Internal Translation Initiation Mediated by the Angiogenic Factor Tie2*

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Tie2 is an endothelium-specific receptor tyrosine kinase required for normal blood vessel maturation. We report that Tie2 mRNA translation is maintained under hypoxic conditions. To identify the mechanism responsible for this, we undertook structure/function analysis of the Tie2 5'-untranslated region (UTR). Transcription start site mapping indicates the existence of a small mRNA isoforms containing unusually long 5'-UTRs (>350 nucleotides) with five upstream open reading frames. We find internal ribosome binding activity that allows the Tie2 mRNA to initiate in a cap-independent fashion. Our data provide a framework for understanding how Tie2 mRNA is translated despite a cumbersome structured 5'-UTR and how its production is secured under unfavorable environmental conditions.

Angiogenesis is an essential step in allowing tumors to grow beyond 1–2 mm in diameter (1). Although the vasculature of most adult tissues is quiescent, during embryogenesis or in pathological conditions such as cancer, a pro-angiogenic setting is established. At least two families of growth factors, vascular endothelial growth factor (VEGF)1 and the angiopoietins, are required for this process. VEGF mediates its effects through VEGFR-1/Flt-1 and VEGFR-2/Flk-1/KDR, two endothelial receptors implicated as central regulators of the vascular system under both normal and abnormal physiological conditions (2–5). VEGF expression is stimulated under hypoxic conditions by the transcription factor hypoxia-inducible factor-1 (HIF-1), which regulates a large range of physiological responses as a consequence of reduced oxygen availability (6, 7).

Angiopoietin receptors belong to a second family of angiogenic factors essential for blood vessel maturation. Angiopoietin-1 (Ang-1) is an agonist of endothelial cell tyrosine kinase receptor Tie2/tek (8). Studies with Tie2 null mice indicate that the angiopoietin/Tie2 signaling system plays a role in the later steps of angiogenesis (9). Consistent with a central role for Tie2 in angiogenesis, germ line-activating mutations in humans are associated with vascular dysmorphogenesis (10). Additionally, the naturally occurring Tie2 antagonist, Ang-2, disrupts angiogenesis in vivo (11). Blocking Tie2 activation with recombinant Ang-2 significantly inhibits tumor growth (12) and is associated with activation of apoptosis (13), possibly due to disruption of Akt signaling (14). Hence, studies aimed at better defining the regulation of Tie2 expression are important in assessing anti-angiogenic therapeutic avenues.

In addition to profound transcriptional effects (6, 7), exposure of cells to hypoxia attenuates protein synthesis by decreasing translation initiation (15, 16). Two main steps of initiation are targets for regulation, either 43 S ribosomal complex formation (by affecting eIF2 phosphorylation status (17)) or the mRNA/ribosome binding step (18). Ribosome recruitment in eukaryotes can occur by two mechanisms, a cap-dependent process and by internal ribosome recruitment. Cap-dependent recruitment occurs through eIF4F-mediated recognition of the m7G-cap structure and involves binding of the 43 S preinitiation complex near the mRNA 5'-end, followed by scanning to the appropriate AUG start codon (18). Barriers to the scanning process, such as mRNA secondary structure and uORFs, impinge in a negative fashion on the translational efficiency of a mRNA species. The cap dependence of an mRNA is thought to be a function of 5'-cap-proximal secondary structure; hence mRNAs with reduced secondary structure will have reduced dependence on eIF4F for initiation, whereas those with increased secondary structure are more dependent on eIF4F for initiation (19, 20). Internal ribosome recruitment allows an mRNA to bypass the cap-dependent initiation requirement for eIF4F. These different mechanisms of initiation provide a layer of gene regulation by which expression of specific mRNAs can be maintained or altered independent of others. Although hypoxia exerts inhibitory effects on cap dependent ribosome binding (16), several mRNAs implicated in angiogenesis remain efficiently translated, including VEGF (21–24), HIF-1α (25), and Tie2 (this report). Whereas VEGF and HIF-1α achieve this by recruiting ribosomes internally to an IRES, the issue of how Tie2 is able to circumvent this translational block has not been addressed previously. Herein, we present functional studies that identify the presence of an IRES element within the Tie2 5'-UTR, highlighting the complexities of Tie2 expression at the level of translation.

EXPERIMENTAL PROCEDURES

Cell Lines, Transfections, and Hypoxia Treatment—Primary human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (Walkersville, MD) and maintained in endothelial growth medium-2 supplemented with growth factors, 2% fetal bovine serum, 50 μg/ml gentamicin, and 50 μg/ml amphotericin according to the manufacturer’s instructions.

