Mechanisms of Translation Initiation of Receptor Tyrosine Kinase Tie2

by

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June 2006

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Your file Votre référence
ISBN: 978-0-494-27827-7
Our file Notre référence
ISBN: 978-0-494-27827-7

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Abstract

Angiogenesis is a process of new blood vessel formation and is the culmination of both mitogenic and tissue remodeling events, resulting in neovascularization. It is a physiological process that is required for, amongst others, normal embryonic development, female reproductive function, and wound healing. Angiogenesis is a tightly regulated process which is balanced by both positive and negative factors. However, in many disease states, including diabetic retinopathy, age-related macular degeneration, rheumatoid arthritis, and several cancers, dysregulation of angiogenesis contributes to disease progression. Previous published reports have implicated the coordinated activities of at least two families of receptor tyrosine kinases (RTKs), the vascular endothelial growth factor receptor (VEGFR) and the Tie receptor families, in this process.

Tie2, an endothelial-specific receptor tyrosine kinase, plays an essential role in normal blood vessel maturation, remodeling, and stability. Tie2 expression is also up-regulated in various cancers indicating a role in tumor angiogenesis. The human Tie2 mRNA transcript contains an unusually long (372 nucleotides) 5' untranslated region (UTR) with five upstream open reading frames (uORFs). In this thesis, we demonstrate that the Tie2 5' UTR promotes cap-independent translation, indicating the presence of functional internal ribosome entry site (IRES). In addition, we illustrate that Tie2 IRES activity is maintained, and even slightly stimulated, under hypoxic conditions when cap-dependent protein synthesis is attenuated. We further show that the Tie2 IRES is functional during quiescence, another condition known to compromise cap-dependent translation. These results present how Tie2 mRNA is translated despite a cumbersome structured 5' UTR and how its production is secured under unfavorable environmental conditions.

We define experimental conditions where the Tie2 IRES is not active, allowing us to assess the contribution of cap-dependent translation to Tie2 protein synthesis. We demonstrate evidence that Tie2 mRNA can be translated via both cap-dependent scanning mechanism and internal initiation. Moreover, we show that the presence of the uORFs within the 5' UTR is inhibitory to downstream translation initiation. Our results suggest that the uORFs serve to decrease the proportion of ribosomes competent for

reinitiation as they traverse the mRNA 5' UTR and thus minimizing interference with the IRES and/or mediating inefficient translation of the potent protein under normal conditions. Like many other cellular IRESes, the entire Tie2 5' UTR appears to be required for maximum IRES activity.

Taken together, our results underscore the complex mechanisms to control gene expression at the level of translation initiation of the Tie2 mRNA.

Résumé

L'angiogenèse est un processus de formation de nouveaux vaisseaux sanguins. Elle est la culmination des événements mitogèniques et de transformations de tissus ayant pour résultat la néo-vascularisation. C'est un processus physiologique qui est requis pour le développement embryonnaire normal, les fonctions reproductrices femelles, et la cicatrisation des blessures. L'angiogenèse est un processus hautement réglé qui est équilibré par des facteurs positifs et négatifs. Cependant, dans plusieurs maladies, comprenant la rétinopathie diabétique, la dégénérescence maculaire liée à l'âge, la polyarthrite rhumatoïde, et plusieurs cancers, la mauvaise régulation de l'angiogenèse contribue à la progression de la maladie. Les rapports publiés antérieurement ont ciblé les activités d'aux coordonnées au moins de deux familles de récepteur de kinase de tyrosine (RTKs) dans ce processus : les récepteurs du facteur de croissance endothélial vasculaire (VEGFR) et les récepteurs Tie.

Le Tie2 est un récepteur de kinase de tyrosine spécifique aux cellules endothéliales. Ce récepteur joue un rôle essentiel dans la maturation naturelle, le remodelage et la stabilité des vaisseaux sanguins. Tie2 est fortement exprimé dans divers cancers indiquant un rôle dans l'angiogenèse tumorale. L'ARN messager de Tie2 contient une région non traduite on qui s'est exceptionnellement longue (372 nucléotides) avec cinq cadres ouverts de lecture en amont. Dans cette thèse, nous démontrons que la région 5 prime non traduite de Tie2 favorise la traduction indépendamment de la structure cap, indiquant la présence d'un site interne fonctionnel pour l'entrée des ribosomes. Nous montrons que l'activité du site interne pour l'entrée des ribosomes de Tie2 est maintenue, et légèrement stimulée, dans des conditions hypoxiques quand la synthèse de protéine dépendante de la structure cap est atténuée. Nous démontrons par la suite que le site interne pour l'entrée des ribosomes de Tie2 est fonctionnel pendant la dormance, une autre condition connue pour compromettre la traduction dépendante a la structure cap. Ces résultats démontrent comment l'ARN messager de Tie2 est traduit même en présence d'une structure encombrante non traduite en 5 prime et comment sa production est sécurisée dans des conditions environnementales défavorables.

Nous définissons des conditions expérimentales dans lesquelles le site interne pour l'entrée des ribosomes de Tie2 n'est pas actif. Ces conditions nous permettant d'évaluer la contribution de la traduction dépendante de la structure cap à la synthèse de la protéine Tie2. Nous démontrons que l'ARN messager Tie2 peut être traduit par un mécanisme de balayage dépendant de la structure cap et par l'initiation interne. D'ailleurs, nous montrons que les cadres ouverts de lecture en amont dans la région 5 prime non traduite de Tie2 inhibitrice l'initiation de traduction en aval. Nos résultats suggèrent que les cadres ouverts de lecture en amont servent à diminuer la proportion de ribosomes compétents pour relancer la traduction pendant que les ribosomes traversent la région 5 prime non traduite de l'ARN messager. Cette action peut servir à minimiser l'interférence avec le site interne d'entrée des ribosomes de Tie2. Il peut également rendre la forte traduction de Tie2 inefficace dans des conditions normales. Comme beaucoup d'autres sites interne pour l'entrée des ribosomes cellulaires, la région 5 prime non traduite de Tie2 semble être requis pour l'activité maximale du site interne d'entrée des ribosomes.

Dans l'ensemble, nos résultats soulignent la complexité des mécanismes génétiques au niveau de l'initiation de traduction de l'ARN messager de Tie2.

Acknowledgements

My first and most earnest acknowledgment must go to my supervisor, Dr. Jerry Pelletier. His patience and guidance allowed me to learn what research is about. This work would not have been possible without his support. Many thanks go to the faculty members of my committee, Drs. Nahum Sonenberg and Arnim Pause for their valuable scientific insight into my research projects. I would also like to thank the CIHR/KM Hunter Foundation and the McGill Cancer Center for their financial support during my studies.

Far too many people to mention individually have encouraged me in so many ways during my studies at McGill. They all have my sincere gratitude. In particular, I would like to thank Maria Discenza (thank you for the excellent translation of the abstract into French), Park Cho, Abba Malina, Betty (Chen-Ju) Lin, and Chung-Hae Lee for their friendship and encouragement. I am also grateful to all current and previous members of the Pelletier Laboratory for their generosity (especially, you Isabelle).

I am also indebted to the staff in the Biochemistry department (Christine, Marlene, and Maureen). Without your assistance in non-lab related matters, I would have been lost.

A special thanks goes to my wonderful parents, who taught me the value of hard work by their example(s). I would like to share this moment of happiness with my family: Mom, Dad, Yun-Hee, Jin-Hee, Yeon-Hee, Abonim, Omonim, Cecilia, Edwin, and Helen. They gave me enormous support during the whole tenure of my studies.

My last, and most heartfelt, acknowledgment must go to my husband, Joseph. His support, encouragement, motivation, and companionship has inspired me and turned my journey through graduate school into a pleasure. For all that, and for being everything I am not, he has my everlasting love.

Dedication

I would like to dedicate this thesis to my family: Mom, Dad, my husband Joseph, my sisters Yun-Hee, Jin-Hee and Yeon-Hee, Abonim, Omonim, Cecilia, Edwin, and Helen. Without their unconditional love, support, and understanding, I would never have completed my present work.

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List of Abbreviations

aa	amino acid
bp	base pair
cDNA	complementary DNA
DNA	deoxyribonucleic acid
IRES	internal ribosome entry site
kb	kilobase
kDa	kilodalton
mRNA	messenger RNA
nt	nucleotide
N-terminal	amino-terminal
nm	nanometer
ORF	open reading frame
poly(A)	polyadenylation
RNA	ribonucleic acid
rRNA	ribosomal RNA
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
uORF	upstream open reading frame
UTR	untranslated region
wt	wild-type

Preface

Included in this thesis are the texts from two published manuscripts for which I am the first author. These papers have been formatted in a manner consistent with the rest of the thesis, and for convenience the references from all the chapters have been placed at the end of the thesis.

Chapter 2

Park, E. H., Lee, J. M., Blais, J. D., Bell, J. C., and Pelletier, J. (2005). Internal translation initiation mediated by the angiogenic factor Tie2. J Biol Chem *280*, 20945-20953.

The bicistronic plasmids Ren/Tie2/FF and Ren/Tie2ΔuAUG/FF were constructed by Joseph M. Lee. Polysome profiling experiments for Figure 2-1 were performed by Jamie D. Blais and real-time RT-PCRs using the polysome fractions were performed by the first author. All other experiments and figures were done solely by the first author.

Chapter 3

Park, E. H., Lee, J. M., and Pelletier, J. (2006). The Tie2 5' untranslated region is inhibitory to 5' end-mediated translation initiation. FEBS Lett *580*, 1309-1319.

The plasmid pGEMCAT/FLUC was constructed by Joseph M. Lee. All other experiments and figures were done by the first author.

Chapter 4

Park, E. H., and Pelletier, J. Translational regulation of Tie2 gene expression during quiescence. In preparation.

All the experiments and figures were done by the first author.

Original Contributions to Knowledge

1. Based on Tie2 transcription start site mapping and sequence analyses, we have identified a major Tie2 mRNA that contains five uORFs.

2. We have demonstrated that the Tie2 5' UTR possesses internal ribosome binding activity that allows the Tie2 mRNA to initiate translation in a cap-independent fashion.

3. We have observed that Tie2 protein synthesis is maintained during hypoxia and quiescence when global protein synthesis is significantly reduced. Our results have indicated that the Tie2 IRES activity plays a role in retaining the protein level constant under these translational repression conditions.

4. We have mapped the Tie2 IRES element and demonstrated that the entire 5' UTR is required for fully functional IRES activity.

5. We have shown that the Tie2 5' UTR is inhibitory to 5' end-mediated translation initiation with ribosome flow decreasing following encounters with each uORF. No single uORF was found to harbor significant *cis*-acting inhibitory activity.

CHAPTER 1

General Introduction

1.1 General Aspects of Angiogenesis

Angiogenesis is a process whereby new blood vessels are formed from previously existing ones. It is a highly regulated process requiring the precise coordination of multiple signaling pathways and is absolutely required for normal embryonic development, female reproductive function, and wound healing. Angiogenesis is a complex and dynamic program dependant on the temporal interplay of cellular proliferation, growth, death, motility, cytoskeletal reorganization, and differentiation signals, amongst others. Pathologically, angiogenesis also contributes to a large number of different and unrelated diseases. An insufficiency of angiogenesis may result in coronary artery disease, peripheral vascular disease, stroke, and delayed wound healing (Carmeliet *et al.*, 1999; Ferrara and Alitalo, 1999; Peters *et al.*, 2004). Excessive angiogenesis is often observed in diseases including arthritis, obesity, atherosclerosis, diabetic retinopathy, age-related macular degeneration, and cancer (Folkman, 2001; Hanahan and Folkman, 1996). Tumor angiogenesis is known to be essential for solid tumor growth and metastasis where pro-angiogenic growth factors produced by the tumor cells overcome other forces that tend to keep existing vessels quiescent and stable (Hanahan and Folkman, 1996). Thus, in healthy individuals, angiogenesis is tightly regulated and is maintained through a balance of pro- and anti-angiogenic factors.

The best characterized of the pro-angiogenic agents is vascular endothelial growth factor (VEGF), which is relatively unique among growth factors in terms of its specificity for the vascular endothelium (Ferrara, 1999). VEGF acts specifically on vascular endothelium because the receptors for VEGF are largely restricted to these cells. It is a critical driver of vascular formation, as it is required to initiate the formation of immature vessels during development as well as in the adults. In addition to the VEGF family, the angiopoietins are another family of growth factors specific for the vascular endothelium. As will be discussed below, the angiopoietin (Ang) family plays an important role in blood vessel maturation and remodeling at the later stage of angiogenesis.

1.2 VEGF, Its Relatives, and Their Receptors

VEGF was initially identified, characterized and purified for its ability to induce vascular leakage and permeability, as well as for its ability to promote vascular endothelial cell proliferation (Dvorak *et al.*, 1995; Dvorak *et al.*, 1999; Ferrara, 1999; Senger *et al.*, 1983). It was originally termed vascular permeability factor as well as VEGF. Another important function of VEGF was later discovered, the stimulation of endothelial cell survival in newly formed blood vessels (Alon *et al.*, 1995; Benjamin and Keshet, 1997). More recently, VEGF has also been implicated in regulating growth control of the lymphatic endothelium (Karkkainen *et al.*, 2000; Makinen *et al.*, 2001). VEGF-mediated angiogenesis is well known to play an important role in tumor growth and metastasis (Hanahan and Folkman, 1996; Toi *et al.*, 1996). To date, five VEGF isoforms, VEGFs A through E have been identified (Eriksson and Alitalo, 1999). These isoforms of the VEGF gene are produced by alternative splicing events from a single gene and differ primarily in heparin binding, which may affect their diffusion rates in the extracellular space. (Breier, 2000; Eriksson and Alitalo, 1999). VEGF ligands have been shown to bind and activate three related receptor tyrosine kinases (RTKs): VEGFR-1 (previously known as Flt-1), VEGFR-2 (previously known as KDR or Flk-1), and VEGFR-3 (previously known as Flt-3) (Eriksson and Alitalo, 1999; Neufeld *et al.*, 1999). Neuropilin-1 has been also identified as an isoform-specific receptor for VEGF, although this receptor is unique among VEGFRs in that it does not possess tyrosine kinase activity and is expressed abundantly in both endothelial and certain non-endothelial cells, including neurons and tumor cells (Soker *et al.*, 1998).

Gene targeting studies in mice have provided insights into the function(s) of VEGF and the VEGF receptors during embryonic vascular development. Loss of a single allele of VEGF (now also called VEGF-A), results in embryonic lethality at embryonic day 11 (E11) (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). This result underscores the importance of VEGF receptor signaling in the developing vasculature. Mice homozygous for VEGFR-1 null allele die between E8.5 and E9.5 due to disorganized blood vessels and misplaced endothelial cells (Fong *et al.*, 1995). Surprisingly, expression of VEGFR-1 lacking the tyrosine kinase domain is sufficient for normal vessel formation and circumvention of lethality (Hiratsuka *et al.*, 1998). Mice homozygous null for VEGFR-2,

die between E8.5 and E9.5 with a defect in endothelial and hematopoietic blood island formation (Shalaby *et al.*, 1995). Subsequent analysis indicated that this is a primary defect which may be caused by the inability of VEGFR-2 cells to localize to the yolk sac from the primitive streak (Shalaby *et al.*, 1997). More recently, it has been suggested that the hematopoietic cell defect can be partially rescued by specific *in vitro* culture conditions, while the defect in endothelial cell differentiation cannot (Hidaka *et al.*, 1999). These results indicate an absolute requirement for VEGFR-2 in endothelial cell differentiation but a conditional role for VEGFR-2 in hematopoietic cell differentiation. VEGFR-3-null embryos experience cardiovascular failure between E10 and E12 from defects in remodeling the primary vessel networks into larger blood vessels suggesting its role during blood vessel development (Dumont *et al.*, 1998). This receptor is most unique based on its expression on lymphatic vessels, for which it seems to be critical and has been linked to human hereditary lymphoedema (Karkkainen *et al.*, 2000; Makinen *et al.*, 2001). VEGF-C and VEGF-D, the ligands for VEGFR-3 seem to be important for lymphatic development. Transgenic overexpression of these ligands lead to lymphangiogenesis and lymphatic metastasis (Jeltsch *et al.*, 1997; Karkkainen *et al.*, 2001). Mice lacking VEGF-B are overtly normal and fertile, but their hearts are reduced in size, suggesting that VEGF-B may play a role in coronary vascularization and growth (Bellomo *et al.*, 2000).

1.3 The Angiopoietins and Their Receptors

1.3.1 The Angiopoietins

Recently, a second family of "growth factors" specific for the vascular endothelium has been identified, with members of this family termed the angiopoietins (Davis *et al.*, 1996; Maisonpierre *et al.*, 1997; Suri *et al.*, 1996). Similar to VEGF, the specificity of the angiopoietins for the vascular endothelium results from the restricted distribution of the angiopoietin receptors (known as Tie receptors) to endothelial cells. However, the actions of the angiopoietins appear to be quite different from those of VEGF. In fact, the angiopoietins play a crucial role in blood vessel maturation and maintenance of

endothelium integrity at the later stage of vascular development (Loughna and Sato, 2001; Yancopoulos *et al.*, 2000). There are four definitive members of the agiopoietin (Ang) family, Angl through 4. All of the known angiopoietins bind primarily to Tie2. It is unclear if there are independent ligands for the second Tie receptor, Tiel. The Angs appear to have opposing actions in endothelial cells, as Angl and Ang4 function as agonists and activate Tie2, while Ang2 and Ang3 behave as context-dependent antagonists (Holash *et al.*, 1999b; Maisonpierre *et al.*, 1997; Suri *et al.*, 1996; Valenzuela *et al.*, 1999). In mouse embryos lacking Angl, the early stages of VEGF-dependent vascular development appear to occur rather normally, resulting in the formation of a primitive vasculature. However, remodeling and stabilization of this primitive vasculature is severely disturbed, leading to embryonic lethality (Suri *et al.*, 1996). Ultrastructural analysis of vessels in these Angl knockout mice demonstrated that endothelial cells fail to associate properly with surrounding supporting cells, which provide the Angl protein that acts on the Tie2 receptor. These embryos appear grossly abnormal by E11 and die by E12.5. These observations indicate that Angl is involved in normal interactions between endothelial cells and their underlying supporting cells, and in the maintenance of vascular stability. Angl is also constitutively expressed in adult tissues where it may continue to play a required stabilizing role for existing vessels (Witzenbichler *et al.*, 1998). Transgenic overexpression of Angl results in profound hypervascularization, more than that reported for VEGF, presumably through promoting vascular remodeling events and/or decreasing normal vascular pruning (Suri *et al.*, 1998).

