additional effect of exon splicing on VEGF regulation. Different spliced mRNAs lead to translation of proteins initiated at different start codons. Previously, we knew that VEGF could be translated from both the AUG 1039 and the CUG 499 codons and that these events were regulated by the two IRES elements, IRES A and B, located in the 5′-UTR. Now, it is clear that the IRES A regulated control of translation initiation is in turn regulated by the exon composition of the mature mRNA.

The existence of L-VEGF, a high molecular weight isoform of VEGF initiated at CUG 499 presents many interesting questions. The 180 extra amino acids encoded in the N-terminal portion of L-VEGF are highly conserved in human, mouse, rat, and cow. While this coding sequence presents no obvious homology with any other known protein, there is evidence of biological importance for this portion of VEGF. A recent report shows that a single nucleotide polymorphism in the VEGF gene (−634C-G) results in a reduction of 17% of L-VEGF and confers an increase risk for motor neuron degeneration in Amyotrophic Lateral Sclerosis (ALS) (37). These results highlight the biological importance of L-VEGF and the use of the CUG alternative initiation codon. It will be now of major interest to understand how L-VEGF exerts biological function.

Differential regulation of alternative initiation codons controls the expression of many growth regulatory genes. FGF-2 mRNA contains four highly regulated CUG codons, three of which are under the control of a single IRES (24, 38–40). The translation initiation of c-Myc can occur at both CUG and AUG codons where the CUG is subject to the main regulation (41–43). We report here that, for VEGF, initiation at the CUG codon is relatively constant whereas the AUG is more highly regulated. Our observation of several isoforms of L-VEGF has also been described by Meiron et al. (21). These proteins, likely initiated at the downstream, in frame CUG 653 and CUG 674 codons, could be the result of leaky scanning mechanisms (44). Similar observations are seen at the AUG in constructs that contain the minimum IRES A sequence (Fig. 5). This particular leaky scanning event, utilizing AUG 1123, is not seen when the full-length 5′-UTR is used, suggesting that the 294 nucleotides of IRES A are sufficient for internal ribosome entry but are not as precise as the complete 5′-UTR for initiating translation at...
FIG. 6. Hypothetical models for IRES control by alternatively spliced sequences and its consequences on protein stoichiometry. A, upper panel, we have demonstrated that the translation initiation of VEGF121 occurs exclusively at the CUG codon. The L-VEGF produced gives rise to a molecule of VEGF (secreted) and a molecule of N-VEGF (intracellular), resulting in a stoichiometric expression of these two proteins. As we show in Fig. 5 that this regulation affects IRES activity, we can hypothesize that the VEGF121 downstream coding sequence inhibits initiation at the AUG codon by down-regulating the IRES efficiency. Middle panel, initiation of VEGF165 occurs as well at the CUG as at the AUG codons. There is approximately as much expression of L-VEGF as VEGF. As one L-VEGF molecule gives rise, after maturation, to one N-VEGF and one VEGF molecules, the quantity of secreted VEGF165 will be higher than the quantity of intracellular N-VEGF. Indeed, N-VEGF is expressed exclusively at the CUG codon whereas VEGF comes from the two alternative AUG and CUG initiation codons. In that case, the VEGF165 coding sequence could raise the inhibition mediated by the VEGF121 coding sequence (see above) or enhance IRES activity. Lower panel, for VEGF189, the initiation at the AUG codon is the most effective. For the same reasons as before, there is in that case, predominant expression of VEGF (secreted) with regard to the N-VEGF (intracellular). Like previously, the VEGF coding sequence between exons 4 and 8 could have an enhancing effect on the translation initiation at the AUG codon by up-regulating IRES activity. B, analysis of RNA-protein interactions by UV cross-linking.

In vitro transcribed RNA probes (10⁵ cpm) corresponding to the sequence from the exon 5 to 8 of the VEGF121, 165, or 189 cDNA (respectively named 1, 2, and 3) were incubated with COS-7 cells S10 extract. After UV irradiation samples were treated with RNase One and analyzed by SDS-PAGE. Arrows indicate the presence of cross-linked proteins. Molecular weight markers are shown. C, working hypothetical model of
AUG 1039. Despite the potential for leaky scanning, the minimal IRES A is sufficient to enable the alternatively spliced coding sequences to exert their effect on translation initiation at AUG 1039.

Through our attempts to find a mechanism whereby the alternatively spliced VEGF mRNA influences start codon selection, we have concluded that this phenomenon is in fact due to the exon content of the mRNA. Modulation of codon choice is not exerted through the VEGF promoter or 3′-UTR. In addition, AUG initiation is not influenced by hypoxic stimulation. In these experiments, artifact of translation initiation, resulting of a lack of splicing, could occur because of the use of cDNA. Many proteins co-transcriptionally associated with the pre-mRNAs are removed during splicing while others, like the exon-junction complex (EJC), bind mRNA in a splicing dependent manner (45). Some hnRNP proteins together with the EJC accompany the mRNAs to the cytoplasm and provide a molecular memory that documents the overall structure of the pre-mRNA, and communicates crucial information to the translational machinery (46–48). The process of splicing has also been ruled out as a participant in AUG selection through our studies with the VEGF minigene (Fig. 4). Taken together, these results solely implicate the different exon content, and various exon/exon junctions, as determinants for codon selection. In addition, this effect is exerted through sequences proximal to the AUG codon, including IRES A but not excluding sequences downstream of AUG 1039 (Fig. 5).