DNA transfections into primary HUVECs were performed using

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1 The abbreviations used are: VEGF, vascular endothelial growth factor; HIF, hypoxia-inducible factor; Ang, angiopoietin; IRES, internal ribosome entry site; UTR, untranslated region; HUVEC, human umbilical vein endothelial cell; EMCV, encephalomyocarditis virus; ORF, open reading frame; uORF, upstream ORF; RT, reverse transcriptase; nt, nucleotide; ODC, ornithine decarboxylase; RACE, rapid amplification of cDNA ends; RPA, RNA protection assay.
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Lipopectin (Invitrogen), as specified by the manufacturer. Briefly, 1.5–1.8 × 10⁶ cells were seeded per 10-cm² plate and grown in endothelial growth medium-2 supplemented with 2% fetal bovine serum. Cells were harvested 24 h post-transfection, and luciferase activity was measured using the dual-luciferase reporter assay system (Promega, Madison, WI). Transient RNA transfections were performed using the cationic lipid reagent, DMRIE-C (Invitrogen). Approximately 1.8 × 10⁶ HUVECs were seeded per 10-cm² plate 24 h prior to transfection. Cells were then transfected with 20 μg of capped and polyadenylated mRNA and harvested 5 h post-transfection. Transfections were performed in duplicate and repeated three times. For polysome analysis and mRNA isolation, extracts prepared from HUVECs grown under hypoxia (>0.01% O₂) and normoxia (20% O₂) for 16 h were fractionated by velocity sedimentation on 10–50% sucrose gradients. All experiments with HUVECs were performed between passages two and five.

**Primer Extension**—Human placental poly(A)⁺ or total RNA was obtained from Clontech (Palo Alto, CA). Total RNA from HUVECs transiently transfected with TRIZol (Invitrogen) according to the manufacturer’s suggested protocol. Oligonucleotides were end-labeled with [γ³²P]ATP (3000 Ci/mmol; PerkinElmer Life Sciences) using T4 polynucleotide kinase and purified after separation on 8% denaturing polyacrylamide gels. A mixture of 2.5 μg of mRNA or 25 μg of total RNA and 2 × 10⁵ cpm of the primer was annealed at 65 °C for 15 min. The extension reaction was performed using SuperScript II (Invitrogen) according to the manufacturer’s instructions. Extension products were separated on an 8% denaturing gel and the products visualized by autoradiography.

**RNAse Protection Assay**—Templates for riboprobe synthesis were prepared by subcloning a 244-bp fragment containing the hTie2 5’-flanking sequence (−467 to −224) into the pBluescript II KS(+) plasmid (Stratagene, La Jolla, CA) to create hTie2UP-Ribo15/pKSHI. [α-³²P]UTP was used for MAXscript in vitro transcription kit (Ambion, Austin, TX) to generate riboprobes from the Xhol-linearized hTie2UP-Ribo15/pKSHI. The radiolabeled RNA was purified by 8% denaturing gel electrophoresis, followed by elution for 12 h at 37 °C in 0.5 mM ammonium acetate, 1 mM EDTA, 0.2% SDS. The efficiency of labeling was determined by scintillation counting, and 1 × 10⁶ cpm of each labeled riboprobe was used per reaction. RNAse protection assay was performed using Ultrascript II (Ambion) according to the manufacturer’s instructions. Experiments were performed and analyzed using an 8% denaturing gel and the products visualized by autoradiography.

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**Northern Blot Analysis**—Total RNA was isolated following transfection with bicistronic reporters. Following extraction of RNA with TRIzol, 21 μg of total RNA was loaded onto a 1% formaldehyde-agarose gel, separated by electrophoresis and transferred to a nylon membrane. Hybridization was in ExpressHyb hybridization solution (BD Biosciences) at 68 °C with a ~400-bp firefly luciferase-specific ³²P-labeled probe. Autoradiography of the membrane was performed at ~80 °C with film (Kodak X-Omat) to visualize the radioactive signal.