Shortly after the discovery of Angl, Ang2 was identified based on homology screening to Angl (Maisonpierre *et al.*, 1997). Interestingly, even though Ang2 binds to the Tie2 receptor with similar affinity to Angl, it can either activate or antagonize Tie2, depending on the cell examined. Mice deficient for Ang2 have a complex phenotype (Gale *et al.*, 2002). These mice are born relatively normal, but soon after the start of feeding, they develop chylous ascites and widespread edema. Many mice die by postnatal day 14 (P14). Transgenic overexpression of Ang2 during embryogenesis also leads to a lethal phenotype, reminiscent of that seen in embryos lacking either Angl or Tie2. These results demonstrate that Ang2 could act as a Tie2 antagonist *in vivo*, at least under some circumstances. In adult animals, Ang2 is normally expressed at low levels but is strongly

induced at sites of active vessel remodeling, such as sprouting or regressing vessels in the ovary and in tumors (Goede *et al.*, 1998; Holash *et al.*, 1999a; Holash *et al.*, 1999b; Maisonpierre *et al.*, 1997; Stratmann *et al.*, 1998; Zagzag *et al.*, 1999). One of the best characterized examples of postnatal vascular regression and remodeling is glioma. Regression of the hyaloid vasculature encasing the lens is coupled to angiogenic sprouting that leads to vascularization of the initially avascular retina (Holash *et al.*, 1999a; Holash *et al.*, 1999b). Neither regression of the hyaloid vasculature nor vascularization of the retina occurs in mice lacking Ang2. These data show that Ang2 provides a key role in destabilizing the vasculature in a manner that is necessary for its subsequent remodeling. However, other defects in the Ang2-knockout mice suggest that it may, in some circumstances, have an agonistic role (Gale *et al.*, 2002; Kim *et al.*, 2000b; Teichert-Kuliszewska *et al.*, 2001). For instance, it is highly expressed in the developing aortic wall, which does not develop properly in mice lacking Ang2. Similarly, lymphatic development is perturbed in these mice (Gale *et al.*, 2002).

Ang3 and Ang4 were discovered by homology cloning by virtue of primary structure similarity to Ang1 and Ang2 and by their ability to bind to Tie2 (Valenzuela *et al.*, 1999). Although mouse Ang3 and human Ang4 are interspecies orthologs, their amino acid identity is relatively low (Valenzuela *et al.*, 1999). To date, their functions are far less characterized than those of Ang1 and Ang2. Ang3 appears to act as an antagonist to human Tie2 whereas Ang4 can act as a strong agonist (Valenzuela *et al.*, 1999). However, Ang3 can act as a strong agonist in its native species (Lee *et al.*, 2004). Moreover, both mouse Ang3 and human Ang4 stimulate corneal angiogenesis in mouse (Lee *et al.*, 2004).

1.3.2 The Tie Receptor Family

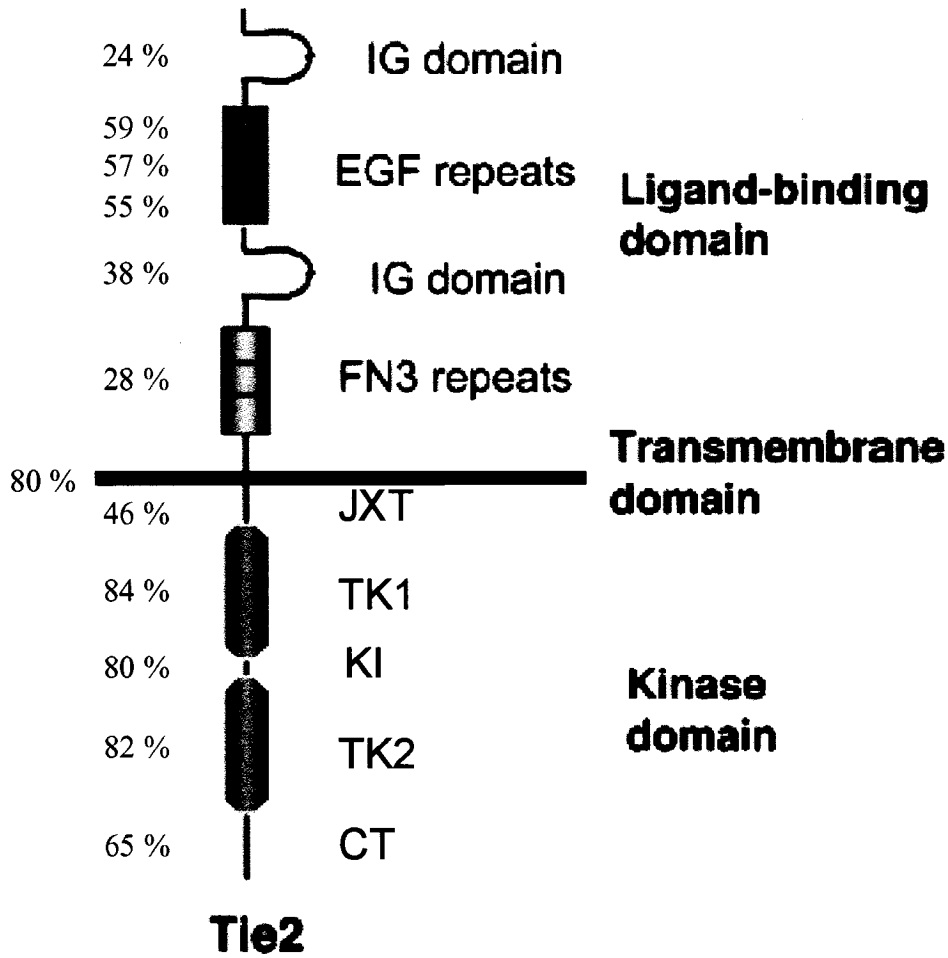
Tie (Tyrosine kinase with Immunoglobulin and Epidermal growth factor homology domains) receptors comprise another family of RTKs that are predominately expressed in vascular endothelial cells. There are two members in this class of RTKs, Tie1 (also known as Tie) and Tie2 (also known as Tek) (Dumont *et al.*, 1992; Iwama *et al.*, 1993; Maisonpierre *et al.*, 1993; Partanen *et al.*, 1992; Sato *et al.*, 1993; Schnurch and Risau,

1993). The angiopoietins have been identified and characterized as ligands for the Tie2 receptor (discussed above), and signaling through Tie2/Ang has been extensively studied (discussed below). The functional significance and signaling partners of Tie1 have not been well as defined due to the lack of an identified ligand for this receptor. Interestingly, recent studies have suggested a novel ligand-independent function for Tie1. This involves shedding of the receptor and heteromeric complex formation with Tie2 (Chen-Konak *et al.*, 2003; Marron *et al.*, 2000a; Marron *et al.*, 2000b; McCarthy *et al.*, 1999; Saharinen *et al.*, 2005; Tsiamis *et al.*, 2002). Although previous studies failed to demonstrate binding of the angiopoietins to Tie1 (Davis *et al.*, 1996; Maisonpierre *et al.*, 1997; Valenzuela *et al.*, 1999), it has recently been reported that Ang1 and Ang4 can, in fact, activate Tie1 (Saharinen *et al.*, 2005).

The highly related Tie receptors share a number of structural features and have unique extracellular domains, consisting of two immunoglobulin-like loops which flank three epidermal growth factor repeats, followed by three fibronectin-type III repeats [Figure 1-1; (Dumont *et al.*, 1993; Iwama *et al.*, 1993; Maisonpierre *et al.*, 1993; Partanen *et al.*, 1992; Sato *et al.*, 1993; Schnurch and Risau, 1993)]. The cytoplasmic regions of the receptors contain highly conserved tyrosine kinase domains including a number of phosphorylation and protein interaction sites.

Both Tie1 and Tie2 receptors are expressed in almost all endothelial cells during embryonic development (Dumont *et al.*, 1995; Dumont *et al.*, 1992; Sato *et al.*, 1993) (Korhonen *et al.*, 1995; Korhonen *et al.*, 1994; Schnurch and Risau, 1993). Phenotypic analyses of Tie1 and Tie2 null mutants suggest that these receptors have distinct roles in blood vessel development, with Tie2 required at an earlier stage than Tie1 (Dumont *et al.*, 1995; Korhonen *et al.*, 1995; Korhonen *et al.*, 1994). Mice lacking Tie1 die before E15 or at P1 (depending on the genetic background) due to extensive hemorrhage and defective microvessel integrity (Puri *et al.*, 1995; Sato *et al.*, 1995). The cellular functions of Tie1 are not clear, although the finding that Tie1 physically interacts with Tie2 in endothelial cells suggests that the receptor participates, in some aspects, in Tie2 signaling. Tie2 null embryos, in contrast, die earlier in embryonic development than

Figure 1-1. Schematic structure of Tie2 receptor tyrosine kinase. Amino acid sequence identities between various domains of Tie2 and Tie1 are shown at the left of the schematic receptor. IG domain, immunoglobulin-like domain; EGF repeats, epidermal growth factor-like repeats; FN3 repeats, fibronectin type3-like repeats; JXT, juxtamembrane domain; TK, tyrosine kinase domain; KI, kinase insert; CT, carboxyl terminal tail.



Adapted and modified from Peters et al. (2004) Recent Prog Horm Res.

those lacking Tie1, with reduction in endothelial cell numbers, abnormal vascular branching, and compromised vascular integrity [discussed below; (Dumont *et al.*, 1994; Sato *et al.*, 1995)]. Subsequently, Tie2 was also shown to be critical for definitive hematopoiesis and induced hematopoietic cell adhesion to the extracellular matrix whereas Tie1 appears to be dispensable for hematopoiesis (Partanen *et al.*, 1996; Rodewald and Sato, 1996; Takakura *et al.*, 1998).

In addition to embryonic expression, both Tie1 and Tie2 receptors are present in quiescent endothelial cells in a range of adult tissues, suggesting their role in the maintenance of quiescent adult vasculature [discussed below; (Korhonen *et al.*, 1995; Korhonen *et al.*, 1994; Wong *et al.*, 1997)]. Furthermore, the levels of these proteins are up-regulated during vascular formation in hormonally induced follicular development (Korhonen *et al.*, 1992; Wong *et al.*, 1997).

It is obvious that both Tie1 and Tie2 play essential roles in normal vascular development. However, exact processes regulated by these receptors still remain unclear. Furthermore, potential functional interaction between these two receptors suggests a far more complex situation for the Tie receptors in the vascular system.

1.4 Tie2 Receptor Tyrosine Kinase

1.4.1 Function of Tie2 in Vascular Development: Lessons from Gene-Targeted Mice

As described earlier, Tie2 is exclusively expressed in endothelial cells and is highly conserved across vertebrate species, predicting the importance of its biological function. In fact, the domain structure of Tie2 is highly conserved from zebrafish to human, with the greatest amino acid homology occurring in the kinase domain (Lyons *et al.*, 1998). During embryonic development, its expression is initially detected in endothelial cells at E7.5, and continues to be expressed in these cells throughout development.

Important biological functions of Tie2 were first revealed in the analysis of Tie2-null mutant mice. Tie2 (-/-) embryos (or embryos homozygous for the Tie2 gene mutation) die between E9.5 and E10.5 with associated cardiac failure, hemorrhage, and other

vascular defects (Dumont *et al.*, 1994; Sato *et al.*, 1995). Ultrastructural examination showed that vessels of Tie2 (-/-) embryos had a decreased number of endothelial cells and decreased contact between endothelial cells and the underlying perivascular cells (pericytes and smooth muscle cells), suggesting a role in the maturation and stabilization of the embryonic vasculature.

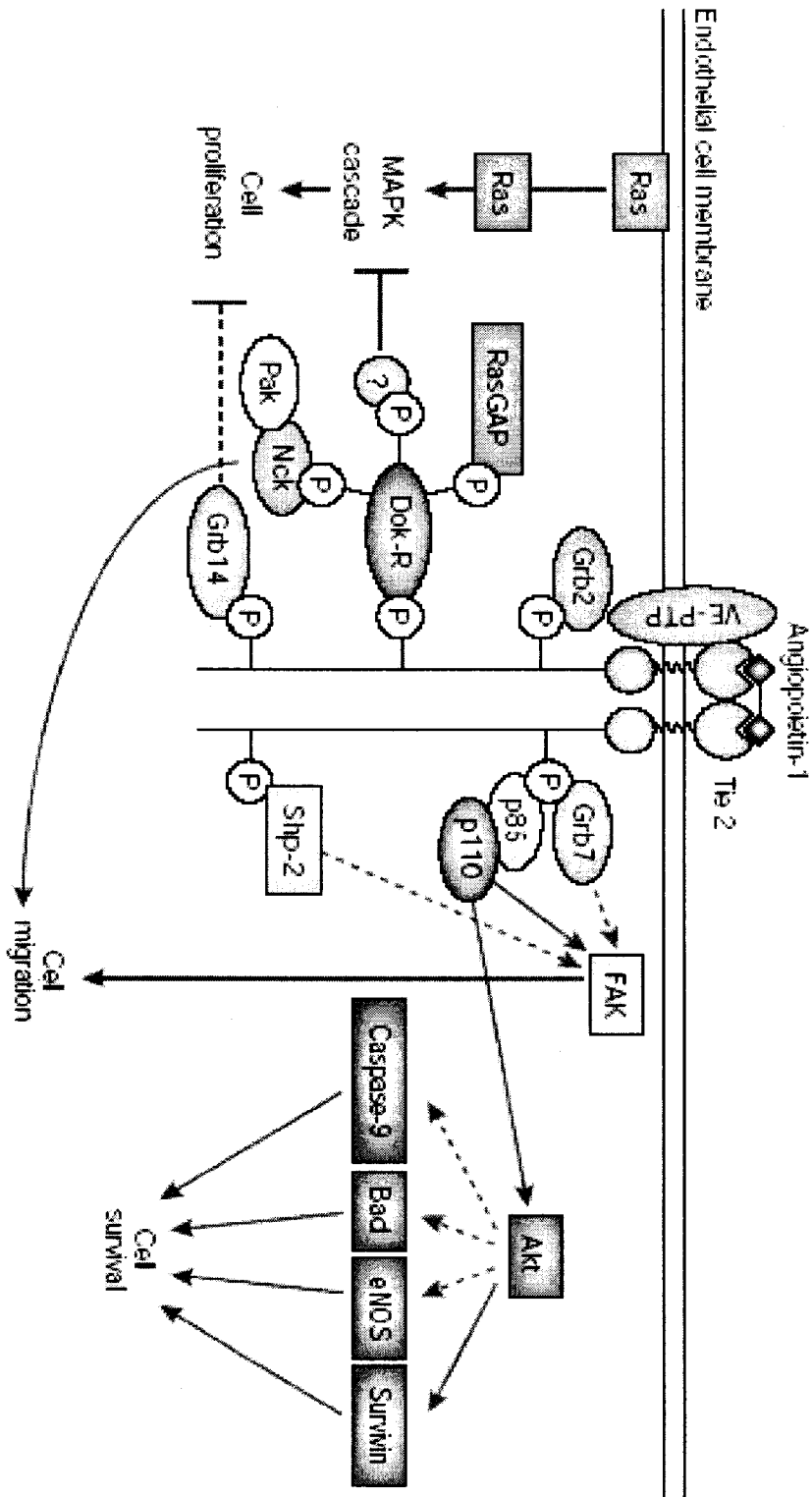
Tie2 function is regulated by ligand binding to the extracellular domain of this receptor. The binding of Angl, an agonist of Tie2, stimulates autophosphorylation of the kinase domain of Tie2. However, unlike other angiogenic growth factors, such as VEGF, Angl does not induce endothelial cell proliferation (Davis *et al.*, 1996). In contrast to Angl, Ang2 does not stimulate Tie2 autophosphorylation but instead blocks Angl-mediated Tie2 activation and endothelial cell migration (Maisonpierre *et al.*, 1997; Teichert-Kuliszewska *et al.*, 2001; Witzenbichler *et al.*, 1998).

1.4.2 Tie2 Signal Transduction Pathway

The discovery and isolation of the angiopoietins have triggered an explosion of intense investigation into the molecular mechanisms that underlie Tie2-mediated signaling. It was initially anticipated that Tie2 might provide a mitogenic signal to endothelial cells as the activated receptor associates with the Grb2, an adaptor protein which has been linked to the activation of Ras and mitogen activated protein kinase (MAPK) cell growth signaling pathways (Huang *et al.*, 1995; Jones *et al.*, 1999). However, subsequent studies have shown that Angl does not activate MAPK nor does it stimulate cellular proliferation (Davis *et al.*, 1996; Koblizek *et al.*, 1998; Kwak *et al.*, 1999; Witzenbichler *et al.*, 1998). Instead, the angiopoietins appear to regulate vascular survival and angiogenic sprouting formation by activating the Tie2 receptor.

Cell survival is controlled by a complex series of biochemical pathways (Figure 1-2). Phosphatidylinositol 3' (PI 3)-kinase has been identified as an important mediator of extracellular survival signals through regulation of the serine-threonine kinase Akt [also known as protein kinase B or PKB; (Downward, 1998; Jones and Dumont, 2000)]. The p85 subunit of PI 3-kinase is associated with the phosphorylated Tie2 receptor, resulting in activation of both PI 3-kinase and Akt (Jones *et al.*, 1999; Kontos *et al.*, 1998).

Figure 1-2. Tie2 signal transduction pathways, showing the known binding partners for Tie2. Solid arrows indicate pathways that are known to exist downstream of Tie2 and dashed arrows indicate pathways that have been inferred from studies on other RTKs. P represents a phosphorylation event. PI 3-kinase consists of two constitutively associated subunits, a p85 regulatory subunit and a p110 catalytic subunit. HCPTPA (human cellular protein tyrosine phosphatase A) has been omitted from this schematic.



Adapted from Jones et al. (2001) Nat Rev Mol Cell Biol.

Activation of Akt in turn stimulates phosphorylation and subsequent inhibition of pro-apoptotic proteins such as Bad and Caspase-9. In endothelial cells, Akt influences the production of nitric oxide through phoshporylation of nitric oxide synthetase to inactivate caspases (Jones *et al.*, 2001a). In addition, the apoptosis inhibitor survivin is upregulated following Angl-mediated Akt activation (Papapetropoulos *et al.*, 2000). Interestingly, Tie2 is constitutively phosphorylated in quiescent endothelium, suggesting that chronic activation of the PI 3-kinase signaling pathway through Tie2 is required for the maintenance and survival of endothelial cells (Wong *et al.*, 1997; Yuan *et al.*, 1999).

Activation of PI 3-kinase and Akt requires a multisubstrate docking site on Tie2 (Jones *et al.*, 1999; Kontos *et al.*, 1998). The role of the receptor in promoting endothelial cell survival is supported by observations in mice lacking Tie2, where mutant endothelial cells are progressively lost (Dumont *et al.*, 1994; Jones *et al.*, 2001b; Partanen *et al.*, 1996; Puri *et al.*, 1999). Moreover, it raises the question as to whether the defects observed in Angl-null mice might actually be due to a loss of contact between the endothelial and smooth muscle cell layers as a direct consequence of endothelial cell apoptosis (Holash *et al.*, 1999a; Patan, 1998; Suri *et al.*, 1996).

