

## Translational Induction of VEGF Internal Ribosome Entry Site Elements During the Early Response to Ischemic Stress

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**Vascular endothelial growth factor-A (VEGF), a powerful factor involved in vasculogenesis and angiogenesis, is translationally regulated through 2 independent internal ribosome entry sites (IRESs A and B). IRESs enable an mRNA to be translated under conditions in which 5'-cap-dependent translation is inhibited, such as low oxygen stress. In the VEGF mRNA, IRES A influences translation at the canonical AUG codon, whereas the 5' IRES B element regulates initiation at an upstream, in frame CUG. In this study, we have developed transgenic mice expressing reporter genes under the control of these 2 IRESs. We reveal that although these IRESs display low activity in embryos and adult tissues, they permit efficient translation at early time points in ischemic muscle, a stress under which cap-dependent translation is inhibited. These results demonstrate the in vivo efficacy of the VEGF IRESs in response to a local environmental stress such as hypoxia.**

**V**ascular endothelial growth factor-A (VEGF) is a primary regulator of blood vessel formation and plays diverse roles in the regulation of both physiological and pathological angiogenesis. VEGF expression is highly regulated in vitro<sup>1</sup> and in vivo<sup>2</sup> at both transcriptional and posttranscriptional levels by hypoxia. Furthermore, VEGF and its receptors are upregulated in ischemic limb.<sup>3</sup>

We have previously shown that 2 independent internal ribosome entry sites (IRES A and IRES B) are present in the 5' untranslated region (UTR) of the human VEGF mRNA and control translation initiation at 2 alternative start codons, AUG 1039 and CUG 499, respectively.<sup>4–6</sup> IRES A allows the maintenance of VEGF translation under hypoxic conditions in a cap-independent manner.<sup>7</sup> IRES B, upstream of CUG

499, directs cap-independent translational initiation of a longer VEGF isoform (L-VEGF).<sup>5</sup>

The activities of these IRESs have been investigated in vivo with transgenic mice using a bicistronic reporter vector strategy, previously described in studies with IRESs from fibroblast growth factor 2 (FGF2),<sup>8,9</sup> c-myc,<sup>10</sup> and encephalomyocarditis virus.<sup>8,10</sup>

## Materials and Methods

### Plasmid Constructions

The mouse VEGF 5'-UTR sequence was cloned by PCR from mouse genomic DNA. We replaced the human VEGF sequence in pCRVL<sup>4</sup> by the mouse IRES A or IRES B sequence. PCR products were obtained by amplification of mouse VEGF 5'-UTR sequence with the primers 5'-AAAGGATCCGCTAGCTCGGGCTGGAG-AAG-3' and 5'-AAACCATGGTTTCGGAGGCCGTCGGGG-GC-3' (IRES A) or 5'-AAAGGATCCCAAGGCAGACTATTCA-GCGGA-3' and 5'-AAACCATGGGCAAGATCCCAAGGG-GGAGG-3' (IRES B). Trimolecular ligation of fragments *Bam*HI-*Xho*I, *Xho*I-*Nco*I of pCRVL with the PCR products digested with *Bam*HI and *Nco*I leads to the replacement of the human VEGF sequence, creating the plasmids pCRAL (containing IRES A) and pCRBL (containing IRES B) (Figure 1).

Full mouse VEGF 5'-UTR, IRES A, and IRES B sequence was also inserted into the pGL3-basic digested by *Bg*III-*Nco*I to generate pGL3AB, pGL3A, and pGL3B, respectively.

Constructs containing the human VEGF IRESs, as well as FGF2 and EMCV IRESs, have been previously described.<sup>4,5,8,10</sup>

### Cell Culture

NIH 3T3 (93061524), L929 (85011425), and B82L (85011408) mouse cell lines were obtained from European Collection of Cell Cultures and cultivated according to their instructions. Cell transfections and IRES activity measurements a Dual-Luciferase Reporter Assay System (Promega) were performed as previously described.<sup>4</sup>

### Transgenic Mice

Transgenic C57Bl/63CBA embryos and adult 6-week-old mice were identified by PCR and Southern blot analysis. Two strains for each construct were chosen based on the following criteria: low copy number of the integrated plasmid, as revealed by Southern blot, and similar expression of the transgene at the RNA level, as determined by quantitative RT-PCR using LucR as the target. LucR expression was similar for IRES A or IRES B constructs (<20% difference between the different lines), whereas all strains expressing IRESs A and B exhibited a 2-fold lower expression compared with other constructs. Every experiment was performed with both lines.