**Metabolic Labeling with [³⁵S]Methionine**—HUVECs were plated at 2.7 × 10⁵ cells per 150-mm plate and incubated for 24 h, followed by a further 12–36-h incubation under either normoxic or hypoxic conditions. Cells were then washed with phosphate-buffered saline and incubated in methionine-free RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum and [³⁵S]methionine (PerkinElmer Life Sciences) 4 h prior to harvesting. Cells were washed three times with phosphate-buffered saline and harvested in lysis buffer (25 mM HEPES (pH 7.4), 157 mM NaCl, 10% glycerol, 2.5 mM EDTA, 2.5 mM EGTA, 0.5% Triton X-100, 5 mM β-mercaptoethanol, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin A). The cell lysates (500 μg of total protein) were precleared with protein G-Sepharose and immunoprecipitations performed with anti-Tie2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-β-actin antibodies (Abcam Inc., Cambridge, MA), and anti-ornithine decarboxylase (ODC) (Sigma) antibodies, respectively. The immunoprecipitates were then subjected to SDS-PAGE, followed by fluorography. Experiments were performed twice at least in triplicate.

**RESULTS**

Tie2 mRNA Is Translated under Hypoxic Conditions—To assess the translational behavior of Tie2 mRNA under hypoxic conditions, we monitored its distribution across polysomes from normoxic and hypoxic treated primary HUVECs (Fig. 1). Under hypoxic conditions, there is a reduction in polysomes accompanied by an apparent increase in amount of free ribosomal subunits (Fig. 1A), as reported previously (23, 25) and consistent with a reduction in protein synthesis associated with this hypoxic biological treatment. Quantitative RT-PCR from fractions collected from the bottom or top half of the polysomes (N/H-4 and N/H-3, respectively), as well as from the top of the gradient (N/H-1) and the region containing the ribosomal subunits (N/H-2), revealed that the distribution of Tie2 and VEGFR mRNAs remain unchanged when cells are transferred from normoxic to hypoxic conditions (Fig. 1B). In contrast, the vast majority of β-actin mRNA appears in heavy polysomes under normoxic conditions (N-4) and redistributes to lighter fractions (H-3, H-2, H-1) when HUVECs are transferred to hypoxic conditions (Fig. 1B). This is similar to what has been reported previously for β-actin mRNA redistribution when cells are exposed to hypoxia (25, 28). We also assessed the change in polysome distribution of a second mRNA encoding ODC. Translation of this transcript is generally cap-dependent (29, 30), except during mitosis (31). As the majority of cells in our experiment were not in mitosis (<5%), we expect ODC to behave as a cap-dependent transcript. The majority of ODC mRNA from HUVECs under normoxic growth conditions was in N-2 or heavy polysomes (N-4). Upon exposure of HUVECs to hypoxia, the majority of the mRNA in the N-4 fraction shifted to lighter polysomes (H-3). These results are consistent with a reduction in cap-dependent protein synthesis associated with hypoxia (23, 25). Like other reports documenting mRNA redistribution among polysomes during hypoxia, we do not observe a complete disaggregation of β-actin (25, 28) or ODC mRNA into free mRNA (Fig. 1B), despite an apparent flattening of the polysome peak (Fig. 1A). We attribute this to the relative insensitivity of using spectrophotometry to monitor polysomes and suspect that there is some residual polysomes in the hypoxia-treated samples.

To assess Tie2 protein levels under hypoxic conditions, we performed Western blot analysis on total protein isolated from HUVECs (Fig. 2A). HIF-2α protein was detected only in extracts prepared from hypoxic cells confirming activation of the hypoxic response (Fig. 2A). In this Western blot analysis, an-
ti-H3 histone was used as a loading control for HIF-2α (Fig. 2A). Consistent with the polysome profiling results (Fig. 1), Tie2 protein was present in both normoxic and hypoxic treated cells (Fig. 2A). (In this experiment, a nonspecific band labeled with an asterisk is used as a loading control for Tie2.) Tie2 protein was actively translated under both normoxic and hypoxic conditions as assessed by metabolic labeling of HUVECs followed by immunoprecipitation with anti-Tie2 antibodies (Fig. 2B). In contrast, synthesis of β-actin and ODC was significantly decreased after exposure of HUVECs to hypoxic stress (Fig. 2B). Taken together these results indicate that translation of Tie2 mRNA is resistant to the reduction in protein synthesis accompanied by hypoxic treatment of HUVECs.