During sprouting angiogenesis, endothelial cells must alter their intercellular architecture to facilitate migration into the surrounding basement membrane following secretion of matrix-degrading proteinases. Numerous studies have shown that activation of Tie2 by Angl results in the stimulation of endothelial cell migration. This role for Angl is consistent with the findings that Tie2- and Angl-null mice have an angiogenic or migratory defect that is manifested as a lack of vessel sprouting and remodeling throughout the embryo (Dumont *et al.*, 1994; Patan, 1998; Sato *et al.*, 1995; Suri *et al.*, 1996). Angl has been shown to stimulate endothelial cell migration, sprouting and tubule formation *in vitro* (Koblizek *et al.*, 1998; Teichert-Kuliszewska *et al.*, 2001) (Hayes *et al.*, 1999; Kwak *et al.*, 1999; Papapetropoulos *et al.*, 1999; Witzenbichler *et al.*, 1998). Angl-induced endothelial cell motility also depends, in part, on PI 3-kinase activity; the inhibition of which reduces tyrosine phosphorylation of the cytoskeletal regulatory focal adhesion kinase (FAK) as well as the secretion of matrix metalloproteinase-2 (MMP-2) (Fujikawa *et al.*, 1999; Jones *et al.*, 1999; Kim *et al.*, 2000a) (Figure 1-2). Cell migration and vessel branching requires, for example, cell-cell

and cell-matrix interactions. Decreased association of the endothelial cell/smooth muscle cells and the underlying matrix is observed in the Ang1-null mutant. Therefore, this is consistent with a role for Ang1 in mediating cell-cell and/or cell-matrix interactions in vessel morphogenesis.

Tie2 can also recruit additional signaling molecules that participate in cellular pathways that affect the shape and migratory properties of cells. Upon tyrosine phosphorylation of Tie2, various molecules interact cytoplasmically with the Tie2 receptor (Figure 1-2). One of these molecules is a docking protein known as Dok-R (also known as $p56^{Dok2}$ and FRIP) that interacts with the phosphorylated Tie2 via its phosphotyrosine binding (PTB) domain, leading to the activation of Dok-R (Jones and Dumont, 1998). Moreover, phosphorylated Dok-R interacts with rasGAP, Nck and Crk. These signaling molecules may be involved in cell migration and proliferation, organization of the cytoskeleton and regulation of Ras signaling (Jones and Dumont, 1998). Tie2 has also been shown to associate with the adaptor protein Grb7 as well as the tyrosine phosphatase Shp2 and both of these signaling molecules can promote adhesion-dependent cell migration through association with activated FAK (Han and Guan, 1999; Huang *et al.*, 1995; Jones *et al.*, 1999; Yu *et al.*, 1998).

Signal transduction is a dynamic process that depends in part on the addition and removal of phosphate groups from the tyrosine residues of cell-surface receptors as well as intracellular signaling proteins. Several tyrosine phosphatases (e.g., Shp2) are expressed in endothelial cells, and differential recruitment of these signaling molecules participate in Tie2 signaling (Figure 1-2). Recruitment of Shp2 to Tie2 seems to modulate receptor activity as disruption of this interaction results in enhanced receptor phosphorylation and hyperactivation of Akt (Jones *et al.*, 2001a). In addition to Shp2, an endothelial cell-specific receptor-type phosphatase, vascular endothelial protein tyrosine phosphatase (VE-PTP; the mouse orthologue of human protein tyrosine phosphatase-ß, HPTPß) and human cellular protein tyrosine phosphatase A (HCPTPA) have also been shown to associate with Tie2 (Fachinger *et al.*, 1999; Huang *et al.*, 1999). Interestingly, the extracellular domain of VE-PTP contains a series of motifs that have been shown to participate in receptor-ligand interactions (Fachinger *et al.*, 1999). It has yet to be determined whether the angiopoietins can simultaneously bind to both Tie2 and VE-PTP

on the surface of endothelial cells, promoting the formation of Tie2/VE-PTP heterodimers. Consequently, VE-PTP could prevent reciprocal phosphorylation of Tie2 and the heterodimers would be functionally inactive. Accordingly, if this ligand were preferentially Ang2 rather than Ang1, this would provide an attractive molecular model for the antagonistic properties of Ang2.

The identification of distinct ligands that are specific for both phosphorylation and dephosphorylation of Tie2 suggests that there is exquisite control over the signaling pathways mediated by this receptor. In fact, deregulation of receptor phosphorylation through an activating mutation in the kinase domain of Tie2, which is known to cause inherited venous malformations, results in the unique activation of signaling pathways involving signal transducer and activator of transcription 1 [STAT1; (Calvert *et al.*, 1999; Korpelainen *et al.*, 1999; Vikkula *et al.*, 1996)]. Elucidation of the signaling that support the function of Tie2 in endothelial cells can ultimately contribute to our understanding of normal vascular development in both the embryo and the adult, as well as in pathological situations with perturbed angiogenesis.

1.4.3 Role of Tie2 in Pathological Angiogenesis

Coordinated expression of Tie receptors and the angiopoietins maintains vascular plasticity, and perturbations in this regulation can contribute to abnormal vascular growth. Gain-of-function mutations at the *TIE2* locus have been identified in some families with inherited venous malformations (Calvert *et al.*, 1999; Vikkula *et al.*, 1996). Moreover, elevated expression of both Tie1 and Tie2 has been observed in the endothelium of the neovasculature in numerous solid tumors as well as in healing skin wounds (Brown *et al.*, 2000; Hatva *et al.*, 1995; Kaipainen *et al.*, 1994; Korhonen *et al.*, 1992; Peters *et al.*, 1998; Salven *et al.*, 1996; Stratmann *et al.*, 1998; Wong *et al.*, 1997). Certain human leukaemia cell lines also express both Tie1 and Tie2 as well as Ang1 (Kukk *et al.*, 1997).

Physiological conditions such as hypoxia play a crucial role in the progression of angiogenesis-related diseases by altering the regulation of certain growth factors and their receptors. In this respect, it has been shown that hypoxia modulates the expression of

Ang2 and Tie1 and that Tie2 is a target of hypoxic activation by the hypoxia inducible factor-2 [HIF2, also known as EPAS1/HRF; (Enholm *et al.*, 1999; McCarthy *et al.*, 1998; Oh *et al.*, 1999; Tian *et al.*, 1997; Willam *et al.*, 2000)]

The expression of the angiopoietins and Tie receptors in a variety of tumor vasculatures indicates that the Tie/Ang pathway(s) might be involved in tumor angiogenesis. In support of this idea, interference of Tie2 signaling pathway using soluble, dominant-negative receptor approaches has already proven to be effective at inhibiting angiogenic growth in tumor-bearing mice (Lin *et al.*, 1998; Lin *et al.*, 1997; Siemeister *et al.*, 1999). Inhibition of Tie2 and possibly Tie1 signaling can therefore provide potential treatments for tumors that do not respond well to anti-VEGF therapy (Millauer *et al.*, 1994).

1.4.4 Transcriptional Regulation of the Tie2 Receptor

Studies involving transgenic mice have revealed that 1.2 kbp of nucleotide sequences upstream of the Tie2 initiator AUG is sufficient to drive Tie2 expression in vascular endothelial cells but insufficient to upregulate Tie2 expression levels during active angiogenesis (Schlaeger *et al.*, 1995). However, in combination with an enhancer sequence present within the first intron, Tie2 expression is specifically and uniformly recapitulated in all vascular endothelial cells throughout embryogenesis and adulthood (Schlaeger *et al.*, 1997). Characterization of the Tie2 basal promoter element has identified a number of *cis*- and *trans*-acting elements required for expression of this receptor (Fadel *et al.*, 1998; Fadel *et al.*, 1999; Hewett *et al.*, 1998; Minami *et al.*, 2003), including two members of the Ets family of transcription factors – ELF1 and NERF2 that are specifically expressed in endothelial cells and promote Tie2 expression (Christensen *et al.*, 2002; Dube *et al.*, 1999; Dube *et al.*, 2001). Other transcription factors have also been implicated. Interestingly, Tie2 transcription through its promoter has been shown to be activated by the transcription factor HIF2 (Tian *et al.*, 1997). Although this observation raises the intriguing possibility that HIF2 or related factors mediate hypoxia-inducible activation of the Tie2 gene during neovascularization and possibly also during embryonic development, this hypothesis has yet to be confirmed.

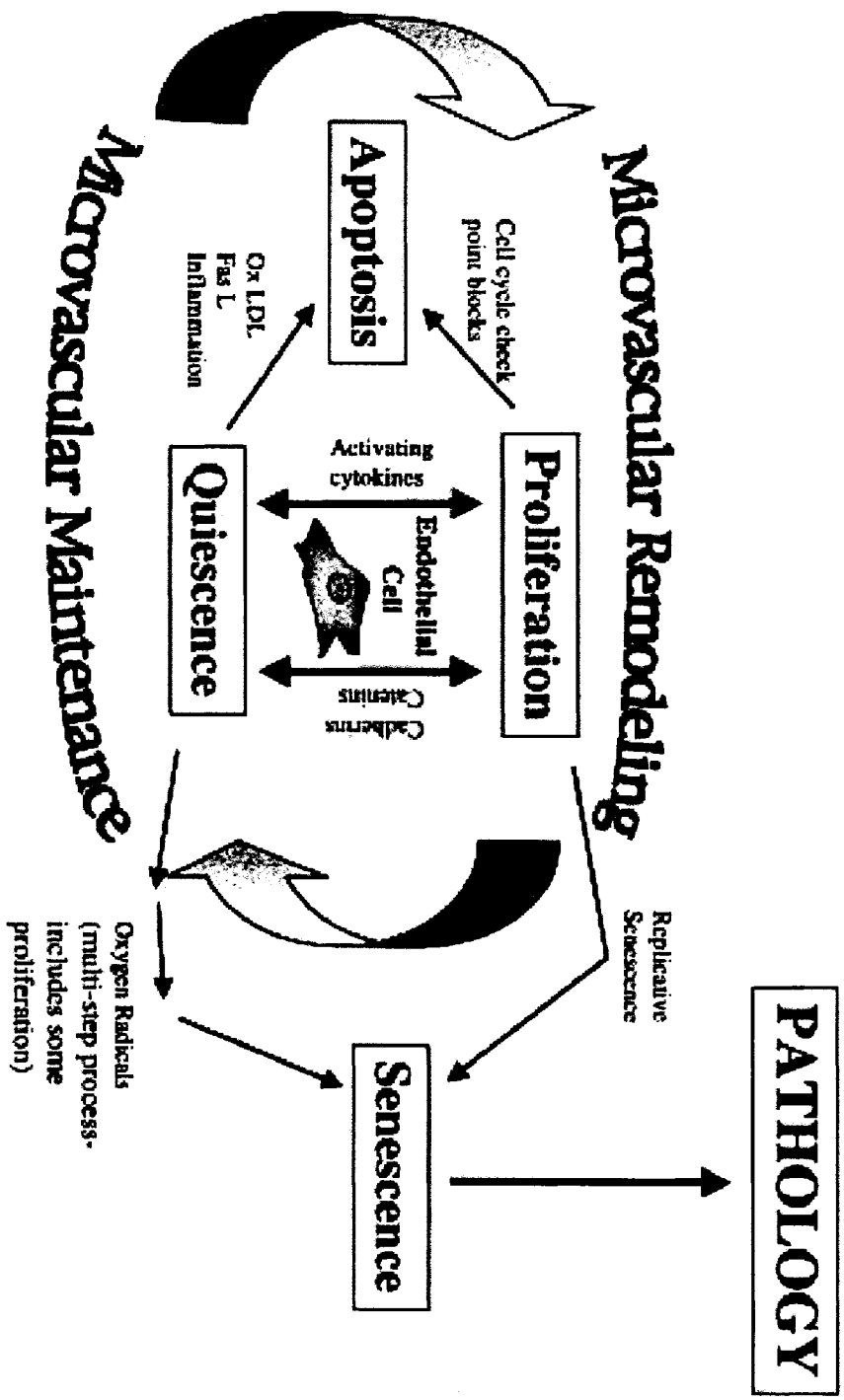
Surprisingly, very little is known about the possible translational control of Tie2 mRNA. In addition, the physical boundary of the Tie2 5' untranslated region (UTR; the RNA sequence between the 7-methyl guanosine cap and the initiation codon of the open reading frame) still remains poorly defined, with only one study defining Tie2 multiple transcriptional start sites by primer extension (Hewett *et al.*, 1998).

1.5 Endothelial Cell Quiescence

Blood vessels are primarily composed of endothelial cells, which interconnect to form the tubes that direct and maintain blood flow and tissue perfusion. To achieve homeostasis, the rate of new blood vessel growth should be well balanced with the loss of old blood vessel due to vascular reorganization and apoptosis. Endothelial cells play a key role in stabilizing and destabilizing vascular structures by balancing cellular processes of proliferation, quiescence, apoptosis, and senescence (Figure 1-3).

In actively growing endothelial cells, proliferation occurs in areas proximal to the tips of the new vessels and is controlled by many factors, including growth factors and proteins involved in extracellular matrix signaling and endothelial cell-cell signaling pathways (Chang *et al.*, 2002). The quiescent state of endothelial cells, a cell cycle arrest between mitosis and S phase, can be induced by different events such as growth factor removal and contact inhibition. Both proliferation and quiescence are reversible processes that would maintain a balance between maintenance and vascular proliferation. Depending on the environmental conditions, these endothelial cells in either quiescence or proliferation can move irreversibly into apoptosis (thus promoting remodeling) or into senescence which represents an irreversible state of cell cycle arrest. Near senescence, endothelial cells exhibit several permanently activated processes such as increased pericellular proteolytic activity, a more disorganized extracellular matrix, an augmented inflammatory adhesion molecule profile, increased adhesion of monocytes, and altered cytoskeletal components controlling cell shape (Chang *et al.*, 2002; Cooper *et al.*, 1994; Vasile *et al.*, 2001; West, 1994). Cells undergoing senescence exit the homeostatic milieu and initiate pathologic conditions for the endothelium.

Figure 1-3. Cellular proliferation, quiescence, apoptosis, and senescence are the four principal cytologic states that set the cutaneous microvascular in a dynamic balance between maintenance and remodeling. Actively growing and quiescent cells are the two reversible processes that would maintain a homeostasis between maintenance and vascular proliferation. Depending on the environmental conditions, endothelial cells in either quiescence or proliferation can move irreversibly into apoptosis (thus promoting remodeling) or into senescence. Cellular processes controlling endothelial cell survival are in a dynamic balance during remodeling and maintenance states of the microvasculature. Molecular mechanisms modulating endothelial cell proliferation and senescence must be balanced against competing processes of apoptosis and quiescence to maintain homeostasis and correct function during states of tissue stress (e.g., inflammation, wound repair, tumor growth, and environmental insults). According to theoretical notions on the contribution of cellular senescence to diseased states, endothelial cells that become senescent are regarded as having exited this homeostatic milieu and to have initiated a pathologic condition for the endothelium.



Adapted from Chang et al. (2002) J Invest Dermatol.

Molecular mechanisms modulating endothelial cell proliferation, quiescence, apoptosis, and senescence must be balanced to maintain homeostasis and correct function during states of tissue stress.

1.5.1 Regulation of Translation during Quiescence

Protein synthesis is one of the major energy-consuming processes inside the cell and is tightly coordinated with cell growth (Hershey, 1991). Regulation at the initiation step is considered to be the rate-limiting step of translation [discussed below; (Mathews *et al.*, 2000)]. In quiescent cells, the rate of protein synthesis is significantly low and some evidence has suggested that the low rate of translation in these cells is due to a lack of function of one or more eukaryotic initiation factors (eIFs). In fact, low levels of eIF2α, eIF4E, and eIF4A were observed in quiescent T cells as compared to proliferating T cells, resulting from a decreased steady state level of their mRNAs and possibly the modifications of eIFs (Mao *et al.*, 1992). A change in the phosphorylation state of several eIFs has also been shown to contribute to the regulation of protein synthesis in several cell lines (Donaldson *et al.*, 1991; Frederickson *et al.*, 1992; Morley and Traugh, 1990).

It is evident that general translation is very inefficient under quiescent conditions; however, it has recently been demonstrated that Tie2 protein is efficiently expressed in adult quiescent endothelial cells and hematopoietic stem cells. Nonetheless, little is known about the mechanisms of Tie2 protein regulation during quiescence to date.

1.5.2 Role of Tie2 in the Normal Adult Vasculature

It has been well documented that angiogenesis plays an important role in normal embryonic development. However, in the adult, once the vasculature has been established, the endothelium remains remarkably quiescent except for pathological and normal physiological processes such as tumorigenesis, wound healing, and female reproduction function (Arai *et al.*, 2004; Engerman *et al.*, 1967). The endothelial cells are among the longest-lived cells in the body outside the central nervous system; in a normal

adult vessel, only 0.01 % of endothelial cells are actively proliferating (Hanahan and Folkman, 1996; Hobson and Denekamp, 1984).

Although, as described earlier, Tie2 has been shown to play an essential role in embryonic vascular development, its role in the adult vasculature has been relatively unexplored. Tie2 is expressed on the endothelium of neovessels in skin wounds and the developing corpus luteum during ovarian folliculogenesis (Peters *et al.*, 2004; Reynolds *et al.*, 1992; Wong *et al.*, 1997). Interestingly, this receptor is also expressed in the quiescent adult vasculature (arteries, veins, and capillaries) in a wide range of adult tissues (Wong *et al.*, 1997). Studies involving transgenic mice expressing marker transgenes driven by the Tie2 promoter have confirmed the broad expression of Tie2 in the adult vasculature (Motoike *et al.*, 2000; Schlaeger *et al.*, 1997). Additionally, Tie2 phosphorylation is detected in normal adult tissues, strongly suggesting a role for Tie2 in the maintenance of quiescent adult vasculature (Wong *et al.*, 1997). More recently, studies in transgenic and knockout (KO) mice suggested a critical role for Tie2 in adult vasculature as well as in later stages of embryonic vascular development (Jones *et al.*, 2001b). However, the molecular mechanism of Tie2 regulation in the adult vasculature has yet been elucidated. Further studies using more effective strategies are required to fully assess its role in the quiescent vasculature.