### Quantitative Real-Time RT-PCR

Total RNA from tibialis muscle, isolated with the SV total RNA Isolation System (Promega), was subjected to quantitative RT-PCR. Additional details are provided in the online data supplement at <http://circres.ahajournals.org>. Quantification was performed as previously described.<sup>9</sup>

### Hindlimb Tourniquet-Induced Ischemia Experiments

Groups of 6- to 8-week-old male mice were subjected to 3 hours of unilateral tourniquet-induced hindlimb ischemia.<sup>11</sup> Reperfusion was obtained by removing the tourniquet after 3 hours of ischemia, and then analyses were performed 1 or 24 hours later.

Blood flow was monitored under anesthesia (milligrams of ketamine per kilogram of body weight/milligrams of xylazine per kilogram of body weight, intraperitoneally) using a laser Doppler imager (Lisca, Perimed) or scintigraphy. Luciferase assays and mRNA extractions were performed on muscle lysates.

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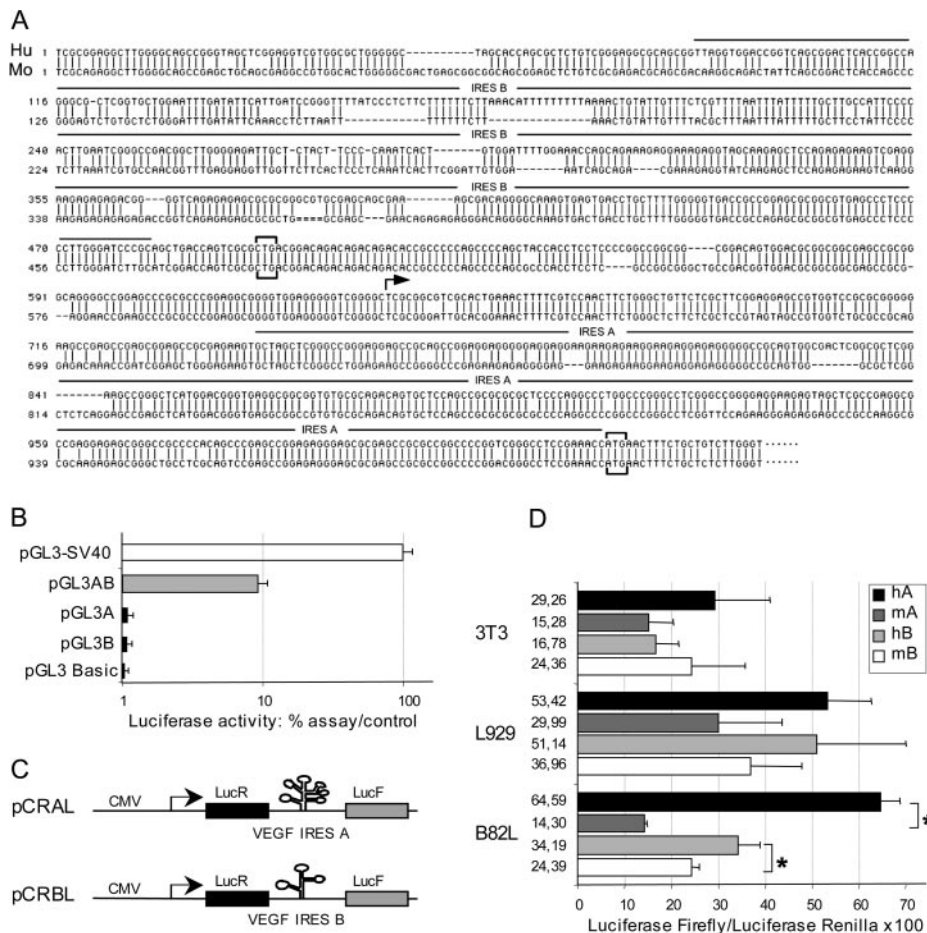
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**Figure 1.** The murine VEGF 5' UTR contains 2 IRES elements. **A**, Alignment of Human (Hu) and mouse (Mo) VEGF 5' UTR sequences. The 2 IRES sequences are designated with lines above them. CUG and AUG start codons are bracketed. The arrow indicates the position of the +1 point of the internal promoter. **B**, Identification of the VEGF cryptic promoter by transfection of promoterless constructs. PGL3-basic and PGL3-SV40 were used as negative and positive controls, respectively. **C**, Schematic representations of bicistronic vectors carrying VEGF IRES A or B. **D**, Comparison of the activities of human IRES A (hA) and B (hB) vs mouse IRES A (mA) and B (mB), as determined by transient transfections ( $n=6$ ) of 3T3, L929, and B82L mouse cell lines. Data are expressed as mean  $\pm$  SEM. \* $P<0.05$ .