The levels of Tie2, VEGF, and β-actin mRNAs in total RNA isolated from HUVECs were also measured by quantitative RT-PCR (Fig. 2D). VEGF mRNA levels increased 70-fold in response to hypoxia, whereas those of β-actin decreased ~4-fold (Fig. 2D). These results are consistent with previously reported effects of hypoxia on VEGF and β-actin gene expres-
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Mapping the Human Tie2 5’-Untranslated Region—In an attempt to understand how Tie2 translation is maintained under hypoxic stress, during a time when overall cap-dependent protein synthesis is attenuated, we undertook a structure/function approach to mapping of the Tie2 5’-UTR. The transcription start sites of the human Tie2 mRNA have been previously defined using 5’-RACE and primer extension (36). The results suggested the presence of 10 transcription initiation sites situated from 269 to 414 nucleotides upstream of the initiator AUG codon (37). However from this report, it is not possible to assess which sites were detected by primer extension versus those identified by 5’-RACE. We re-mapped the transcription initiation sites in the human Tie2 mRNA to identify transcription start sites. Primer extension analysis was performed with three oligonucleotides (hTie2-UP-6, hTie2-UP-7, hTie2-UP-1), which together span the entire length of the Tie2 5’-UTR (Fig. 3A). No specific products were visualized with hTie2-UP-6 or hTie2-UP-7, indicating that these likely reside too far downstream of the transcription start site(s). However, two distinct extension products were obtained with hTie2-UP-1 (Fig. 3B). These fragments map to nts −372 and −377 of the Tie2 5’-UTR (Fig. 3D, encased within gray boxes).

To complement the primer extension results and determine whether we could detect the same start sites, we performed RNase protection experiments. The probe used extended from −467 to −224 (Fig. 3D, generated by primers hTie2-UP-4 and hTie2-UP-5). Two major protected fragments of 127 and 149 nucleotides were observed (Fig. 3C). These mapped to positions −372 and −350 of the Tie2 5’-UTR (Fig. 3D). We note that the start site at nucleotide −350 may have been missed in the primer extension analysis, due to high background in this size range of the gel (Fig. 3C). Taken together, the RNase protection and primer extension results indicate that a major Tie2 transcription site lies at nucleotide −372. This site also lies within 2 nucleotides of the identified murine Tie2 transcription initiation site (37). Sequence analysis of over 300 5’-RACE PCR products demonstrated sequence contiguity with the Tie2 genomic DNA sequence, indicating the absence of a frequent splicing event within the Tie2 5’-UTR. Hence, the Tie2 mRNA isoforms identified here contain several features predicted to be inhibitory to a cap-dependent initiation mechanism, notably a lengthy 5’-UTR (372 nts), five uORFs, and an AUG codon embedded with the last uORF (Fig. 3D). In this report, we further characterize the translational properties of the −372 isom.

The Tie2 5’-UTR Mediates Internal Initiation—One mechanism by which Tie2 could be efficiently translated under hypoxic conditions (Fig. 2B), a situation that reduces cap-dependent translation initiation (16, 26), would be if Tie2 translation proceeded by internal ribosome recruitment to the 5’-UTR. To test this, the Tie2 5’-UTR was positioned within the intercistronic spacer between the firefly and Renilla luciferase coding regions (Fig. 4A). We also generated a construct in which all uAUGs had been removed by mutating AUG to UUG, to assess their potential contribution to IRES activity (Fig. 4A). As a negative control for these experiments, we utilized a deletion mutant of the EMCV 5’-UTR that does not support internal ribosome binding (Fig. 4A, construct Ren/ΔEMC/FF). The length of the ΔEMC sequence is −440 bp, similar to the size of the Tie2 5’-UTR used in these experiments (372 bp). In addition, we generated constructs in which the human immunodeficiency virus TAR element (TAR(+III)) was positioned upstream of the Renilla luciferase. This allowed us to probe whether firefly luciferase expression was independent of Renilla expression and not a consequence of ribosome reinitation (Fig. 4A). The stable secondary structure of this element (DG = −49.9 kcal/mol) is expected to act as a physical barrier to 5’-end-mediated initiation and decrease expression of the Renilla luciferase protein (27).

Transfection experiments with HUVECs revealed that expression vectors containing the TAR(+III) element upstream of the Renilla luciferase initiation codon displayed a 5-fold re-

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\(^2\) E.-H. Park and J. Pelletier, unpublished data.
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**Fig. 3. Determination of the transcription start site(s) of human Tie2 mRNA.** A, schematic representation of the human Tie2 5′-UTR. The uORFs are depicted as well as their relative reading frames by different shades of gray. Asterisks above an uORF indicate the presence of an internal AUG, in-frame with the uORF initiator AUG. B, mapping of the transcription start sites of the human Tie2 gene by primer extension. Total RNA from HUVECs, human placental poly(A)+ RNA, and yeast tRNA were analyzed using oligonucleotide hTie2-UP-1 as a primer. Sequencing reactions were used as molecular markers. The two major products (arrows) terminate at adenine residues and are encased in the gray boxes in D. The smaller products observed from the human placenta mRNA preparations were not reproducibly seen among different experiments. C, RNase protection mapping of the human Tie2 mRNA. The RNase protection assay (RPA) of human placental poly(A)+ RNA or yeast RNA was performed as described under “Experimental Procedures.” Sequencing reactions were used as molecular markers. The arrows indicate the size and position of migration of the protected fragments. D, nucleotide sequence upstream of the human Tie2 initiation codon. The Tie2 translation start codon is designated as +1, and five uORFs are indicated in bold and italics. The locations of the oligonucleotides used for primer extension and RPA are shown. The transcription start sites determined by primer extension and RPA are indicated by gray boxes and gray circles above the nucleotide, respectively.