1.5.3 Role of Tie2/Ang1 during Hematopoietic Stem Cell Quiescence

Stem cells have a self-renewal capacity as well as the capacity to differentiate into single or multiple lineages distinguishable from progenitor cells. Hematopoietic stem cells (HSCs) are derived from the para-aortic splanchnopleural mesoderm and the aorta-gonad-mesonephros regions, and move to fetal liver and finally to the spleen and bone marrow (BM) at late embryonic stages (Cumano *et al.*, 1996; Medvinsky and Dzierzak, 1996). The fate of HSCs is controlled by the specific microenvironment known as the stem cell niche. Key features of HSCs in the niche are that they are quiescent and adhere to surrounding cells (Arai *et al.*, 2005). In fact, the quiescent state is important for the maintenance of HSCs (Arai *et al.*, 2004). However, true stem cells must also enter the cell cycle and proliferate, resulting in differentiation to replace damaged or senescent

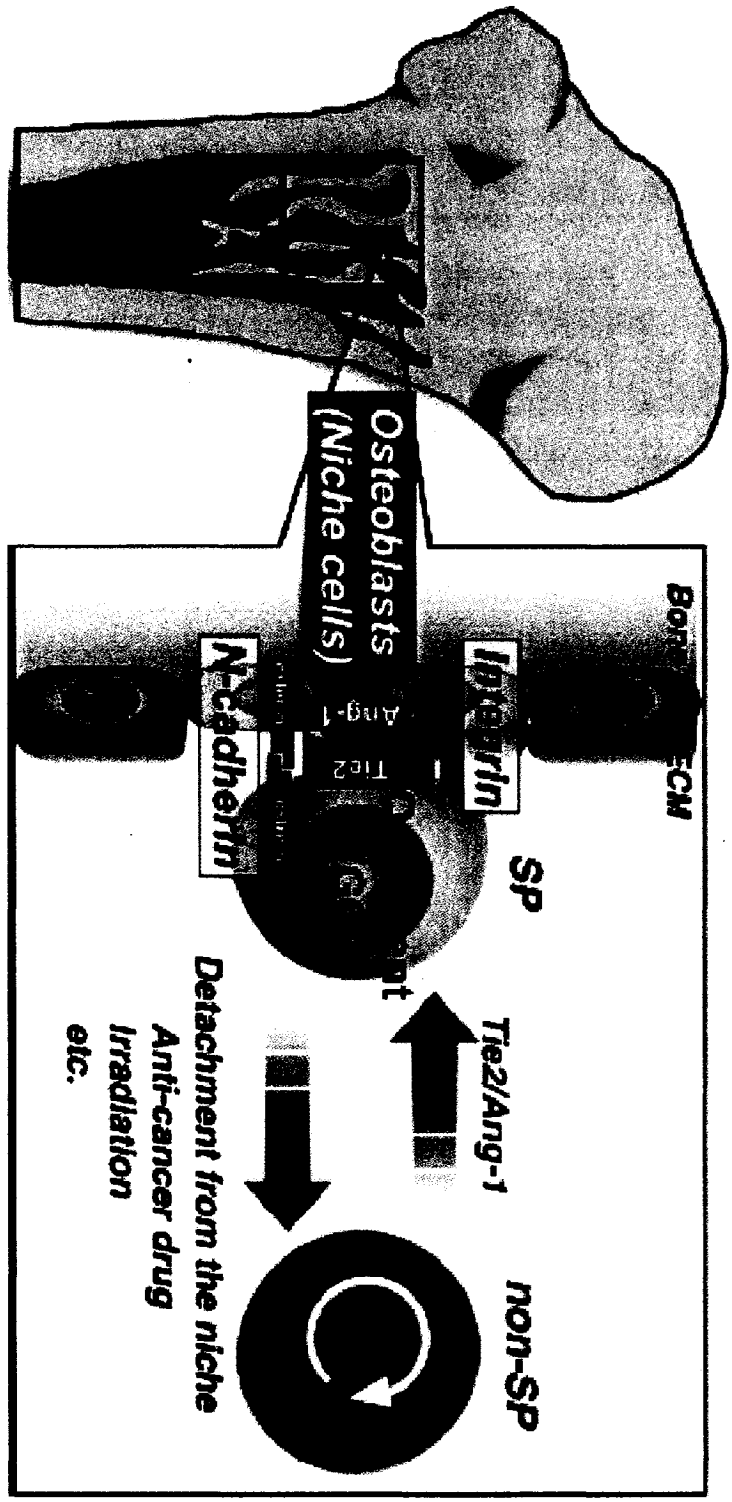
cells (Bonde *et al.*, 2004). Interaction of the HSCs with their niche is critical for adult hematopoiesis in the BM (Arai *et al.*, 2004). Moreover, adult stem cells possess resistance to various physiologic stresses, a characteristic required for the maintenance of hematopoiesis throughout life (Hess *et al.*, 2004).

Recently, Tie2/Angl signaling has been implicated in regulating quiescence of HSCs in the BM niche [Figure 1-4; (Arai *et al.*, 2004)]. Tie2 is expressed in quiescent HSCs that adhere to osteoblasts (OBs) on the bone surface. These HSCs are anti-apoptotic and comprise a side-population (SP) of HSCs. Angl is mainly produced by OBs in adult BM and activates Tie2 on the HSCs. The interaction of Tie2 with Angl promotes tight adhesion of HSCs to the OBs, resulting in the maintenance of long-term repopulating activity of HSCs by preventing cell division. In addition, Angl induces the ability of HSCs to become quiescent, which subsequently leads to enhanced survival of HSCs from various stresses. Therefore, it appears that the Tie2/Angl signaling plays a critical role in the maintenance of HSC quiescence in the adult BM microenvironment.

1.6 Internal Ribosome Entry Sites in Cellular mRNAs

A series of tightly regulated events allow the recruitment of ribosomal subunits to the mRNA to support the proper initiation of protein synthesis. Two distinct mechanisms have evolved in eukaryotic cells to initiate translation: cap-dependent initiation and internal ribosome recruitment. The cap-dependent scanning mechanism commences with 5' end-dependent recruitment of 40S ribosomal subunits to the mRNA. The 40S subunit carrying the initiator methionine-tRNA and certain eukaryotic initiation factors is thought to scan the mRNA in a 5' to 3' direction until an appropriate start codon is encountered at which stage a 60S subunit joins to form an 80S ribosome that can decode the mRNA into protein (Hershey and Merrick, 2000; Kozak, 1989). However, a subset of mRNAs can be translated via internal initiation, a process that involves direct binding of ribosome to specific mRNA regions. This translation initiation mechanism is generally independent of recognition of the 5'-mRNA end and involves direct recruitment of the 40S ribosomes to the vicinity of the initiation codon.

Figure 1-4. Model of the regulation of adhesion and quiescence of HSC by Tie2/Angl signaling in the adult BM niche. $Tie2^+$ HSCs specifically located in the stem cell niche and adhered to $Angl^+$ OBs in adult BM. Angl produced by OBs activates Tie2 on the HSCs and promotes tight adhesion of HSCs to the niche, resulting in quiescence and enhanced survival of HSCs.

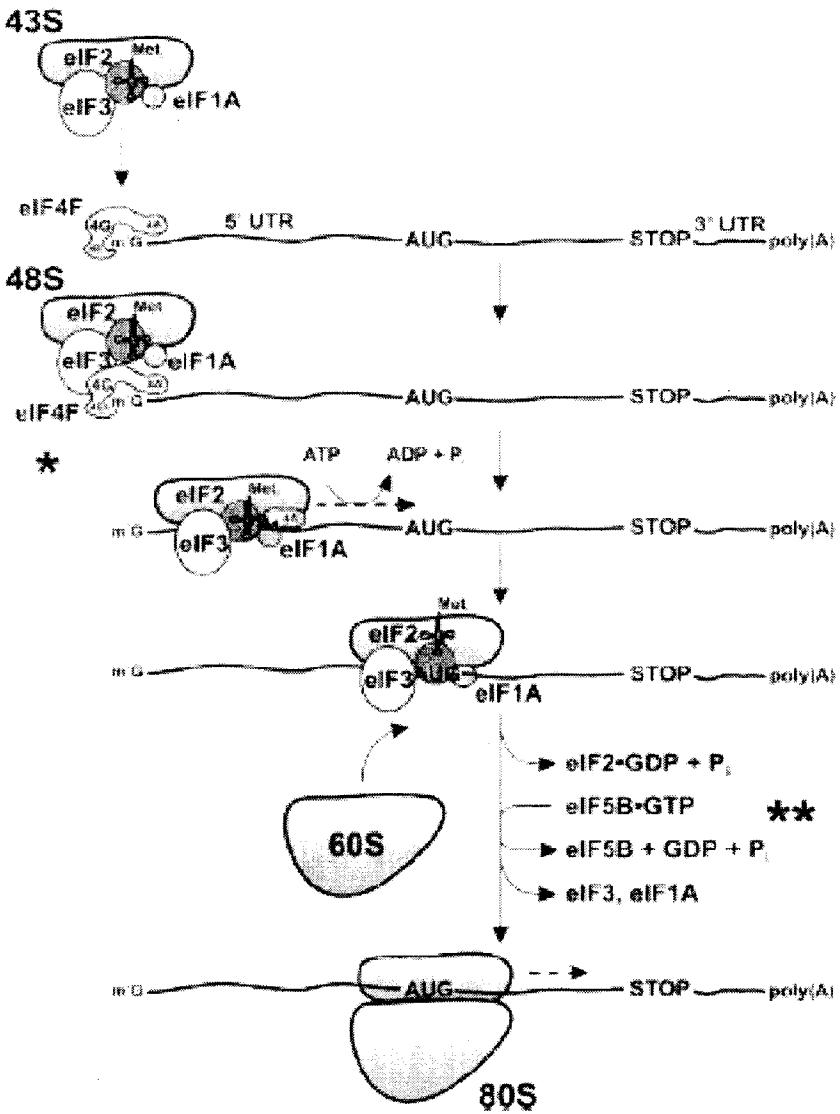


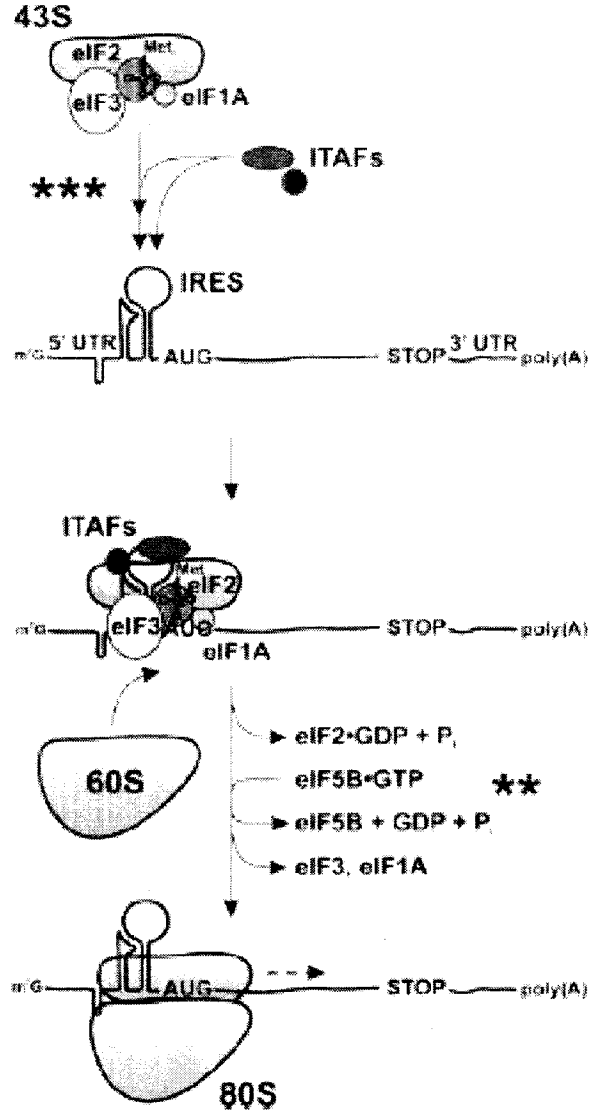
Adapted from Arai et al. (2005) Trends Cardiovasc Med.

1.6.1 The Canonical Scanning Mechanism of Translation Initiation

Most vertebrate mRNAs are functionally monocistronic and contain a 5'-terminal m^7GpppN (where N can be any nucleotide) cap structure. The initiation codon used as the start site for protein synthesis is preceded by a 5' UTR in which length, nucleotide composition, and structure can determine the efficiency and the mechanism by which a given mRNA is translated (Hershey and Merrick, 2000). Genetic and biochemical evidence has shown that most mRNAs in eukaryotic cells recruit ribosomes by a scanning mechanism in which a 43S "preinitiation complex", composed of a 40S subunit bound to eIF2-GTP-Met-tRNA$_i$, eIF1A, and eIF3, is recruited to the capped 5' end [Figure 1-5, left panel; (Donahue, 2000; Hershey and Merrick, 2000; Kozak, 1989)]. Binding of the 43S complex to mRNA involves recognition of the 5' capped mRNA by the eIF4E (cap-binding) subunit of eIF4F cap-binding complex and is greatly enhanced by the poly(A)-binding protein (PABP) bound to the 3' poly(A) tail (Gingras *et al.*, 1999; Sachs and Varani, 2000). eIF4F comprises eIF4E, eIF4A, and eIF4G subunits. eIF4A is a DEAD box RNA helicase/RNA-dependent ATPase, which is thought to unwind the secondary structure in the mRNA 5' UTR. The helicase activity of eIF4A is strongly enhanced by eIF4B. eIF4G is a large polypeptide and functions as a molecular scaffold protein that binds eIF3, eIF4A, eIF4E, and PABP and coordinates their activities (Gingras *et al.*, 1999; Hentze, 1997). It is thought that the recruitment of 43S complex is mediated by interactions between the eIF4G subunit of cap-bound eIF4F and the eIF3 component of the 43S complex, as well as between the mRNA and eIF4G, eIF3 and the 40S subunit (Lamphear *et al.*, 1995). Following complex recruitment, eIF1 and eIF1A act synergistically to enable scanning of the 43S complex along the mRNA template in a 5' to 3' direction from its initial binding site to the initiation codon (Pestova *et al.*, 1998a). Upon base pairing between the initiation codon of mRNA and the anticodon of initiator tRNA, the resulting ribosomal 48S complex contains eIF1, eIF1A, eIF3 and eIF2-GTP-Met-tRNAi. eIF5 induces hydrolysis of eIF2-bound GTP, leading to displacement of eIF2-GDP; the inactive eIF2-GDP is recycled to the active eIF2-GTP by eIF2B, a guanine nucleotide exchange factor (Hershey and Merrick, 2000). Finally, eIF5B mediates joining of the 60S large ribosomal subunit to the 40S subunit, resulting in

Figure 1-5. Schematic representation of the cap-dependent ribosome scanning (left panel) and internal initiation (right panel) pathways for the formation of 80 S initiation complexes. The scanning pathway for translation initiation postulates a three-step mechanism by which the 40 S ribosome approaches the initiation codon. At a first step, a 43 S initiation complex (comprising a 40 S subunit, eIF2·GTP·Met-tRNA$_i$, and eIF3) binds to the capped 5'-mRNA end, thus leading to the formation of the 48 S complex (note that eIF1A facilitates the Met-tRNA$_i$ binding to the 40 S ribosome). At a second step, the 40 S ribosome with associated initiation factors and Met-tRNA$_i$ scans downstream along the 5' UTR of the mRNA in search of the initiation codon. At a third step, the scanning complex encounters and recognizes the initiation AUG codon (usually this is the first AUG met by the scanning complex). This recognition is followed by the release of the initiation factors, and subsequently the 40 S subunit is joined by a 60 S subunit to form an 80 S ribosome (subunit joining is catalyzed by the initiation factor eIF5B). Note that this scheme is a simplified representation of the pathway and omits additional initiation factors participating in the process. The internal initiation pathway postulates a one-step (or in some cases two-step) mechanism by which the 40 S ribosome approaches the initiation codon. This translation initiation mechanism is generally independent of the recognition of the 5'-mRNA end and involves direct recruitment of the 40 S ribosome to the vicinity of the initiation codon (directed by an IRES element). The 40 S recruitment is assumed to be accompanied by the simultaneous recognition of the initiation codon. In certain cases, however, the 40 S ribosome is also assumed to be able to scan downstream of the internal landing "place" to locate the initiation codon. Single asterisk, the fate of eIF4F bound to the cap structure after the formation of the 48 S complex is unclear. It is assumed that it can either fall apart with at least eIF4A continuing to be part of the scanning complex, or even all 3 proteins (eIF4E, eIF4G, and eIF4A) can stay with the scanning ribosome. Double asterisks, the exact sequence of events and timing of the initiation factors release are not known. It is also not clear whether the mechanism of ribosomal subunit joining in the case of cap-dependent and cellular mRNA internal initiation pathways is similar or not. Triple asterisks, the subset of canonical initiation factors as well as ITAFs required for internal initiation varies with different viral and cellular IRES elements.





Adapted from Komar and Hatzoglou (2005) J Biol Chem.

formation of an 80S ribosome in which initiator tRNA is positioned in the ribosomal P site and is now competent to begin protein synthesis (Pestova *et al.*, 2000).

1.6.2 Internal Initiation

Some twenty years ago, it was reported that prokaryotic, but not eukaryotic, ribosomes could bind circular RNA molecules, suggesting that eukaryotic ribosomes enter mRNAs exclusively via their free 5' ends (Konarska *et al.*, 1981; Kozak, 1979; Kozak, 1980). However, later on, an alternative mechanism of translation initiation has emerged existing in eukaryotic cells. A *cis*-element was identified in the 5' UTR of viral and certain cellular mRNAs enabling ribosomes to be recruited directly to the mRNAs without the aid of a cap-structure. This specific mRNA structural element is termed an internal ribosome entry site (IRES) or ribosome landing pad (RLP); the former term is generally used today. The internal initiation mechanism is generally independent of recognition of the 5'-mRNA end and involves direct recruitment of the 40S ribosome to an internal RNA structure (Figure 1-5, right panel).

1.6.2.1 Discovery of IRES elements

The existence of IRES-driven translation initiation was first reported for two members of picornaviruses, poliovirus and encephalomyocarditis virus [EMCV; (Jang *et al.*, 1988; Pelletier and Sonenberg, 1988)]. Picornavirus mRNAs are uncapped and consequently translation initiation is cap-independent. In addition, they possess long 5' UTRs of approximately 600 to 1200 nucleotides, which are highly structured and harbor multiple upstream AUGs (uAUGs). These characteristics are considered as strong barriers to a cap-dependent scanning mechanism (Belsham and Sonenberg, 1996; Iizuka *et al.*, 1995; Jackson and Kaminski, 1995). However, the translation of most picornavirus RNAs is very efficient, even when cap-dependent protein synthesis is inhibited in the host cell (Belsham and Sonenberg, 1996). The presence of IRESes within the 5' UTRs of poliovirus was experimentally addressed by Pelletier and Sonenberg (Pelletier and Sonenberg, 1988). They developed a bicistronic mRNA translation assay, where the

bicistronic mRNAs encoded two open reading frames (ORFs) of reporter genes (also called cistrons) and the viral 5' UTR inserted within the intercistronic region. The first cistron measured cap-dependent initiation, whereas the second reflected the existence of internal initiation of translation. Using this assay, it was demonstrated that the picornavirus 5' UTR was able to confer internal initiation, independent of the 5' end (Jang *et al.*, 1988; Pelletier and Sonenberg, 1988): translation of the downstream cistron occurred even when translation of the upstream cistron was abolished, and was dependent on the integrity of the inserted IRES. Mapping of the sequences required for picornavirus IRES activity showed that approximately a 450 nucleotide stretch of RNA is needed for internal entry of the 40S ribosomal subunit, precisely at an AUG codon immediately downstream of an oligopyrimidine tract at the 3' end of the IRES: Small deletions or insertions, and even substitution of single nucleotides in the element, severely reduced its activity (Jang and Wimmer, 1990; Kuhn *et al.*, 1990; Nicholson *et al.*, 1991). It is clear that the functional capacities of viral IRESes depend directly on their secondary and tertiary structures. RNA structure prediction studies demonstrate that these IRESes fold into complex coordinated structures comprising several distinct domains.

With the discovery of IRES elements in picornavirus RNAs, functional IRES elements have been identified in an increasing number of viral RNAs and thus far, all the members of picornavirus family have been found to contain IRES elements. Furthermore, internal ribosome recruitment has also been shown in capped viral RNAs [e.g., hepatitis C virus (HCV)] as well as uncapped RNAs. Table 1-1 provides a list of RNA viruses and one DNA virus, Kaposi's sarcoma-associated herpesvirus, whose genomes contain IRES elements.