## Statistical Analysis

Statistical comparisons were made by analysis of variance (ANOVA; Fisher's protected least squares difference) and values were considered to be significant when  $P<0.05$ .

## Results and Discussion

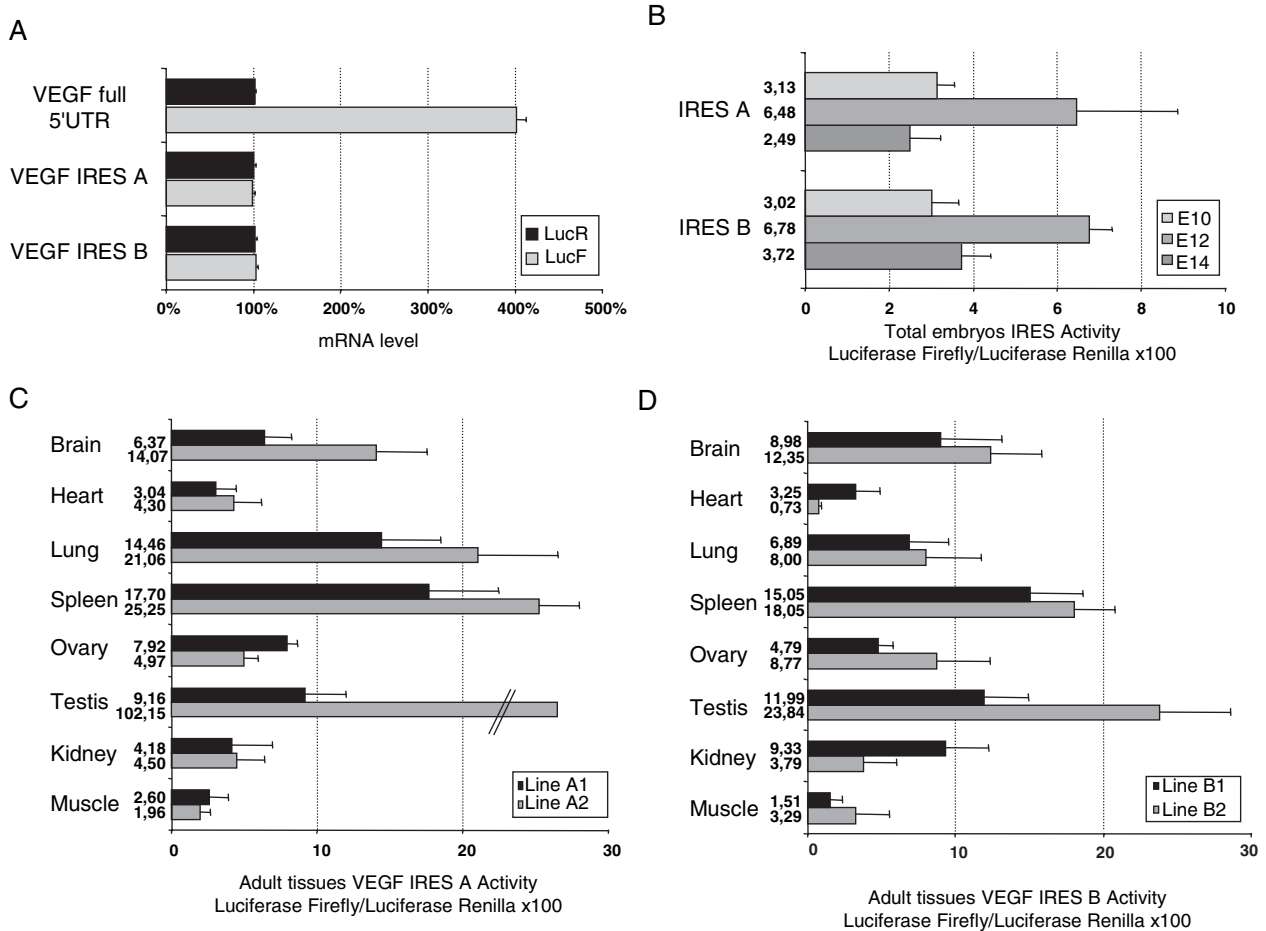
### Murine VEGF 5' UTR Contains Two IRES Elements