Production in Renilla luciferase activity relative to the constructs lacking this stem-loop barrier (Fig. 4B, compare lanes 1–3 to lanes 4–6). Very low levels of firefly luciferase activity were detectable from cells transfected with Ren/ΔEMC/FF or TAR(III)/Ren/ΔEMC/FF, since the ΔEMC 5′-UTR does not allow for efficient reinitiation or for internal ribosome recruitment (Fig. 4B, lanes 1 and 4). However, significant and comparable firefly luciferase activities were detected in extracts from cells transfected with Ren/Tie2/FF and TAR(III)/Ren/Tie2/FF (Fig. 4B, compare lane 2 with lane 5) or with Ren/Tie2ΔaUG/FF and TAR(III)/Ren/Tie2ΔaUG/FF (Fig. 4B, compare lane 3 with lane 6). These results indicate that the Tie2 5′-UTR mediates the production of firefly luciferase independent of Renilla luciferase, as would be expected if an IRES was present.

**IRES Activity Is Maintained during Hypoxia**—One function of the Tie2 IRES might be to maintain efficient translation during hypoxia (Fig. 1). To assess whether the putative Tie2 IRES could function in a bicistronic context under hypoxic conditions, we transfected HUVECs with Ren/ΔEMC/FF or Ren/Tie2ΔaUG/FF and placed these cells under normoxic or hypoxic conditions (Fig. 4C). Luciferase activities were then measured in cell extracts. We observed that the relative Renilla luciferase activity decreased ~2-fold when cells were exposed to hypoxia (Fig. 4C). This effect is likely an underestimation of the true decrease in protein synthesis, since it relies on measuring enzyme activity, some of which will be already present prior to the start of hypoxic treatment. Expression of the firefly luciferase cistron was increased slightly under hypoxic conditions in Ren/Tie2ΔaUG/FF (Fig. 4C). The hypoxic/normoxic ratio indicates that the potential Tie2 IRES activity was even slightly stimulated under hypoxic conditions (Fig. 4C). These results indicate that the putative Tie2 IRES is functional during hypoxic treatment.

Northern blot analysis indicates that bicistronic transcripts produced in vivo from Ren/ΔEMC/FF, Ren/Tie2/FF, and Ren/Tie2ΔaUG/FF were of comparable quality (Fig. 4D). No major degradation product was visible that could account for the differential expression of firefly luciferase from Ren/Tie2/FF and Ren/Tie2ΔaUG/FF, relative to Ren/ΔEMC/FF (Fig. 4B).

Another alternative interpretation for the observed results would be the existence of cryptic splice sites upstream of the firefly luciferase coding region that could eliminate a portion, or all of, the upstream Renilla sequences. Subsequent translation of a spliced transcript could then lead to increased firefly luciferase levels, without the need to invoke the existence of an IRES. As a test for this, we performed RT-PCR on total RNA isolated from HUVECs that had been transfected with Ren/Tie2/FF or Ren/Tie2ΔaUG/FF (Fig. 5A). PCR primers were designed that targeted the 5′-UTR of Renilla and within the firefly luciferase coding region (Fig. 5A, left panel). A single DNA product was obtained following RT-PCR of RNA from transfected cells and corresponds in size to the expected full-length PCR product encompassing the Renilla ORF and Tie2...
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5'-UTR (Δ1.7 kbp) (Fig. 5A). PCR products were not observed when reverse transcriptase was omitted from the RT-PCR (Fig. 5A). From this experiment, no evidence of splicing was obtained as assessed by the lack of smaller PCR products (whose amplification should be favored during the PCR) (Fig. 5A).