1.6.2.2 Cellular IRES

Soon after the first IRES activity was found in the picornavirus 5' UTR, the first cellular IRES was identified in the mRNA encoding the immunoglobulin heavy chain binding protein [BiP; (Macejak and Sarnow, 1991; Sarnow, 1989)]. The modification of several translation initiation factors involved in the efficient recruitment of 40S subunits to capped mRNAs has been demonstrated in virus-infected cells (Cuesta *et al.*, 2000; Gingras *et al.*, 1996; Gradi *et al.*, 1998; Kuyumcu-Martinez *et al.*, 2004). Particularly, the

Table 1-1 Internal ribosome entry sites in viral genome

Virus	Reference
Poliovirus	Pelletier and Sonenberg 1988
Rhinovirus	Borman and Jackson 1992
Encephalomyocarditis virus	Jang et al. 1988
Foot-and-mouth disease virus	Kuhn et al. 1990
Hepatitis C virus	Tsuklyama-Kohara et al. 1992
Classic Swine Fever Virus	Rijnbrand et al. 1997
Bovine viral diarrhea virus	Poole et al. 1995
Friend murine leukemia virus *gag* mRNA	Berlioz and Darlix 1995
Moloney murine leukemia virus *gag* mRNA	Vagner et al. 1995b
Rous sarcoma virus	Deffaud and Darlix 2000
Human immunodeficiency virus *env* mRNA	Buck et al. 2001
Plautia stali intestine virus	Sasaki and Nakashima 1999
Rhopalosiphum padi virus	Domier et al. 2000
Cricket paralysis virus	Wilson et al. 2000b
Kaposi's sarcoma-associated herpesvirus	Grundhoff and Ganem 2001; Bieleski and Talbot 2001

Adapted from Hellen and Sarnow (2001) Genes Dev.

proteolysis of eIF4G by viral-encoded proteases has been shown to separate eIF4G into N-terminal eIF4G/eIF4E/PABP and C-terminal eIF4G/eIF4A/eIF3 complexes, resulting in the uncoupling of cap recognition from ribosome-binding and helicase functions of eIF4F (Gradi *et al.*, 1998; Lamphear *et al.*, 1995; Lamphear *et al.*, 1993). Consequently, translation of most capped cellular mRNAs is severely inhibited under these circumstances. However, BiP mRNAs were efficiently translated during the viral infection, suggesting that translation initiation by an internal ribosome-binding mechanism can occur in cellular eukaryotic mRNAs. Recent studies have identified additional IRES-containing cellular mRNAs in poliovirus-infected cells in which 3-5 % of the cellular mRNAs remained associated with polyribosomes (Johannes *et al.*, 1999; Johannes and Sarnow, 1998). Indeed, in the past few years, IRES elements have been detected in an increasing number of cellular mRNAs from various species, and the list continues to expand (Table1-2). So far, IRES elements are found mainly in genes involved in development, differentiation, cell cycle progression, cell growth, apoptosis, and stress.

1.6.2.3 Features in Cellular IRES Elements

Common features found in IRES-containing 5' UTRs are that they are longer than 5' UTRs which lack IRES elements, contain relatively high GC nucleotide content, and often harbor multiple AUG triplets (Iizuka *et al.*, 1995). These features, in fact, have also been shown to impede the 5'-end-dependent scanning of 43S ribosomal subunits (Kozak, 1986a; Pelletier and Sonenberg, 1985).

Based on deletion mapping analyses, cellular IRES elements often appear to be located toward the 3' end of the 5' UTRs of mRNAs. Juxtaposition of an IRES with an open reading frame will position the 43S ribosomal subunit close to the AUG start codon, allowing 80S formation without the need to scan long distances from the initial binding site to the initiation codon. To date, no nucleotide sequence motif has been identified among cellular IRES elements even though many cellular IRESes have been recently discovered. Most viral IRES elements contain an oligopyrimidine-rich sequence near the 3' border of the IRES and it is often critical for IRES function [e.g., enterovirus; (Liu *et al.*, 1999)]. Most cellular mRNA IRESes, however, do not contain such an

Table 1-2 Internal ribosome entry sites in cellular mRNAs

mRNA/IRES	Protein function	Conditions leading to induction
Apaf-1	Activator of apoptosis	Apoptosis
XIAP	Inhibitor of apoptosis	Apoptosis/irradiation/serum deprivation
c-*myc*	Oncogene	Apoptosis/development/genotoxic stress/cell cycle/tissue specific
DAP5	Initiation factor	Apoptosis
Protein kinase C5	Regulates NADPH oxidase activity	Apoptosis/G_2/M phase of cell cycle
Reaper	Activator of apoptosis	Apoptosis/heat shock
Hsp70	Chaperone	Apoptosis/heat shock
Bcl-2	Inhibitor of apoptosis	Apoptosis
HIAP2/c-IAP1	Inhibitor of apoptosis	Apoptosis/irradiation/ER stress
Survivin	Inhibitor of apoptosis	Apoptosis/G_2/M phase of cell cycle
betaPix-b(L)	Rho family GTPase	Brain development
Antennapedia	Homeotic protein	Development, tissue specific
Ultrabithorax	Homeotic protein	Development, tissue specific
PDGF2/c-Sis	Growth factor	Differentiation
AML1/RUNX1	Transcription factor	Differentiation
MYT2	DNA binding	Differentiation
ODC	Ornithine decarboxylase	G_2/M phase of cell cycle
PITSLRE kinase	Cyclin-dependent kinase	G_2/M phase of cell cycle
NAP1L1	Nucleosome assembly	G_2/M phase of cell cycle
NPM1	Nucleophosmin	G_2/M phase of cell cycle
ABETA	Amyloid _A4 precursor	G_2/M phase of cell cycle
Cyr61	Angiogenic inducer	G_2/M phase of cell cycle
GRP58	Chaperone	G_2/M phase of cell cycle
hnRNPA/B	mRNA binding	G_2/M phase of cell cycle
hSNM1	DNA repair	G_2/M phase of cell cycle
Human Sp3	Transcription factor	G_2/M phase of cell cycle
p27^{Kip1}	Inhibitor of cyclin dependent kinases	G_2/M phase of cell cycle
Notch antagonist Hairless	Transcriptional repressor	G_2/M phase of cell cycle
VEGF	Growth factor	Hypoxia
HIF-1α	Transcription factor	Hypoxia
Cat-1	Cationic amino acid transporter	Amino acid starvation/ER stress/hypoxia Glucose starvation
AT1R	Angiotensin receptor	Serum deprivation
BAG-1	Co-chaperone	Heat shock
BiP	Chaperone	Heat shock
Ure2	Nitrogen regulator	Heat shock
Rbm3	RNA binding	Mild hypothermia
FGF-2	Growth factor	Hyperglycemia/tissue specific
FGF-1	Growth factor	Tissue specific
N-*myc*	Oncogene	Neuronal tissue
NRF	Transcription factor	Cell specific

Adapted from Komar and Hatzoglou (2005) J Biol Chem.

oligopyrimidine tract. Therefore, in the absence of a consensus IRES and sequence motif, it appears that an important feature of the IRES is a complex secondary and tertiary structure which can be recognized by the translation machinery. Based on computer-aided analysis, Le and Maizel suggested a secondary structure motif that may be common to several cellular IRES elements: a Y-shaped double-hairpin structure followed by a smaller single-hairpin structure upstream of the start AUG codon (Le and Maizel, 1997). This and a similar motif were found in a variety of cellular IRES elements including the IRESes encoded in BiP, FGF2, *Antennapedia*, VEGF, and PDGF/*c-sis* (Huez *et al.*, 1998; Le and Maizel, 1997; Sella *et al.*, 1999). There is no experimental evidence to support a functional role for this proposed motif in internal initiation and the structural features of cellular IRES elements still remain largely unknown.

1.6.2.4 Structure-function Relationships in Cellular IRESes

IRES elements in viral RNA genomes contain higher ordered structures whose integrities are essential for the assembly of a competent 80S initiation complex (Hellen and Sarnow, 2001). Small deletions and point mutations in viral IRESes can have profound effect on internal initiation. In contrast, deletions within many cellular IRESes rarely disable the element completely. These observations imply that the structure-function relationship is not as rigid in these cellular IRESes as in the viral IRESes. In fact, in many cases, separate, non-overlapping sections of cellular IRESes have been shown to promote internal initiation albeit not as efficiently as the entire IRES (Chappell *et al.*, 2000a; Chappell and Mauro, 2003; Coldwell *et al.*, 2000; Huez *et al.*, 1998; Jopling *et al.*, 2004; Kullmann *et al.*, 2002; Miskimins *et al.*, 2001; Stoneley *et al.*, 1998; Yang and Sarnow, 1997). For instance, a small 9-nt segment from the 5' UTR of the homeodomain gene, *Gtx,* can promote internal initiation. Further, multiple copies of this sequence module significantly enhance IRES activity (Chappell *et al.*, 2000a). Interestingly, this short IRES module is perfectly complementary to the 18S rRNA and has recently been shown to facilitate protein synthesis by direct binding to the 18S rRNA in eukaryotic cells (Dresios *et al.*, 2006). This suggests that the base-pairing interaction between the 9-nt element and 18S rRNA leads to ribosome recruitment and translation initiation similar to the way initiation proceeds in prokaryotes (Dresios *et al.*, 2006; Yusupova *et al.*, 2001).

Surprisingly, the same nine nucleotide sequence element also can function as a translational repressor when present in the 5' UTR of a capped monocistronic mRNA (Hu *et al.*, 1999). So far, there is no clear explanation for this discrepancy and it remains to be answered.

Despite the above-mentioned differences between viral and cellular IRESes, mutational analyses of some cellular IRESes have provided evidence that structural modules are involved in cellular internal initiation. For example, *c-myc* IRES function was relatively unaffected when a single structural motif in the 5' UTR was changed by mutations or deletions. However, the IRES was disabled by two mutations or deletions that disrupt separate structural regions, suggesting a role of combined structural modules for *c-myc* IRES activity (Le Quesne *et al.*, 2001). Moreover, since *c-myc* protein synthesis can be mediated by cap-dependent initiation as well as by internal initiation, the structure of the IRES may well be dynamic to accommodate the scanning ribosome (Stoneley *et al.*, 2000b; West *et al.*, 1998). The IRES of the cationic amino-acid transporter gene, *cat-1* is another example in which the RNA structure has been implicated for the IRES function. Disruption of RNA-RNA interactions between the 5' and 3' end of the IRES prevents inducible internal initiation, whereas the restoration of this structural feature re-establishes the activity of the inducible IRES (Yaman *et al.*, 2003). On the other hand, RNA structure can also have a negative impact on cellular internal initiation. For both the *c-myc* and *cat-1* IRESes, evidence has been presented that a pseudoknot can inhibit the activity of the IRES. Moreover, in the *cat-1* IRES, this structural feature has been implicated in the regulation of *cat-1* internal initiation (Le Quesne *et al.*, 2001; Yaman *et al.*, 2003).

Evidence, to date, suggests that the combined effect of both structural and functional features of IRES elements is involved in ribosome recruitment. Exactly how these motifs combine to promote internal initiation remains to be determined.

1.6.2.5 Regulation of Translation by Cellular IRESes

Translation initiation factors (except for eIF4E) utilized in 5' end cap-dependent translation are also required for the IRES-mediated initiation in picornaviruses. This has been well documented by 40S ribosome-binding assays in the EMCV IRES (Pestova *et*

al., 1996). In this system, a fragment of eIF4G possessing RNA-binding properties, along with all of the canonical initiation factors (except for eIF4E), were sufficient to promote binding of 40S ribosomes at the initiator AUG. It is most likely that cellular IRESes also require these initiation factors (except eIF4E). Cellular IRESes enable translation of the mRNAs under various stress conditions when cap-dependent protein synthesis is impaired, as observed with viral IRES elements (Table 1-2). A growing body of evidence suggests that cellular IRESes are involved in the regulation of gene expression under such conditions.

Amino-acid starvation in mammalian cells results in a significant increase in the serine phosphorylation of eIF2α (Ser51; leading to a reduced concentration of ternary complex) and dephosphorylation of eIF4E (leading to a reduced concentration of active eIF4F), which results in decrease in global protein synthesis. However, the translation of *cat-1* mRNA is stimulated via an IRES under these conditions (Fernandez *et al.*, 2001). It has been proposed that induction of *cat-1* IRES activity requires both the translation of a small upstream open reading frame (uORF) within the IRES and phosphorylation of eIF2α (Yaman *et al.*, 2003). According to this model, translation of the uORF causes structural remodeling of the IRES, leading to the attainment of an active IRES conformation. Induction of *cat-1* IRES activity is also dependent on the phosphorylation of eIF2α. A time lag between this phosphorylation event and the increased translation of *cat-1* has led to speculation that an essential ITAF is synthesized during this period (Fernandez *et al.*, 2002a; Fernandez *et al.*, 2002b; Yaman *et al.*, 2003).

Internal initiation has also been implicated in the regulation of gene expression during the cell cycle. Two cell cycle regulating PITSLRE protein kinase isoforms, $p110^{PITSLRE}$ and $p58^{PITSLRE}$ are translated from a single mRNA, but the synthesis of $p58^{PITSLRE}$ increases considerably in cells arrested in G2/M phase when cap-dependent protein synthesis is inhibited. Studies have indicated that an IRES element is located just upstream of the $p58^{PITSLRE}$ initiation codon, and its activity is cell cycle regulated, permitting translation of $p58^{PITSLRE}$ at the G2/M phase (Cornelis *et al.*, 2000). Evidence has also been presented for a G2/M-regulated IRES in the ornithine decarboxylase gene (Pyronnet *et al.*, 2000).

During apoptosis (programmed cell death) there is a considerable inhibition of cap-dependent protein synthesis, which is accompanied by a caspase-dependent cleavage of translation initiation factors [e.g., eIF2α, eIF4B, eIF4G, and the p35 subunit of eIF3; (Clemens *et al.*, 2000)]. However, the synthesis of certain proteins remains unaffected by these changes. For instance, translation of *c-myc* mRNA is maintained during apoptosis via IRES activity (Stoneley *et al.*, 2000a). Other cellular IRESes that stimulate internal initiation in apoptotic cells include those of the death-associated protein 5 (DAP5/p97), X chromosome-linked inhibitor of apoptosis protein (XIAP), inhibitor of apoptosis protein 2 (HIAP2/c-IAP1), a pro-apoptotic protein Reaper, anti-apoptotic proteins Bcl-2, protein kinase Cδ, and the apoptotic protease activating factor 1(Apaf-1) mRNAs (Coldwell et al., 2000; Henis-Korenblit et al., 2000; Hernandez et al., 2004; Holcik and Korneluk, 2000a; Morrish and Rumsby, 2002; Sherrill et al., 2004; Van Eden et al., 2004; Warnakulasuriyarachchi et al., 2004). These IRESes probably have reduced requirements for the integrity of the initiation factors such as eIF2α, eIF4B, eIF4G, and the p35 subunit of eIF3.

Efficient translation of several other mRNAs is maintained by functional IRES activity in a number of other cellular conditions such as hypoxia and heat shock. It has been well established that global cap-dependent translation is significantly reduced under these stress conditions (Coldwell *et al.*, 2001; Kraggerud *et al.*, 1995; Rhoads and Lamphear, 1995). The IRESes in the vascular endothelial growth factor (VEGF) and hypoxia inducible factor-1α (HIF1α) genes promote the translation of the corresponding mRNAs during hypoxia, allowing the accumulation of these gene products and the activation of the downstream signal transduction under hypoxic conditions (Huez *et al.*, 1998; Lang *et al.*, 2002; Stein *et al.*, 1998). Synthesis of the Bag-1 isoform, p36 which is involved in the protein refolding response, is mediated by the Bag-1 IRES element following heat shock (Coldwell *et al.*, 2001). During cell cycle arrest by serum starvation, XIAP and $p27^{kip1}$ proteins are efficiently translated by internal ribosome entry (Holcik *et al.*, 1999; Miskimins *et al.*, 2001).

Clearly cellular IRESes are implicated in the regulation of gene expression in various physiological states and enable cells to respond to these conditions against the background of a general reduction in protein synthesis. In addition, many cellular IRESes

show cell-type specific variation and, in some cases, IRES activity is developmentally controlled (Creancier *et al.*, 2001; Creancier *et al.*, 2000; Jopling *et al.*, 2004; Jopling and Willis, 2001; Mitchell *et al.*, 2003; Pickering *et al.*, 2003; Stoneley *et al.*, 2000b; Ye *et al.*, 1997). For instance, there is significant variation in FGF2 and *c-myc* internal initiation in different tissues during murine embryogenesis. Interestingly, the efficiency of the *c-myc* IRES is very low in adult tissues suggesting that repression of the IRES may play a role in the downregulation of *c-myc* expression in differentiated and quiescent cells (Creancier *et al.*, 2001). Conversely, FGF2 internal initiation is very efficient in the adult brain, a tissue in which this growth factor is known to play a significant role (Creancier *et al.*, 2000). Cell-type specific or extract-dependent limitation of IRES activity has been previously demonstrated for a number of IRESes. For example, the IRES activity of the PITSLRE protein kinase is cell-cycle regulated and is not detectable in *in vitro* translation assays in rabbit reticulocyte lysates (Cornelis *et al.*, 2000). The *c-myc* IRES is not active in reticulocyte lysates possibly due to lack of cell-type specific *trans*-acting factors and lack of a nuclear experience (Stoneley *et al.*, 2000b). Moreover, the hepatitis C virus (HCV) IRES is functional in rabbit reticulocyte lysates and Krebs extracts, but not in wheat germ extracts (Pestova *et al.*, 1998b), most likely due to incompatibility between the HCV IRES and wheat germ ribosomes and/or wheat germ eIF3. It has been suggested that the activity of an IRES element could be modulated by non-canonical transacting-factors (discussed below). For example, there is evidence that the neuronal-specific ITAF nPTB (the neuronal-enhanced version of polypyrimidine tract binding protein 1) may be responsible for enhanced Apaf-1 internal initiation in cell lines of neuronal origin (Mitchell *et al.*, 2003).

1.6.2.6 Internal Initiation *Trans*-acting factors (ITAFs)

In addition to their requirements for canonical eukaryotic initiation factors, the efficiency of some viral IRESes is improved by noncanonical initiation factors known as ITAFs (Martinez-Salas *et al.*, 2001). As shown in Table 1-3, all known ITAFs are cellular RNA-binding proteins of diverse functions in cells (Andino *et al.*, 1999; Belsham and Sonenberg, 1996; Stoneley and Willis, 2004; Vagner *et al.*, 2001). For example, the levels of ITAF expression were shown to correlate with pathogenic properties and tissue

Table 1-3 IRES-*trans*-acting factors and the corresponding IRESes with which they interact

ITAF/trans-*acting factor*	*IRESes interacts with*
PTB (hnRNP1)	EMCV, FMDV, TMEV, PV1, HRV, HCV, HAV, human T-lymphotrophic virus type 1, Apaf-1, IGF-IR, BAG-1
N-PTB (neuronal PTB)	TMEV, Apaf-1
La	EMCV, HCV, PV1, human T-lymphotrophic virus type 1, HIV-1 (gag RNA), XIAP, Bip/Grp78, coxsackievirus B3
Unr	HRV, Apaf-1
ITAF45	FMDV
HnRNPE2 (PCBP2)	PV1, HRV, Coxsackievirus B3, c-*myc*
HnRNPE1 (PCBP1)	PV1, c-*myc*
HnRNPC1/C2	PDGF2/c-sis, XIAP, c-*myc*
HnRNPL	HCV
HnRNPK	c-*myc*
DAP5	DAP5, c-*myc*, Apaf-1, XIAP
GAPDH	HAV
Nucleolin	HRV, PV1
ELAV/Hu	p27
Ribosomal protein S9	HCV, CSFV
Ribosomal protein S5	HCV

Adapted from Stoneley and Willis (2004) Oncogene

specificity of picornaviruses. The properties and tissue distribution of ITAFs were suggested to determine the biological properties of a variety of viruses that use the IRES-dependent translation initiation (Andino *et al.*, 1999; Belsham and Sonenberg, 1996; Vagner *et al.*, 2001).