While it is apparent that differences in exon content between VEGF121 and VEGF165/189 control AUG-initiated translation, it is not obvious whether or not this is a positive influence by the mRNAs containing exons 6 and/or 7 or a negative effect of either the lack of exon 7 or the presence of exon 5/8 junction. While this phenomenon could be due to differential binding of specific transactivating or repressing factors on the alternative mRNA sequences, our results do not support this hypothesis. On the other hand, sequence analysis has proposed that different tertiary structure can be formed for VEGF121 versus VEGF165 and -189, which are dictated by the alternatively spliced sequence (Fig. 6, C and D). These differences are mechanistically intriguing since they purport that the exon/exon junctions specific of each messenger interact with sequences located upstream and downstream of AUG 1039. Since this codon is under the dependence of the IRES A and differentially regulated by the alternative splice variants, we find the different tertiary structure models to be an attractive hypothesis. The predicted hypothetical models suggest that a disruption of IRES A local structure can lead to a modulation of its activity and/or conformational changes around the AUG start codon, potentially modifying its accessibility. These hypotheses are in agreement with the observation that the exon 5/8 junction, which binds just downstream of the AUG, negatively regulates usage of this codon. Conversely, the exon 6/7 junction, shown to positively influence AUG usage, interacts upstream of the AUG and possibly preserves IRES A folding and activity (schematic depicted in Fig. 6A).

In fact, RNA structures may influence the presentation of primary sequence motifs and thus affect their accessibility for interaction with cytoplasmic factors or ribosomes. For example, in the analysis of the Apaf-1 IRES, it was shown that disruption of particular RNA structures by either trans-acting factors or mutation enhances IRES activity (49). It has been proposed that trans-acting proteins can act as RNA chaperones either to maintain or to attain the correct three-dimensional structure of the IRES that is required for efficient assembly of the 48 S complex.

In the case of the arginine/lysine transporter cat-1, translation of a small upstream open reading frame unfolds an inhibitory structure in the mRNA leader eliciting a conformational change and allowing the leader to form an inducible IRES (50). This study suggests that regulation of dynamic RNA interactions is a mechanism for regulating IRES activity.

In our case, the proposed mechanism of alternative splicing controlling translation start site selection leads to different quantities of VEGF and N-VEGF depending upon the VEGF isoform being expressed. In the case of VEGF121, the exclusive initiation at the CUG codon leads to stoichiometric expression of VEGF and N-VEGF (Fig. 6A). On the other hand, expression of VEGF165 and -189 isoforms leads to the creation of a surplus of VEGF compared with N-VEGF (Fig. 6A). In cells expressing multiple VEGF isoforms, the ratio of VEGF to N-VEGF is more complex. The biological relevance of such complexity is not fully understood. Indeed, Carmeliet et al. (18) demonstrate in transgenic mice that a reduction in total VEGF level due to the absence of VEGF164 and -188 results in lethal vascular defects. While this is a consequence of a decrease in secreted VEGF, an imbalance between VEGF and N-VEGF could participate in the observed phenotype. Nevertheless, the function of N-VEGF cleavage fragment is intriguing.

In conclusion, we have showed that alternative translation initiation of VEGF, which gives rise to a series of protein isoforms is a result of a complex interaction between the spliced mRNA, IRES sequences, and multiple, in frame start codons. This newest level in the regulation of VEGF expression couples pre-mRNA splicing with selection of the translation initiation codon. The existence of such concomitant post-transcriptional regulations awaits discovery in other messengers encoding growth regulatory proteins. Intriguingly, FGF-2, another major angogenic factor regulated by alternative polyadenylation and mRNA stability, is also regulated at the translational level (24, 51, 52). The evolution of such transcriptional and post-transcriptional regulatory networks permits the synthesis of a complex repertoire of proteins from a single mRNA. In this report, we have demonstrated that it is also possible to interweave an additional layer of complexity to translational regulation by enabling different alternatively spliced mRNAs to vary the regulation of codon start site selection through modulation of IRES activity.

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VEGF121 tertiary structure (see “Materials and Methods”). Each color box corresponds to complementary sequences schematized on the linear sequence in panel E. The ΔG of the base pairing between VEGF coding sequence and exon 5/8 junction is −47.89 kcal/mol. D, working hypothetical model of VEGF165/189 tertiary structure (see “Materials and Methods”). Each color box corresponds to complementary sequences schematized on the linear sequence in panel E. The ΔG of the base pairing between sequences of IRES A and exon 6/7 junction is −77.24 kcal/mol. E, linear VEGF189 cDNA sequence from IRES A to the stop codon (positions 745–1685). ATG 1039 is red underlined. Each color box corresponds to complementary sequences potentially involved in the formation of structures represented in panels C and D.
Control of the Vascular Endothelial Growth Factor Internal Ribosome Entry Site (IRES) Activity and Translation Initiation by Alternatively Spliced Coding Sequences
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