To confirm that the results obtained in Fig. 4 were not due to the presence of a cryptic promoter within the Tie2 5'-UTR, we generated expression vectors in which the Tie2 5'-UTR was placed upstream of the firefly luciferase gene in a promoterless plasmid backbone (Fig. 5B). A firefly reporter construct containing SV40 promoter (SV40/Luc) was used as a positive control. Transfection of these reporter constructs into HUVECs showed that pGL-Basic (negative control) and hTie2/Luc constructs produced similar levels of low luciferase activity (Fig. 5C). On the other hand, SV40/Luc yielded over 100 times higher luciferase activity compared with hTie2/Luc and pGL-Basic (Fig. 5C). These results indicate the absence of a detectable cryptic promoter activity within the Tie2 5'-UTR.

To confirm and extend these results, we performed a series of mRNA transfection experiments. We generated capped and polyadenylated mRNAs from bicistronic constructs containing: (i) the EMCV 5'-UTR with the deleted uAUGs (negative control), (ii) the poliovirus IRES (positive control), and (iii) the Tie2 5'-UTR containing or lacking all uAUGs (Fig. 6A). Following transfection of mRNAs into HUVECs, firefly and Renilla production was assessed by Western blotting (Fig. 6B) or by measuring luciferase activities (Fig. 6C). Western blotting experiments revealed that production of Renilla luciferase was similar among the four transfected mRNA species, whereas the levels of firefly luciferase differed among expression constructs (Fig. 6B). No firefly luciferase was observed from cells transfected with TAR(+III)/Ren/AEMC/FF (lane 3), consistent with the absence of an IRES in this construct. In contrast, transfection of TAR(+III)/Ren/Polio/FF mRNA into HUVECs produced high levels of firefly luciferase (lane 4). Firefly luciferase was also detectable from cells transfected with TAR(+III)/Ren/Tie2/FF and TAR(+III)/Ren/Tie2ΔuAUG/FF mRNAs, with slightly more being produced from the construct lacking uAUGs (Fig. 6B, compare lane 1 with lane 2).

These results were confirmed by monitoring Renilla and firefly luciferase activities (Fig. 6C). The FF/Ren ratio obtained from HUVECs transfected with TAR(+III)/Ren/Polio/FF mRNA was ~100-fold greater than that obtained from cells transfected with TAR(+III)/Ren/AEMC/FF mRNA, consistent with the presence of an IRES in the former transcript (Fig. 6C). The FF/Ren ratio of TAR(+III)/Ren/Tie2ΔuAUG/FF trans-
The presence of an IRES within the Tie2 5'-UTR provides an explanation for how this mRNA is able to escape the reduction in translation imposed by hypoxia. Exposure of cells to hypoxic conditions has been shown to be associated with a decrease in cap-dependent translation as a result of increased formation of the eIF4E/4E-BP1 inhibitory complex (16, 43). In addition, increased phosphorylation of eIF2α associated with hypoxia also contributes to decreased general protein synthetic rates but increases the production of a specific set of mRNAs (15).

The idea that the Tie2 IRES allows an escape from translation repression during hypoxia is supported by our experiments in which the Tie2 IRES activity was maintained, and even slightly stimulated, under hypoxic conditions (Fig. 4C).

The Tie2 mRNA 5'-UTR contains five uORFs, an unusually high number for a eukaryotic transcript (Fig. 3D). The primer combination used to assess the polysome distribution of the hTie2 mRNA in Fig. 1B spanned five uORFs and the first 60 nucleotides following the initiation codon (Fig. 3D). Our quantitative RT-PCR results demonstrate that the Tie2 transcript obtained using these primers is associated with polysomes under both normoxic and hypoxic conditions, indicating that translating Tie2 mRNA contains this complex 5'-UTR with its five uORFs. We note that in both DNA and RNA transfections, expression of firefly luciferase is increased when all of the Tie2 uAUGs are deleted (Figs. 4B and 6). A simple interpretation of these results is that some (or all) of the uORFs reside between the IRES and the initiator ATG and are encountered by the internally initiated ribosome. This could have the consequence
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FIG. 6. Messenger RNA transfection into HUVEC cells. A, schematic representation of mRNA species used in mRNA transfections. B, Western blot analysis of extracts from HUVECs transfected with bicistronic mRNAs shown in A. Cell lysates were analyzed by immunoblotting with anti-Renilla luciferase and anti-firefly luciferase antisera. The nature of the transfected mRNAs is indicated above the lanes. C, luciferase activities were measured from HUVEC lysates following RNA transfection. The relative FF/Ren ratio obtained for each mRNA construct is shown. The ratio of the negative control, TAR(+III)Ren/Tie2/FF, was arbitrarily set to 1. The results represent the average of three independent experiments performed in duplicate, and the error bars denote the error of the mean.