Before developing transgenic mice, we wanted to discern whether or not the human IRES elements, A and B, were functionally conserved in the mouse. We cloned and sequenced the mouse 5' UTR (Figure 1A). Comparison between human and mouse sequences highlighted an important conservation, particularly for the regions surrounding the start codons, the +1 start point of the internal promoter as well as the IRESA and, to a lesser extent, IRES B. By using a promoterless vector experiment, we verified the existence of a cryptic promoter in the 5' UTR of VEGF gene<sup>12</sup> but no transcriptional activity in the 2 IRES regions (Figure 1B and the online data supplement). Bicistronic reporter vectors containing mouse VEGF IRESs have been constructed as shown in Figure 1C. These constructs were assayed alongside their human counterparts. The mouse and human IRES activities were not significantly different in 3T3 and L929 cell lines; however, the mouse IRESs had lower activities in

the B82L cell line (Figure 1D). This discrepancy could be attributable to differential affinities of IRES binding negative or positive transacting factors.

### VEGF IRES A and B Activities in Transgenic Mice

Because a difference was pointed out between human and mouse IRES activities, transgenic mice were generated with constructs containing either murine VEGF IRES A, IRES B, or the complete 5' UTR. To rule out the presence of either cryptic promoters or splice sites in each individual IRES, bicistronic mRNA integrity was verified by using a quantitative real-time PCR strategy on each cistron<sup>9</sup> (Figure 2A). As expected, the lines expressing IRES A or B gave LucR/LucF mRNA ratios of 1. In contrast, mice expressing the full-length 5' UTR constructs exhibited a ratio of 4.2. This result coupled with the in vitro experiments (Figure 1B and Tet-off experiments in the online data supplement) demonstrated the conservation of the cryptic promoter previously identified between human IRES A and IRES B.<sup>12</sup> Thus we restricted our studies to mice with only IRES A or B. Both of the 2 VEGF IRESs display similar expression levels in embryos and patterns of expression in adult mouse tissues. Interestingly, a peak was found at embryonic day (E12), which corresponds to a period of intense angiogenesis (Figure 2B). In adult



**Figure 2.** VEGF IRES activities in transgenic mice. A, Determination of cryptic promoter activity by measurement of LucR and LucF mRNA levels in the different transgenic mice. B, Transgenic embryos were harvested at 10, 12, and 14 days postcoitum (E10, E12, and E14), and IRESs activities were measured (n=6). C and D, Different organs from 2 independent transgenic lines expressing pCRAL (IRES A) (C) or pCRBL (IRES B) (D) were harvested from adult transgenic mice, and IRES activities were measured (n=10). Data were expressed as mean±SEM.

tissues, IRES activities overall displayed a low efficacy. Interestingly, a slight tissue specificity was observed for both IRESs, in contrast to the strong tissue specificity reported for the FGF2 IRES<sup>8</sup> (Figure 2C and 2D).