Specific functional RNA-protein interactions between cellular IRESes and known ITAFs have been also identified for a number of cellular IRESes and some of these proteins have been shown to be required for efficient internal ribosome entry (Table 1-3). For instance, the Apaf-1 IRES can form a specific RNA-protein complex with the ITAFs, upstream of N-ras (UNR) and polypyrimidine tract-binding protein (PTB) (Mitchell *et al.*, 2003). Remodeling of the Apaf-1 structure upon interaction with UNR protein promotes binding of PTB, and these events lead to stimulation of Apaf-1 internal initiation. Similarly, PTB and poly-(rC)-binding protein 1 (PCBP1) interact with the Bag-1 IRES and stimulate its activity (Pickering *et al.*, 2003; Pickering *et al.*, 2004). Members of the poly-(rC)-binding protein family have also been shown to stimulate the activity of the *c-myc* IRES (Evans *et al.*, 2003). Therefore, structural remodeling by the RNA chaperone activities of ITAFs such as PTB, UNR, and PCBP1 may prove to be a common theme in the mechanism of cellular IRES. However, it should be noted that these factors do not affect all cellular IRESes.

One striking feature of many of these ITAFs is that they are predominantly located in the nucleus. However, since these proteins are known to shuttle between the nucleus and cytoplasm they can clearly influence internal initiation. It was suggested that the relative levels of ITAFs present in the cytoplasm vary under different stress conditions and their cell/tissue distribution could significantly modulate the level of IRES-mediated translation. Furthermore, the nucleocytoplasmic trafficking of ITAFs may be particularly relevant in the light of observation that exposure to the nuclear compartment is essential for efficient internal initiation on some cellular IRESes (Holcik *et al.*, 2003; Shiroki *et al.*, 2002; Stoneley *et al.*, 2000b).

1.6.2.7 De-regulation of Internal Ribosome Entry and Disease

Cellular IRESes have been identified in a number of genes that are involved in cell growth, proliferation, apoptosis and angiogenesis. It has been suggested that deregulated

function of these IRESes may contribute to the development and progression of cancer and other diseases (Holcik, 2004; Martin *et al.*, 2003; Teshima-Kondo *et al.*, 2004). In fact, with respect to tumor progression, the cellular IRESes of angiogenic factors, such as VEGF and FGF2, have been shown to mediate protein synthesis under hypoxic conditions when cap-dependent translation is compromised (Akiri *et al.*, 1998; Huez *et al.*, 1998; Pickering and Willis, 2005; Stein *et al.*, 1998; Vagner *et al.*, 1995). As a tumor grows, it reaches a point where the centre of the growth becomes hypoxic. Overexpression of VEGF and FGF2 driven by IRES-mediated translation under such conditions would lead to neovascularization of the tumor. In addition, as described earlier, HIF1α harbors an IRES that is active during hypoxia (Lang *et al.*, 2002). Translation of HIF1α mRNA through an IRES-mediated mechanism would allow HIF1α to accumulate under hypoxic conditions, and consequently, activate VEGF transcription.

The *c-myc* protein is frequently found to be overexpressed in many types of cancer. Aberrant translational regulation through *c-myc* IRES-mediated mechanism has been identified in the human neoplasia multiple myeloma (MM) (Paulin *et al.*, 1996). The analysis of several cell lines obtained from MM patients has indicated that *c-myc* protein levels were significantly enhanced due to a single C to U change in the *c-myc* IRES (Paulin *et al.*, 1998). Subsequently, the same sequence substitution in the IRES was identified in 42 % of bone marrow samples collected from MM patients (Chappell *et al.*, 2000b). It is known that the C to U transition can stabilize the formation of RNA-protein complexes containing the *c-myc* IRES (Paulin *et al.*, 1998). Recently, a member of the poly-(rC)-binding protein family hnRNPK has been shown to interact with the mutant IRES more strongly than the wild-type sequence. Moreover, this ITAF stimulates the activity of the mutant IRES to a greater extent. Hence, the increased affinity of hnRNPK for the mutant IRES may contribute towards enhanced *c-myc* internal initiation in MM (Evans *et al.*, 2003).

Expression of the anti-apoptotic XIAP protein is increased during acute low doses of ionizing irradiation. This up-regulation of the XIAP translation is mediated by an IRES within the XIAP 5' UTR and results in increased resistance to radiation in cancer cell (Holcik *et al.*, 1999; Holcik *et al.*, 2000b).

1.7 Translational Control by Upstream Open Reading Frames

The regulation of mRNA translation is a key component of gene expression control. Translation itself is regulated by a variety of mechanisms at three different steps: initiation, elongation, and termination. However, the most critical and rate limiting step in translation is initiation, which involves the formation of an elongation-competent 80S ribosome at a start codon either by the ribosome scanning mechanism or by a cap-independent mechanism (discussed above).

The regulation of initiation can be mediated by *cis*-acting elements within the 5' UTR of transcripts such as stem-loop structures and upstream AUGs (uAUGs)/upstream ORFs (uORFs). The effect of stem-loops on initiation is determined by the stability of the structure, its location within the 5' UTR, and the ability of the stem-loop to bind specific regulatory proteins (Cazzola and Skoda, 2000). When stable stem-loop structures are located near the 5'-cap, translation efficiency is dramatically reduced due to interference with assembly of the preinitiation complex. mRNAs with stable stem-loop structures located further downstream are associated with reduced translation efficiency. This is achieved by opposing the unwinding activity of the ribosome-associated helicase and thereby hindrance of conventional scanning of 40S subunits. Alternatively, there are examples of 5' UTRs containing stem-loops located near a start codon which can enhance the recognition of the preceding AUG.

In the majority of eukaryotic mRNAs, the first AUG downstream from the 5' cap is considered as the translation start site. However, in some mRNAs, AUGs/uORFs are commonly found upstream of the start of translation. Depending on the database considered, between 10 and 50 % of human mRNAs have been estimated to contain uAUGs or uORFs (Kozak, 1987a; Meijer and Thomas, 2002; Yamashita *et al.*, 2003). Interestingly, uAUG codons are noticeably common in certain classes of genes, including two-thirds of oncogenes and many other genes involved in the control of cellular growth and differentiation (Kozak, 1987a; Kozak, 1991; Morris, 1995; Morris and Geballe, 2000). Despite the availability of large amounts of genomic and expressed sequence data, it is difficult to estimate the accurate number of genes with uORFs. Only a minority of

database entries are based on careful mRNA mapping data with annotations that identify the precise start of the transcript leader.

Experimental evidence suggests that uAUGs/uORFs usually diminish translation of the ORF by reducing the number of ribosome reaching and initiating at the authentic or main AUG start codons (Cazzola and Skoda, 2000; Gray and Wickens, 1998; Hinnebusch, 1997; Kozak, 2002; Meijer and Thomas, 2002; Morris and Geballe, 2000). Ribosomes reaching the main AUG of these mRNAs do so mainly via context-dependent leaky scanning and/or reinitiation mechanisms, although it is widely believed that these are inefficient mechanisms (Kozak, 2002; Meijer and Thomas, 2002).

1.7.1 Context-dependent Leaky Scanning

For a uORF to function as a regulatory element, its initiation codon must be recognized, at least, at certain times by the scanning 40S ribosomal subunit and associated initiation factors. When uORF recognition is regulated by a so-called leaky-scanning mode of regulation, ribosomes either ignore the upstream AUG codon and scan past it or recognize it and initiate translation, depending on the conditions. The most efficient context for initiation of protein translation is known as the Kozak sequence ($GCC^{A}/_{G}CC\underline{AUG}G$), which was initially identified as a consensus sequence delineating the AUG start codon of vertebrate mRNA (Kozak, 1981; Kozak, 1984; Kozak, 1986b; Kozak, 2002). Two positions within this sequence, -3 and +4 (the A of the AUG codon is designated +1) are the most critical for determining the strength of the initiator and hence translation efficiency. A strong consensus sequence contains both of these important nucleotides, whereas an adequate sequence contains only one of them. It is thought that most, if not all ribosomes, will initiate at these optimal AUGs. The AUG codons initiating the main ORF of a messenger have a good or an adequate sequence context in 95-97 % of the cases. This percentage is lower for uAUGs (43-63 %) (Suzuki *et al.*, 2000). The relatively frequent presence of a uAUG within a good or adequate context suggests that the translational machinery can deal with these sequence elements in 5' UTRs and that the uAUGs might be involved in the translational control of mRNAs. In fact, some critical regulatory genes, such as cytokines, growth factors, kinases, and

transcription factors often produce mRNAs in which the 5' UTR sequence is GC-rich or burdened by uAUGs, suggesting that these encumbered 5' sequences are nature's way of limiting the synthesis of potent proteins that would be harmful if overproduced (Kozak, 1991).

1.7.2 Reinitiation

Reinitiation is another mechanism that allows ribosomes to reach and initiate at downstream AUG codons. Initiation may occur at the AUG of a uORF, but following termination, a proportion of the ribosomal 40S subunits remains attached to the template and resume scanning, gradually regaining competence to reinitiate translation at a downstream AUG codon. Reinitiation is considered to be a rare and inefficient event, although the incidence of this mechanism may be much greater, since only a few mRNAs with uORFs have been adequately examined (Meijer and Thomas, 2002).

Several features of mRNA may influence the efficiency of this process in higher eukaryotes. For instance, increasing the intercistronic spacing between the termination codon of the uORF and the downstream ORF, up to approximately 50 to 80 nucleotides, reduces or eliminates the inhibitory effects of uORFs. In some cases, enhanced reinitiation is observed (Child *et al.*, 1999; Kozak, 1987b). In addition to length, the sequence of the intercistronic region can affect reinitiation. In the case of the CCAAT/enhancer binding protein alpha (C/EBPα) mRNA, substitution of a single nucleotide in the 7-nucleotide intercistronic region significantly increases reinitiation (Lincoln *et al.*, 1998). Similarly, replacement of the intercistronic region in the maize *Lc* gene with a sequence of similar length greatly augments reinitiation (Wang and Wessler, 1998). Following translation of a uORF, the ribosome may encounter RNA sequences or structures that promote dissociation of the ribosome from mRNA and eliminate the potential for reinitiation (Geballe and Sachs, 2000). Although the ribosome remains attached to the mRNA, it presumably needs to be reloaded with initiation factors (such as eIF2-GTP and Met-tRNA$_i^{Met}$) before it can reinitiate downstream. Longer intercistronic spacing may augment reinitiation by allowing more time for ribosomes to acquire the necessary factors, as appears to be the case for *GCN4* (Grant *et al.*, 1994).

The length of a uORF is also a major limitation on reinitiation in eukaryotes-i.e., the efficiency of downstream reinitiation is reduced when the uORF is lengthened (Kozak, 2002). The reason why reinitiation is usually restricted by the size of the uORF is unknown, but a possible explanation is that certain initiation factors dissociate from the ribosome only gradually during the course of elongation. If elongation period is short, the factors required for reinitiation may still be present when the 40S subunit resumes scanning. Reinitation can be also controlled by sequence around the termination codon [e.g. *GCN4*, discussed below; (Grant and Hinnebusch, 1994; Miller and Hinnebusch, 1989)].

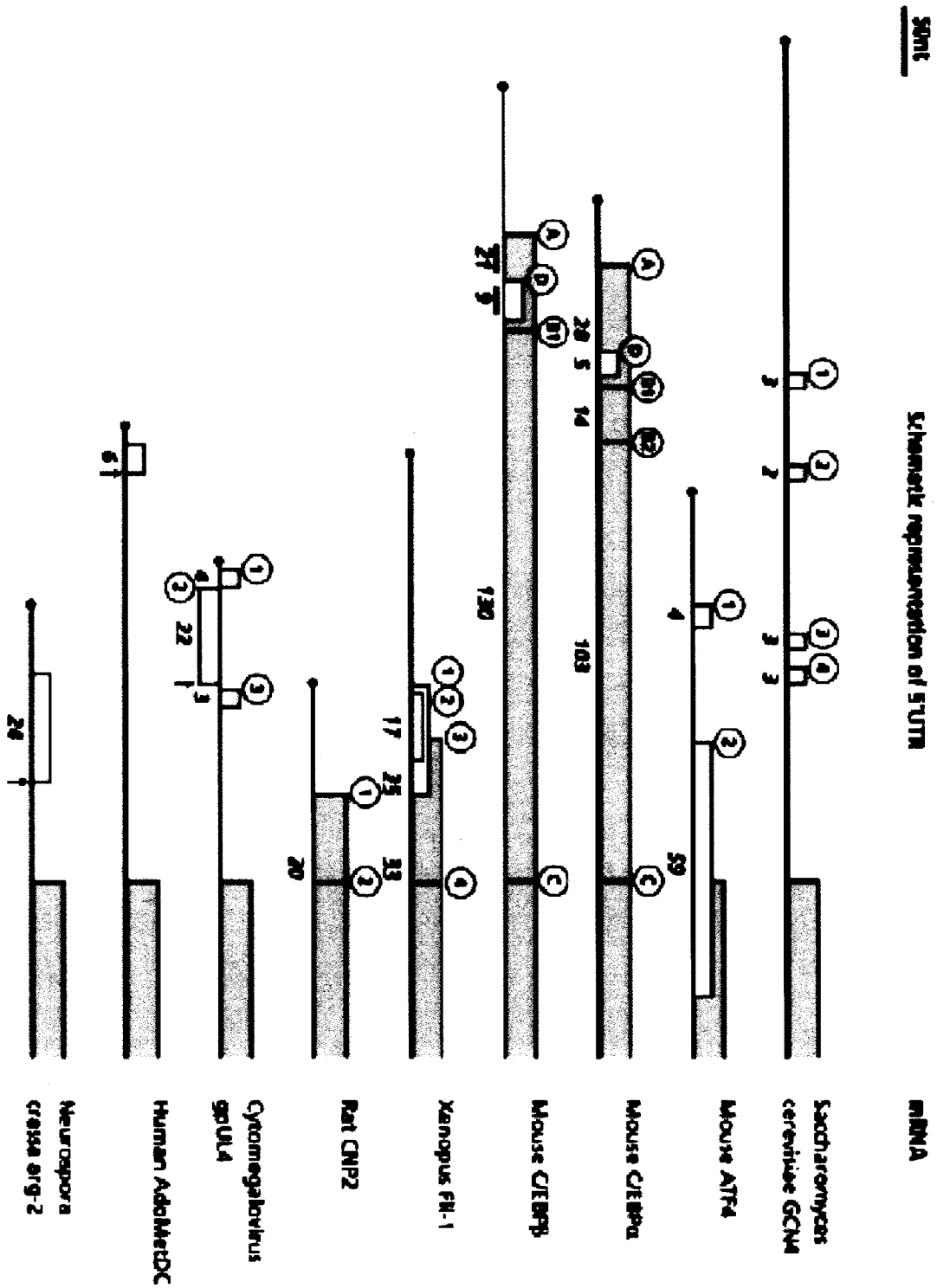
Elucidating the controls that influence the fate of the ribosome and its reinitiation potential after translation of a uORF will be critical for understanding the regulatory mechanisms used by many uORFs.

1.7.3 Examples of uAUG-mediated Translational Control

1.7.3.1 Stress-mediated control of initiation

The best understood example of translational control via reinitiation is observed in *Saccharomyces cerevisiae GCN4* mRNA (Hinnebusch, 2005; Morris and Geballe, 2000). The *GCN4* gene is a transcription factor that activates the expression of a large group of genes involved in amino acid biosynthesis. During amino acid starvation, general protein synthesis is reduced; however, translation of *GCN4* mRNA is greatly enhanced. This differential enhancement of *GCN4* translation in response to amino acid starvation is controlled by four small uORFs within the 590 nt long 5' UTR (Figure 1-6). In fact, the combined activities of the different uORFs regulate the expression of *GCN4*. After translating uORF1, approximately 50 % of the initial ribosomes can still continue to scan and reinitiate at a downstream ORF. On the other hand, uORF4 by itself has a strong inhibitory effect and abolishes the translation of the *GCN4* by approximately 99 %. The sequences around the termination codon of the uORFs determine the efficiencies of reinitiation- i.e., uORF4 has a relatively GC-rich sequence surrounding its termination codon, while the same region of uORF1 is AU rich (Miller and Hinnebusch, 1989). In

Figure 1-6. Schematic representation of uORF-containing 5' UTRs. Main ORFs are depicted by grey boxes, whereas uORFs are indicated by white boxes. When more than one upstream initiation site is present, they are numbered at the left upper corner of the initiation site. The numbers under the uORFs indicate the number of amino acids in the encoded peptides. Ribosome stalling sites are indicated with upward-pointing arrows (↑).



Adapted from Meijer and Thomas (2002) Biochem J.

addition, a rare proline codon at the end of uORF4 plays a role in preventing reinitiation after translating this uORF.

Under conditions of amino acid starvation, the high level of uncharged tRNAs activates the phosphorylation of eIF2α by the GCN2 kinase (Qiu *et al.*, 2000). The phosphorylation of eIF2α prevents the GDP↔GTP exchange and therefore inhibits general translation. However, translation of *GCN4* is stimulated by a surprisingly sophisticated reinitiation-dependent mechanism under these stress conditions. The weak inhibitory activity of uORF1 works together with strong inhibition by the downstream uORFs. Initiating ribosomes enter at the 5' cap of the mRNA, translate uORF1, and resume scanning after translation termination of the uORF1. In order to be able to reinitiate, these ribosomes have to regain the ability to initiate by rebinding eIF2-GTP-Met-tRNA$_i$. Under normal conditions, most of the ribosomes are able to reinitiate upon reaching uAUG4. After termination of uORF4, ribosomes will leave the mRNA, and therefore not translate the *GCN4* ORF. When general protein synthesis is inhibited, only a limited amount of ribosomal complexes can initiate and terminate at uORF4. The rest of the complexes will not be loaded with eIF2-GTP-Met-tRNA$_i$ in time for initiation at uORF4, and will be reloaded during the scanning of the remaining 150 nt of the 5' UTR, enabling translation of the *GCN4* ORF.