of reducing translation initiation, due to ribosomes dropping off during re-initiation, or bypassing the initiation codon of the major ORF, due to an inability to re-acquire a ternary complex. Another possibility for the role of the Tie2 uORFs in translation initiation could be to simply prevent the majority of ribosomes that have initiated by a cap-dependent mechanism from reaching the Tie2 initiation codon, thereby acting as a damper to prevent 5'-end-mediated initiation from interfering with IRES activity on the Tie2 mRNA template. Alternatively, the structure of the Tie2 IRES may be dynamically affected by scanning ribosomes. In this situation, ribosomes initiated in a cap-dependent fashion could affect Tie2 5'-UTR secondary structure and impact on IRES function. Such a model has been implicated in the function of the cap-1 IRES, where disruption of RNA-RNA interactions between the 5'- and 3'-end of an IRES prevents inducible internal initiation. The cap-1 IRES activity is restored upon inhibition of 5'-end mediation initiation (44). Whether a similar scenario occurs on the Tie2 IRES needs to be experimentally addressed and may indicate that translation initiation on the Tie2 mRNA is more dynamic and complex than expected.

Our findings indicate that Tie2 belongs to a class of mRNA transcripts harboring IRES activity to circumvent the reduction of protein synthesis imposed during hypoxic stress. Other mRNAs in this category include c-myc (45–48), HIF-1α (25), and VEGF (21–24). The presence of IRESs in the 5'-UTRs of two mRNAs encoding major angiogenic factors (VEGF and Tie2) suggest that this translation initiation mechanism plays an important role in the control of angiogenesis. The advantage of IRES-dependent expression during this process might be to allow specific gene expression during a time when global translation is reduced. We would expect that additional genes whose expressions are maintained during hypoxia represent excellent candidates for harboring IRESs. Additionally, the Tie2/angiopoietin-1 signaling system has been implicated in regulating quiescence of hematopoietic stem cells in the bone marrow niche (49). Tie2 expression needs to be maintained during quiescence, a physiological state associated with reduced protein synthesis (50). Hence, the Tie2 IRES described herein may serve to circumvent the translation block imposed by quiescence to keep Tie2 protein levels constant.