### VEGF IRES A and B Activity Is Highly Induced in Ischemic Muscle

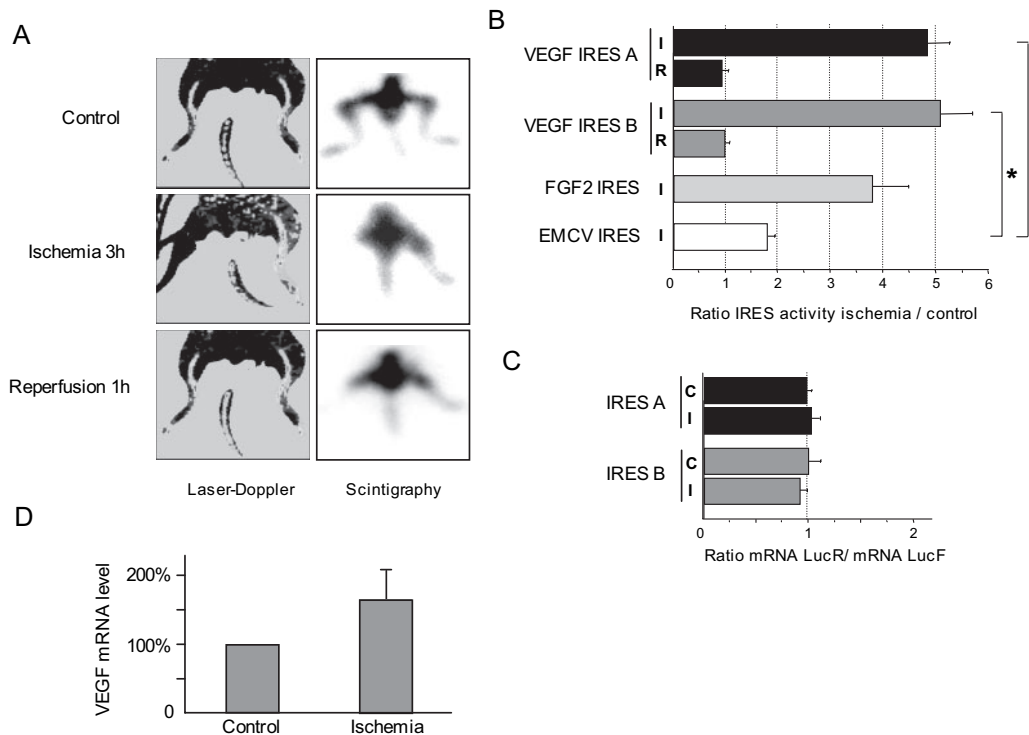
Ischemic skeletal muscle displays a significant stimulation of VEGF expression, which in turn plays a critical role in the angiogenic response.<sup>3</sup> Because IRESs allow protein expression under stress conditions that inhibit cap-dependent translation, we investigated the consequences of ischemic stress on VEGF IRES-dependent translation in vivo. Laser Doppler and scintigraphy experiments were performed to ensure the ischemic state of the whole left limb during the 3 hours of tourniquet-induced ischemia, with the unaffected right limb serving as a control (Figure 3A). To quantitate the ischemic induction of IRES activity, we calculated the ratio of reporter activities between the ischemic limb and the control limb. Three hours after muscle tissue was subjected to severe hindlimb ischemia, we found both IRES A and B were 5 times more active in the ischemic limb versus the control limb (Figure 3B). Furthermore, the FGF2 IRES activity was

4-fold stimulated, whereas the EMCV IRES was only 2-fold activated. Thus the angiogenic growth factors VEGF and FGF2 seem to have evolved IRES elements that not only enable their translation by 5'-cap-independent mechanisms but also allow enhancement of translation under ischemia, a stress condition whose response requires the expression of these factors. Finally, after 24 hours of reperfusion, activities of both VEGF IRESs returned to preischemic values (Figure 3B). We verified the integrity of the bicistronic mRNAs in the limb muscle by real-time PCR and found a marginal fluctuation in the level of message under ischemic conditions between lines A and B (Figure 3C).

Endogenous VEGF mRNA levels in the ischemic muscle never exceeded 150% of that found in the control muscle, as determined by quantitative RT-PCR (Figure 3D). Although mRNA stabilization and/or transcriptional induction presumably play a role in the stress response, the enhancement of IRES-dependent translation represents a rapid and efficient mechanism by which protein synthesis can be induced as a result of the application of an acute stress.

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**Figure 3.** VEGF IRES activities are upregulated in ischemic muscle. A, Ischemic state and blood flow were assessed by laser Doppler and scintigraphy analysis. B, Fold induction of IRES activities corresponding to the ratio of IRES activities in muscle ischemic/control (I) or reperused/control (R) ( $n \geq 6$ ). C, Bicistronic mRNA integrity was determined by mRNA levels for LucF and LucR in ischemic (I) and control (C) muscles ( $n \geq 4$ ). D, Endogenous VEGF mRNA level determined by quantitative RT-PCR. \* $P < 0.05$

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### Disclosures

None.

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**KEY WORDS:** VEGF ■ translation ■ internal ribosome entry site ■ transgenic mice ■ ischemia