Expression of activating transcription factor 4 (*ATF4*) is regulated in a similar manner to that of *GCN4* under eIF2α phosphorylation (Harding et al., 2000; Lu et al., 2004; Vattem and Wek, 2004). The mouse *ATF4* 5' UTR (272 nt) contains two uORFs and the second one overlaps with the *ATF4* ORF (Figure 1-6). Translation of *ATF4* increases upon stress-induced eIF2α phosphorylation (by a PKR-like ER kinase, PERK), whereas global protein synthesis is inhibited under these conditions (Lu et al., 2004). *ATF4* translation is enhanced during cellular stress when both uORFs are present. Scanning ribosomes initiate translation efficiently at both uORFs. The ribosomes that had translated uORF1 efficiently reinitiate translation at downstream AUGs (Lu et al., 2004; Vattem and Wek, 2004). In unstressed cells in which the level of eIF2α phosphorylation is low, ribosomes scanning downstream of uORF1 reinitiate at the uORF2, an inhibitory element that blocks *ATF4* expression. In stressed cells, high levels of eIF2α phosphorylation increase the time required for the scanning ribosomes to become

competent to reinitiate translation. This favors reinitiation at the *ATF4* ORF over the inhibitory uORF2. These features are similar to those observed in *GCN4* in yeast. The translational control of *ATF4* is not identical with the translational control of *GCN4*. Whereas mutation of uAUG1 of *GCN4* leads to a severe inhibition of translation, mutation of uAUG1 of *ATF4* mRNA has a stimulatory effect. uORF1 of *ATF4* is less effective than the uORF1 of *GCN4* in overcoming the inhibitory effect of the second uORF.

1.7.3.2 Synthesis of nested proteins

Another regulatory function of uAUGs is the possibility of initiation at different AUG codons, resulting in the synthesis of different proteins. C/EBPs are a family of transcription factors that regulate the expression of tissue specific genes during differentiation of a variety of cell types. C/EBPα and C/EBPβ mRNAs have a common structure (Figure 1-6). Both mRNAs contain one uORF in a reading frame different from the C/EBP ORF (initiation at site D). Several potential translation initiation sites are present in these mRNAs, leading to the production of several isoforms (Calkhoven *et al.*, 2000; Lin *et al.*, 1993; Ossipow *et al.*, 1993). The truncated isoforms lack different parts of the N-terminus of the full-length protein and do not contain the activation domain. Therefore the isoform ratio is important for the activity of these proteins. Translational control determines the isoform ratio and is dependent on the presence of the out-of-frame uORF immediately upstream of the major translation start site (B1 in Figure 1-6). Translation initiation at B1 can occur by combination of leaky scanning past the uORF AUG codon and reinitiation after uORF translation (Calkhoven *et al.*, 2000; Lincoln *et al.*, 1998). In contrast to its inhibitory effect on initiation at B1, the uORF enhances initiation at site C (Figure 1-6), generating a truncated form of this transcription factor (Calkhoven *et al.*, 1994; Calkhoven *et al.*, 2000). In this case, ribosomes bypass the start sites B1 (and B2 in the C/EBPα mRNA) after translating the uORF and thereby gain access to site C. The isoform ratio is not only modulated by uORFs, but also by translation initiation factor activity. Both high eIF2 activity and eIF4E overexpression lead to elevated production of the truncated C/EBP forms and a decrease of the full-length protein (Calkhoven *et al.*, 2000). This is due to stimulated translation initiation at

site D, reducing initiation at site B1 and increasing reinitiation at site C. Therefore, the uORF in the C/EBP mRNAs is critical for the regulation of the isoform ratio and consequently for the transcriptional regulation of C/EBP target genes.

The proto-oncogene *fli-1* is a transcription factor of the ets family that is important for differentiation and cell migration during early embryonic development. Two different Fli protein isoforms of 51 and 48 kDa are synthesized from the same transcript by using two in-frame initiation codons, respectively (Sarrazin *et al.*, 2000). The synthesis of these two isoforms is regulated by two short uORFs that both overlap the initiation codon of the 51 kDa isoform (Figure 1-6). Translation termination at the stop codon of the uORFs stimulates the synthesis of both Fli-1 isoforms.-i.e., the translation of the uORFs stimulates 48 kDa protein synthesis, by allowing downstream reinitiation at the 48-kDa AUG codon. The synthesis of the 51 kDa isoform is stimulated by the blockage caused by the terminating ribosomes, which causes the upstream scanning ribosomal subunits to pile up and increase upstream initiation (Sarrazin *et al.*, 2000).

One of the mRNAs encoding 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP2) directs the synthesis of two protein isoforms: CNP1 and CNP2 (O'Neill *et al.*, 1997). CNP1 translation is initiated at an internal AUG codon. The CNP2 isoform is initiated at AUG1 and has a 20-amino-acid extension at the N-terminus (Figure 1-6). Although several tissues contain the CNP2 transcript, the CNP2 polypeptide is only present in myelinating cells in the adult rat brain, whereas all tissues produce the shorter isoform, CNP1. Initiation of the upstream codon is tissue-specific and was not further studied.

1.7.3.3 Stalling of Ribosomes at uORFs

S-Adenosylmethionine decarboxylase (AdoMetDC) is a key enzyme in the pathway of polyamine biosynthesis. The translation rate of *AdoMetDC* mRNA depends on cell type, cellular polyamine content, and, for T lymphocytes, growth status (Hill and Morris, 1992; Mach *et al.*, 1986; Ruan *et al.*, 1996; White *et al.*, 1990). This mRNA contains a uORF in the 5' UTR that plays an important role in feedback control by polyamine levels and in cell-specific regulation, although by different mechanisms [Figure 1-6; (Hill and Morris, 1993; Ruan *et al.*, 1994; Ruan *et al.*, 1996)]. The *AdoMetDC* uORF encodes a

hexapeptide (MAGDIS) and its amino acid sequence, especially aspartic acid (D) and isoleucine (I), is critical for translational control (Mize *et al.*, 1998).

In the presence of polyamines, the uORF functions as a negative regulatory element; inhibition is partially relieved under low polyamine levels (Ruan *et al.*, 1996). Studies have demonstrated that elevated polyamines inhibit synthesis of the uORF-encoded peptide by stabilizing an intermediate in the termination process. The complete nascent peptide linked to the tRNA decodes the final codon. This complex causes stalling of the translating ribosome and thus blocks translation of the associated downstream cistron (Raney *et al.*, 2000; Raney *et al.*, 2002).

The extent of translational repression of *AdoMetDC* mRNA by the uORF is also dependent on cell type (Hill and Morris, 1992). In resting normal T cells and T-cell lines with normal cellular levels of polyamines, ribosome loading on the *AdoMetDC* mRNA is suppressed to a point where the mRNA is largely associated with single ribosomes, whereas in many cell lines of nonlymphoid origin, molecules of this mRNA can contain 5 to 10 ribosomes. This cell-specific translation of *AdoMetDC* mRNA depends not only on the amino acid sequence of the uORF-encoded peptide but also on the close proximity of the uORF initiation codon to the cap in the wild-type mRNA. Initiation at the uAUG, which is located only 14 nt from the cap, is relatively infrequent in non-lymphoid cells, and the translation efficiency of *AdoMetDC* mRNA in these cells is therefore relatively efficient (Ruan *et al.*, 1994). Whether lymphoid cells have a more stringent control over polyamine synthesis remains to be known. The suggested association of polyamines with the MAGDIS peptide is reminiscent of the influence of arginine on translation of the *arg-2* mRNA.

The *Neurospora crassa arg-2* gene encodes the small subunit of the arginine-specific carbamoyl phosphate synthetase. The *arg-2* mRNA contains an evolutionarily conserved uORF encoding a 24-amino-acid peptide, the arginine attenuator peptide (AAP; Figure 1-6). Similar uORFs are also found in the 5' UTRs of the corresponding mRNAs in *S. cerevisiae* and several other fungi (Wang and Sachs, 1997). The *arg-2* uORF is located approximately 40 nucleotides downstream of the closest transcription start site, and its presence is necessary for regulation by arginine (Wang *et al.*, 1998b; Wang and Sachs, 1997). In the presence of low arginine concentrations, most ribosomes

bypass the suboptimal uAUG codon by leaky scanning and translate the downstream cistron. In the presence of high arginine concentrations, ribosomes translating the uORF stall in association with the AAP, creating a blockade to scanning ribosomes that subsequently load on the mRNA. Stalling on the uORF is reversible and the inhibition of *arg-2* translation is released when arginine is depleted (Wang *et al.*, 1998b). This sequence-dependent ribosome stalling is similar to that observed with *AdoMetDC*, yet with significant differences. AAP causes the ribosome stalling even when the uORF termination codon of the *arg-2* gene is removed, indicating that termination itself is not necessary for *arg-2* regulation and this AAP can act at the elongation or termination step.

Human cytomegalovirus early glycoprotein, *gpUL4* contains a 22-codon upstream open reading frame (uORF2) that represses translation of the downstream cistron [Figure 1-6; (Degnin *et al.*, 1993)]. The uORF2 AUG is frequently bypassed by a leaky scanning mechanism because of its suboptimal context (Cao and Geballe, 1995). However, once the uORF2 is translated, the ultimate tRNA is not released and the ribosomal complex, including peptidyl-tRNA, forms a barrier for upstream scanning 40S ribosomal subunits (Cao and Geballe, 1996). Therefore, translation of the *gpUL4* ORF is strongly inhibited. The peptide is supposed to interact with the ribosome and/or ribosome-associated translation factors.

In these examples, stalling of the scanning 43S ribosomal complexes is due to slow elongation or termination of ribosomes translating a uORF. Stalling is not necessarily dependent on the presence of uORFs, as 43S complexes can be stalled on an adenine-rich element in the 5' UTR of Pabp1 mRNA (Bag, 2001). Moreover, the translational repression of *HAC1* (a transcription factor that controls the unfolded protein response) mRNA by ribosome stalling is due to interaction of the 5'- and 3'-ends of the mRNA. This example shows that secondary structure can be responsible for stalling as well (Ruegsegger *et al.*, 2001).

1.8 Summary

The expansion or remodeling of pre-existing blood vessels, known as angiogenesis, is a highly regulated process. Angiogenesis is critical not only during normal vascular

development, but also in the progression of several disease states, including cancers, diabetes, and tissue ischaemia. Tie2 signaling is involved in multiple steps of the angiogenic remodeling processes during development, including destabilization of existing vessels, endothelial cell migration, tube formation, and the subsequent stabilization of newly formed tubes by mesenchymal cells. Tie2 is also present in the quiescent adult vasculature, implicating its role in the maintenance of blood vessels. Beyond this critical role in blood vessel development, recent studies suggest a wider role for Tie2 in lymphangiogenesis and hematopoiesis. Although the importance of Tie2 signaling has been well documented, the molecular mechanisms of how Tie2 gene expression is regulated are not clear.

1.9 Thesis Proposal

The Tie2 receptor tyrosine kinase plays an essential role in angiogenesis. Since this receptor was first identified in the early 1990s, numerous studies have been devoted to understanding its important biological functions. However, very little is known about the possible translational control of Tie2 mRNA. We have observed that despite a significant decrease in the mRNA levels, Tie2 mRNA translation is maintained under hypoxia, when overall protein synthesis and cap-dependent translation is inhibited. Moreover, we found that Tie2 protein levels in quiescent endothelial cells are comparable to those in proliferating endothelial cells. In fact, recently, Arai *et al.* have shown that Tie2/Ang1 signaling pathway plays a crucial role in maintaining HSC quiescence in the BM niche (Arai *et al.*, 2004). These observations led us to investigate the molecular mechanisms of Tie2 gene regulation, especially at the translational level to understand how Tie2 translation is maintained under unfavorable environment conditions.

Based on our mapping assays and sequence analyses of the Tie2 5' UTR, we observed that the Tie2 5' UTR is unusually long and contains many potential *cis*-acting elements such as uORFs and IRESes. These features are also found in other cellular mRNAs involved in angiogenesis such as VEGF, FGF-2, and PDGF mRNAs (Bernstein *et al.*, 1997; Huez *et al.*, 1998; Johannes *et al.*, 1999; Stein *et al.*, 1998; Vagner *et al.*, 1995). Nevertheless, no clear molecular mechanism of Tie2 gene regulation has yet been

revealed. Therefore, we decided to investigate if Tie2 mRNA harbors any functional *cis*-acting elements and if so, how they regulate Tie2 gene expression under different physiological conditions.

In summary, we explored the molecular mechanisms of Tie2 translational regulation by its 5' UTR in order to gain a better understanding of the complex events controlling Tie2 protein expression. In the discussion, I will explain our proposed model whereby both IRES-mediated and cap-dependent initiation can occur in the Tie2 5' UTR.

CHAPTER 2

Internal Translation Initiation Mediated by the Angiogenic Factor Tie2

2.1 Abstract

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' UTR. Transcription start site mapping indicates the existence of a several 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.

2.2 Introduction

Angiogenesis is an essential step in allowing tumors to grow beyond 1-2 mm in diameter (Risau, 1997). 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) 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 (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996; Griffioen and Molema, 2000; Shalaby *et al.*, 1995). VEGF expression is stimulated under hypoxic conditions by the transcription factor hypoxia-inducible factor 1 (HIF1), which regulates a large range of physiological responses as a consequence of reduced oxygen availability (Maxwell *et al.*, 2001; Semenza, 2002).

The angiopoietins belong to a second family of angiogenic factors essential for blood vessel maturation. Angiopoietin-1 (Ang1) is an agonist of endothelial cell tyrosine

kinase receptor Tie2/tek (Suri *et al.*, 1996). Studies with Tie2 null mice indicate that the angiopoietin/Tie2 signaling system plays a role in the later steps of angiogenesis (Dumont *et al.*, 1994). Consistent with a central role for Tie2 in angiogenesis, germ line-activating mutations in humans are associated with vascular dysmorphogenesis (Vikkula *et al.*, 1996). Additionally, the naturally occurring Tie2 antagonist, Ang2, disrupts angiogenesis *in vivo* (Maisonpierre *et al.*, 1997). Blocking Tie2 activation with recombinant Tie2 significantly inhibits tumor growth (Lin *et al.*, 1998) and is associated with activation of apoptosis (Jones *et al.*, 2001b), possibly due to disruption of Akt signaling (Papapetropoulos *et al.*, 2000). 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 (Maxwell *et al.*, 2001; Semenza, 2002), exposure of cells to hypoxia attenuates protein synthesis by decreasing translation initiation (Arsham *et al.*, 2003; Koumenis *et al.*, 2002). Two main steps of initiation are targets for regulation - either 43S ribosomal complex formation [by affecting eIF2 phosphorylation status (Clemens, 2001)] or the mRNA/ribosome binding step (Gingras *et al.*, 1999). 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 m^7G-cap structure and involves binding of the 43S preinitiation complex near the mRNA 5' end, followed by scanning to the appropriate AUG start codon (Gingras *et al.*, 1999). 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-dependency of an mRNA is thought to be a function of 5' cap proximal secondary structure; hence mRNAs with reduced secondary structure will have reduced dependency on eIF4F for initiation, whereas those with increased secondary structure are more dependent on eIF4F for initiation (Gehrke *et al.*, 1983; Svitkin *et al.*, 2001). 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 (Arsham *et al.*, 2003), several mRNAs

implicated in angiogenesis remain efficiently translated, including VEGF (Akiri *et al.*, 1998; Huez *et al.*, 1998; Miller *et al.*, 1998; Stein *et al.*, 1998), HIF1α (Lang *et al.*, 2002), and Tie2 (this report). Whereas VEGF and HIF1α achieve this by recruiting ribosomes internally to an IRES, the issue of how Tie2 is able to circumvent this translational block has not been previously addressed. 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.

2.3 Materials and Methods

Cell lines, Transfections, and Hypoxia Treatment

Primary human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (Walkersville, MD) and maintained in endothelial growth media-2 (EGM-2) supplemented with growth factors, 2 % fetal bovine serum (FBS), 50 μg/mL gentamicin, and 50 μg/mL amphotericin according to the manufacturer's instructions.

DNA transfections into primary HUVECs were performed using Lipofectin (Invitrogen; Carlsbad, CA), as specified by the manufacturer. Briefly, 1.5-1.8x10^5 cells were seeded per 10 cm^2 plate and grown in EGM-2 medium supplemented with 2 % FBS. Cells were harvested 24 hr post-transfection and luciferase activities 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 1.8x10^6 HUVECs were seeded per 10 cm^2 plate 24 hr prior to transfection. Cells were then transfected with 20 μg of capped and polyadenylated mRNA and harvested 8 hr post-transfection. Transfections were performed in duplicate and repeated 3 times. For polysome analysis and RNA isolation, extracts prepared from HUVECs grown under hypoxia (>0.01 % O_2) and normoxia (20 % O_2) for 16 hrs 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 HUVEC cells was isolated using TRIzol (Invitrogen) according to the manufacturer's suggested protocol. Oligonucleotides were end-labeled with [γ-^{32}P]ATP (3000 Ci/mmol; PerkinElmer Life and Analytical Sciences; Wellesley, MA) using T4 polynucleotide kinase and purified after separation on 8 % denaturing polyacrylamide gels. A mixture of 2.5 μg of mRNA or 25 μg total RNA and $2x10^5$ 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; LaJolla, CA) to create hTie2UP-Ribo15/pKSII. [α-^{32}P]UTP (800 Ci/mmol) was used with the MAXIscript *in vitro* transcription kit (Ambion; Austin, TX) to generate riboprobes from the XhoI-linearized hTie2UP-Ribo15/pKSII. The radiolabeled cRNA was purified by 8 % denaturing gel electrophoresis, followed by elution for 12 hr at 37 °C in 0.5 M ammonium acetate, 1 mM EDTA, 0.2 % SDS. The efficiency of labeling was determined by scintillation counting and 1 x 10^5 cpm of each labeled riboprobe was used per reaction. RNAse protection assays were performed using the RPAIII System (Ambion) according to the manufacturer's instructions. Briefly, the labeled riboprobe was hybridized to 5 μg of human placental Poly[A]$^+$ for 16 hr at 42°C. Following hybridization, unprotected single-stranded RNA was digested with a mixture of RNaseA (2.5 units/mL) plus RNaseT1 (100 units/mL). The resulting protected products were separated on an 8 % denaturing polyacrylamide gel and visualized by autoradiography.

Sucrose Gradients and Polysome Analysis

Polysome analyses were performed on HUVECs at 70 % confluence. Cells were treated with 0.1 mg/mL cycloheximide for 3 min, and harvested in lysis buffer [15 mM

Tris-HCl (pH 7.4), 300 mM NaCl, 15 mM $MgCl_2$, 1 % Triton X-100, 0.1 mg/mL cycloheximide, 333.3 units/mL RNAse inhibitor (Ambion; Austin, Texas)] at 4 °C. After a brief centrifugation, the extract was layered on 10-50 % sucrose gradients in buffer [15 mM Tris-HCl (pH 7.4), 300 mM NaCl, 15 mM $MgCl_2$]. Gradients were centrifuged at 39,000 rpm for 1.5 hr at 4 °C, and fractions were collected across the gradient and snap frozen in liquid nitrogen. Total RNA or RNA from the indicated pooled fractions following sucrose gradient centrifugation was isolated using TRIzol (Invitrogen).

Generation of Monocistronic and Bicistronic Constructs

The generation of bicistronic constructs was based on pRΔDEF (Johannes *et al.*, 1999). First, a fragment corresponding to the hTie2 5'-UTR was generated by PCR amplification of the hTie2 5'-UTR cDNA and subcloned into pGL3-BASIC (Promega). In the second step, the fragments containing hTie2 5'-UTR and amino-terminal coding region of firefly luciferase were introduced into pRΔDEF by replacing the ΔEMCV 5' UTR and the first 121-bp of firefly luciferase. Bicistronic constructs containing a stable stem-loop structure [TAR(+III) element (Parkin *et al.*, 1988)] were generated by inserting the stem-loop element 232 bp upstream of the *Renilla* coding region of the above-mentioned bicistronic constructs. For all of the recombinant constructs generated in this study in which a reporter was placed downstream of uORF5, its AUG codon was placed in-frame with the predicted Tie2 AUG codon embedded in uORF5. The accuracy of all constructs was confirmed by sequencing.