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REFERENCES
1. Risau, W. (1997) Nature 386, 671–674
2. Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhooke, A., Harker, K., Eberhard, C., Dedegor, C., Pawling, J., Moon, L., Cullen, D., Risau, W., and Nagy, A. (1996) Nature 380, 435–439
3. Ferrara, N., Carver-Grone, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., Powell-Braxton, L., Hillan, K. J., and Moore, M. W. (1996) Nature 380, 439–442
4. Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X. F., Breitman, M. L. (1995) Nature 376, 62–66
5. Grillo, H. S., and Molema, G. (2000) Pharmacol. Rev. 52, 237–268
6. Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. (2001) Curr. Opin. Genet. Dev. 11, 293–299
7. Semenza, G. L. (2002) Trends Mol. Med. 8, 562–567
8. Suri, C., Jones, P. F., Patel, S., Bartunkova, S., Maisonneuve, P. C., Davis, S., Sato, T. N., and Yancopoulos, G. D. (1996) Cell 87, 1171–1180
9. Dumont, D. J., Grabow, G. H., Puri, M. C., Gertsenstein, M. Auerbach, and Breitman, M. L. (1994) Genes Dev. 8, 1897–1909
10. Vikkula, M., Boon, L. M., Carraway, K. L., 3rd, Calvert, J. T., Diamonti, A. J., Goumnerova, L., Pasyk, K. A., Marshuk, D. A., Warman, M. L., Cantley, L. C., Mulliken, J. B., and Olsen, B. R. (1996) Cell 87, 1181–1190
11. Maisonneuve, P. C., Suri, C., Jones, P. F., Bartunkova, S., Wiegand, S. J., Radziejewska, J., Compton, D., McClain, J., Allrich, T., Papadopoulos, N., Daly, T. J., Davis, S., Sato, T. N., and Yancopoulos, G. D. (1997) Science 277, 55–60
12. Lin, P., Buxton, J. A., Acheson, A., Radziejewska, J., Maisonneuve, P. C., Yancopoulos, G. D., Channon, K. M., Hale, L. P., Dewhirst, M. W., George, S. E., and Peters, K. G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8829–8834
13. Jones, N., Voskas, D., Master, Z., Zarao, R., Jones, J., and Dumont, D. J. (2001) EMBO Rep. 2, 438–445
14. Papapetropoulos, A., Pultorak, M., Mabbouhi, K., Kalh, R. G., O'Connor, D. S., Li, F., Alteri, D. C., and Sessa, W. C. (2000) J. Biol. Chem. 275, 9102–9105
15. Koumenis, C., Nazcki, L., Kotzitzinsky, M., Rastani, S., Diehl, A., Sonenberg, N., Chinnaswamy, A., and Weisberg, H. G. (2002) Mol. Cell. Biol. 22, 7405–7416
16. Arsham, A. M., Howell, J. J., and Simon, M. C. (2003) J. Biol. Chem. 278, 29655–29660
17. Clemens, M. J. (2001) J. Cell. Mol. Med. 5, 221–239
18. Gintas, A. C., Raught, B., and Sonenberg, N. (1999) Annu. Rev. Biochem. 68, 913–963
19. Gehrke, L., Auron, P. E., Quigley, G. J., Rich, A., and Sonenberg, N. (1983) J. Biochemistry 22, 5157–5164
20. Srikrishna, V. P., Palkova, Z., and Cuthbertson, G. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7722–7727
21. Stein, I., Bun, E., Kain, D., and Keshet, E. (1998) Mol. Biol. Cell 18, 3112–3119
22. Miller, D. L., Dibbens, J. A., Damert, A., Risau, W., Vadas, M. A., and Goodall, G. J. (1998) FEBS Lett. 434, 417–420
23. Lang, K. J., Kappel, A., and Amdoll, G. J. (2002) Mol. Cell. Biol. 12, 1792–1801
24. Mennes, F., and Dranitsaris, G. (2002) Mol. Cell. Biol. 22, 1181–1190
25. Zaal, J. A., and Korsten, M. (2002) Biochim. Biophys. Acta 1555, 125–134
26. Rousseau, D., Kaspary, R., Rosenwald, I., Gehrke, L., and Sonenberg, N. (1996) EMBO J. 7, 2831–2837
27. Gorlach, A., Canzian, C., Kvitkova, I., Vogt, L., Wengler, R. H., and Warman, M. L. (1990) Biochim. Biophys. Acta 1006, 125–134
28. Levy, A. P., Levy, N. S., Wegner, S., and Goldberg, M. A. (1995) J. Biol. Chem. 270, 13333–13340
29. Mandriota, S. J., and Pepper, M. S. (1998) Circ. Res. 83, 852–859
30. Hewitt, P. W., Daff, E. L., and Murray, J. C. (1998) Biochem. Biophys. Res. Commun. 252, 546–551
31. Fadel, B. M., Boulou, S. C., and Quertermous, T. (1998) Biochem. J. 330, 353–354
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38. Peters, K. G., Kuntos, C. D., Lin, P. C., Wong, A. L., Rao, P., Huang, L., Dewhirst, M. W., and Sankar, S. (2004) Recent Prog. Horm. Res. 59, 61–71
39. Park, Y. S., Kim, N. H., and Jo, I. (2003) Microvasc. Res. 65, 125–131
40. Willam, C., Koehe, P., Jurgensen, J. S., Grafe, M., Wagner, K. D., Bachmann, S., Frei, U., and Eckardt, K.-U. (2000) Circ. Res. 87, 370–377
41. Christensen, R. A., Fujikawa, K., Madore, R., Oettgen, P., and Varticovski, L. (2002) J. Cell. Biochem. 85, 505–515
42. Lin, S., Shyu, K. G., Lee, C. C., Wang, B. W., Chang, C. C., Liu, Y. C., Huang, F. Y., and Chang, H. (2002) Biochem. Biophys. Res. Commun. 296, 710–715
43. Tinton, S. A., and Buc-Calderon, P. M. (1999) FEBS Lett. 446, 55–59
44. Yaman, I., Fernandez, J., Liu, H., Caprara, M., Komar, A. A., Koromilas, A. E., Zhou, L., Snider, M. D., Scheuner, D., Kaufman, R. J., and Hatzoglou, M. (2003) Cell 114, 519–531
45. Johannes, G., and Sarnow, P. (1998) RNA (N. Y.) 4, 1500–1513
46. Nanbru, C., Prats, A. C., Drosogmans, L., Defrance, P., Huez, G., and Prats, A. C. (1997) J. Biol. Chem. 272, 32061–32066
47. Arai, F., Hirao, A., Ohmura, M., Sato, H., Matsuoka, S., Takubo, K., Ito, K., Koh, G. Y., and Suda, T. (2004) Cell 118, 149–161
48. Hofmann, G. E., and Hand, S. C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8492–8496
49. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275