Real Time RT-PCR Analysis

Relative expression levels were assessed using 200-300 ng RNA sample by real-time, one-step RT-PCR using the Roche Diagnostics LightCycler Instrument and the Roche Diagnostics LightCycler RNA Master SYBR Green I kit according to the manufacturer's instruction. The level of 28S rRNA was used as a reference value for quantitation within samples to determine the relative amounts of specific mRNA. The sequences of oligonucleotides used for RT-PCR are as follows: for Tie2, hTie2-L: 5' ATTGCGAGATGGATAGGGCTTGAG 3'; hTie2-R: 5' ACCTTCCACAGTTCCAGAAAGGAG 3'; hTie2(1273-1289): 5'

GCCGCTACCTACTAATG 3'; hTie2(1612-1596): 5' GTGATTAACGGGTTTGT 3' ; for VEGF, hVEGF-L: 5' CTTTCTGCTGTCTTGGGTGCATTG 3'; hVEGF-R: 5' CATGGTGATGTTGGACTCCTCAGT 3'; for βactin, hβactin-L: 5' GATGACCCAGATCATGTTTGAGACC 3'; hβactin-R: 5' GACTCCATGCCCAGGAAGGAAGGC 3'; and for 28S rRNA, h28SrRNA-L: 5' TACCACAGGGATAACTGGCTTGTG 3'; and h28SrRNA-R: 5' TAATCCCACAGATGGTAGCTTCGC 3'.

Northern Blot Analysis

Total RNA was isolated following transfection with bicistronic reporters. Following extraction of RNA with TRIzol (Invitrogen), 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; Palo Alto, CA) at 68 °C with a ~400-nt firefly luciferase-specific ^{32}P-labeled probe. Autoradiography of the membrane was performed at -80 °C with film (Kodak X-OMAT) to visualize the radioactive signal.

Metabolic Labeling with ^{35}S-Methionine

HUVECs were plated at 2.7x10^5 cells per 150 mm plate and incubated for 24 hr, followed by a further 12-36 hr incubation under either normoxic or hypoxic conditions. Cells were then washed with phosphate-buffered saline and incubated in methionine-free RPMI 1640 medium (Sigma; St. Louis, MI) supplemented with 10 % fetal calf serum and ^{35}S-methionine (PerkinElmer Life and Analytical Sciences; Wellesley, MA) 4 hr prior to harvesting. Cells were washed three times with phosphate-buffered saline and harvested in lysis buffer (25 mM HEPES (pH7.4), 137 mM NaCl, 10 % glycerol, 2.5 mM EDTA, 2.5 mM EGTA, 0.5 % Triton X-100, 5 mM β-mercaptoethanol, 2 ug/mL Aprotinin, 2 ug/mL Leupeptin, 2 ug/mL PepstatinA). The cell lysates (500 ug of total protein) were pre-cleared 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-ODC (Sigma; Saint Louis, MI) antibodies, respectively.

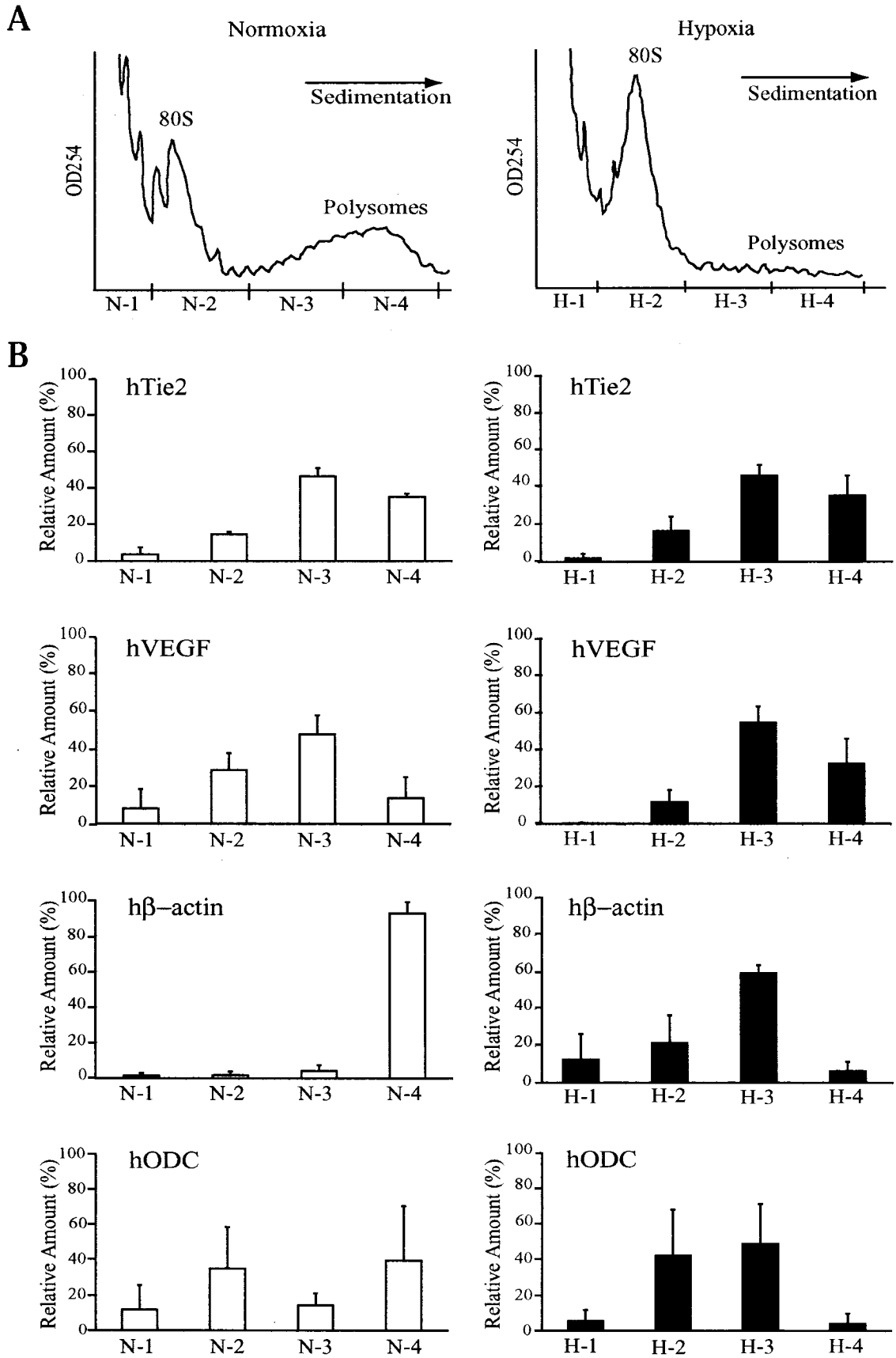
The immunoprecipitates were then subjected to SDS-PAGE, followed by fluorography. Experiments were performed twice at least in triplicate.

2.4 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 human umbilical vein endothelial cells (HUVECs; Figure 2-1). Under hypoxic conditions, there is a reduction in polysomes accompanied by an apparent increase in amount of free ribosomal subunits (Figure 2-1A), as previously reported (Lang *et al.*, 2002; Stein *et al.*, 1998) and consistent with a reduction in protein synthesis associated with this physiological 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 VEGR mRNAs remain unchanged when cells are transferred from normoxic to hypoxic conditions (Figure 2-1B). 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 hypoxia (Figure 2-1B). This is similar to what has been previously reported for β-actin mRNA redistribution when cells are exposed to hypoxia (Gorlach *et al.*, 2000; Lang *et al.*, 2002). We also assessed the change in polysome distribution of a second mRNA encoding ornithine decarboxylase (ODC). Translation of this transcript is generally cap-dependent (Rousseau *et al.*, 1996; Seidel and Ragan, 1997), except during mitosis (Pyronnet *et al.*, 2000). 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 (Lang *et al.*, 2002; Stein *et al.*, 1998). Like other reports documenting mRNA redistribution among

Figure 2-1. Effect of hypoxia on Tie2 expression in HUVECs. **A.** A_{254} profile of polysomes obtained from HUVECs under normoxic or hypoxic conditions. **B.** RNA was purified from the indicated pooled fractions in [A] and quantified using real-time RT-PCR. For each gene, the relative amount of each pooled fraction was calculated as a percentage of its total in the polysomes. The results are an average of three independent experiments and the standard deviations are shown. Primers used for quantitative RT-PCR of the hTie2 transcript were hTie2(443-423) ($^{5'}$GATGAATTGCGAGATGGATAGGGCTTGAG$^{3'}$)(targeting nts -257 to -285) and hTie2UP-5'2 ($^{5'}$ACCTTCCACAGTTCCAGAAAGGAG$^{3'}$) (targeting nts +43 to +66).

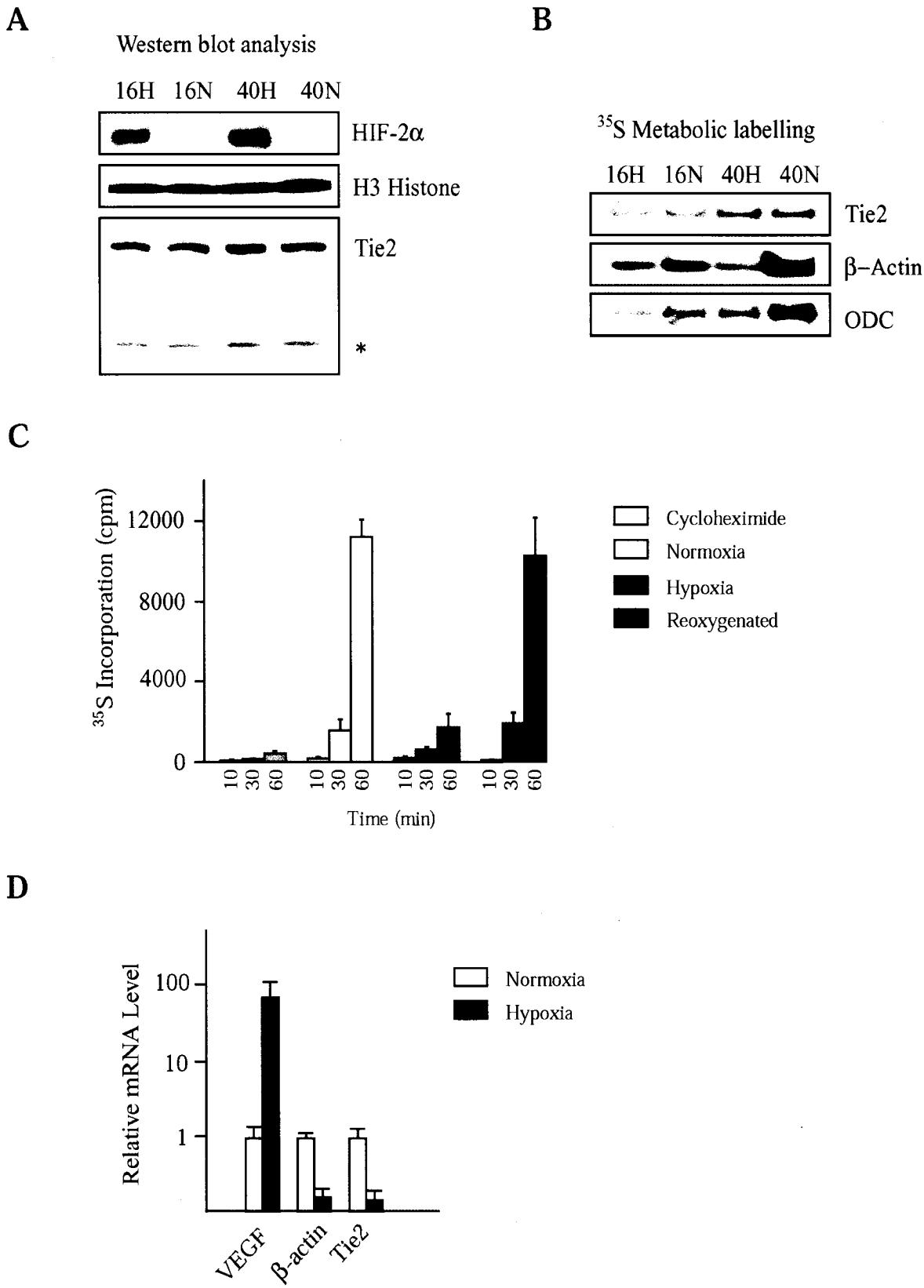

A
Normoxia
Hypoxia
80S
Sedimentation
Polysomes
OD234
OD254
N-1
N-2
N-3
N-4
H-1
H-2
H-3
H-4
B
hTie2
hVEGF
hβ–actin
hODC
Relative Amount (%)
0
20
40
60
80
100

polysomes during hypoxia, we do not observe a complete disaggregation of β-actin (Gorlach *et al.*, 2000; Lang *et al.*, 2002) or ODC mRNA into free mRNA (Figure 2-1B), despite an apparent flattening of the polysome peak (Figure 2-1A). 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 (Figure 2-2A). HIF2α protein was detected only in extracts prepared from hypoxic cells confirming activation of the hypoxic response (Figure 2-2A). In this western blot analysis, anti-H3 histone was used as a loading control for HIF2α (Figure 2-2A). Consistent with the polysome profiling results (Figure 2-1), Tie2 protein was present in both normoxic and hypoxic treated cells (Figure 2-2A). [In this experiment, a non-specific 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 (Figure 2-2B). In contrast, synthesis of β-actin and ODC were significantly decreased after exposure of HUVECs to hypoxic stress (Figure 2-2B). To gauge the extent to which translation in HUVECs is suppressed by hypoxia, we measured the rate of [^{35}S]methionine incorporation into newly synthesized proteins in HUVECs under normoxic or hypoxic conditions (Figure 2-2C). Under hypoxic conditions, protein synthesis is reduced by 85 % in HUVECs, in comparison to HUVECs under normoxic or reoxygenated conditions following hypoxic stress (Figure 2-2C). 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 (Figure 2-2D). VEGF mRNA levels increased 70-fold in response to hypoxia, whereas those of β-actin decreased ~4 fold (Figure 2-2D). These results are consistent with previously reported effects of hypoxia on VEGF and β-actin gene expressions (Forsythe *et al.*, 1996; Levy *et al.*, 1995; Shweiki *et al.*, 1992). Under hypoxic conditions, Tie2 mRNA levels decreased ~4 fold, consistent with the results reported by Mandriota and Pepper (Mandriota and Pepper, 1998).

Figure 2-2. Tie2 protein production is maintained under hypoxic conditions. **A.** Tie2 protein expression in HUVECs exposed to hypoxia (H; 1 % O_2) or normoxia (N; 20% O_2) for 16 and 40 hrs. Upper panel: Western blot analysis performed using polyclonal anti-HIF2α (Novus Biologicals, Inc.; Littleton, CO) antibodies. Middle panel: Western blot analysis performed using histone H3 antisera (Cell Signaling; Beverly, MA). Lower panel: Western blot analysis performed using anti-Tie2 (Santa Cruz Biotechnology, Inc.; Santa Cruz, CA) antibodies. A non-specific band (indicated by an asterisk) was used as a loading control. **B.** Metabolic labeling of Tie2 under hypoxic conditions. HUVECs were metabolically labeled with ^{35}S-methionine under normoxia (N) or hypoxia (H) and immunoprecipitated with anti-Tie2, anti-β-actin, or anti-ODC antisera. Immunoprecipiates were resolved by SDS/PAGE and visualized by fluorography. **C.** Metabolic labeling using ^{35}S-methionine in HUVECs under conditions of normoxia, hypoxia, or reoxygenation. HUVEC cells were preincubated for 40 hr under hypoxia or normoxia or incubated for 40 hr under hypoxia, followed by 1 hr of normoxia (reoxygenated). Radiolabelled methionine incorporation under normoxia is also presented in the presence of 20 ug/ml cycloheximide. Cells were incubated in the presence of ^{35}S-methionine for 10, 30 or 60 minutes, then immediately harvested. Trichloroacetic acid precipitation of cell lysates was performed in duplicate and used to determine the incorporation of [^{35}S]methionine into protein. Each measurement was normalized with respect to total protein as determined by the Lowry protein assay. The results represent the average of three independent experiments performed and the error bars denote the error of the mean. **D.** Total RNA was isolated from cells and used as template for real-time RT-PCR. The relative mRNA amounts were normalized to the abundance of the 28S rRNA and averaged for three independent experiments.


A
Western blot analysis
16H 16N 40H 40N
HIF-2α
H3 Histone
Tie2
*
B
35S Metabolic labelling
16H 16N 40H 40N
Tie2
β–Actin
ODC
C
35S Incorporation (cpm)
12000
8000
4000
0
10 30 60 10 30 60 10 30 60 10 30 60
Time (min)
Cycloheximide
Normoxia
Hypoxia
Reoxygenated
D
Relative mRNA Level
100
10
1
VEGF
β-actin
Tie2
Normoxia
Hypoxia

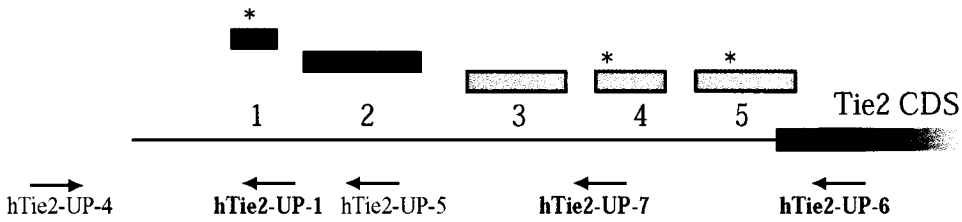
Mapping the Human Tie2 5' Untranslated Region.

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 (Hewett *et al.*, 1998). The results suggested the presence of 10 transcription initiation sites situated from 269 to 414 nucleotides upstream of the initiator AUG codon (Fadel *et al.*, 1998). 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 (Figure 2-3A). 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 (Figure 2-3B). These fragments map to nts –372 and –377 of the Tie2 5'UTR (Figure 2-3D, encased within grey boxes).

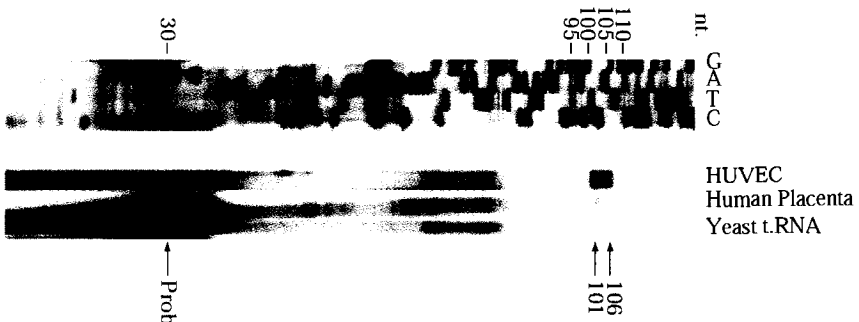
To complement the primer extension results and determine if we could detect the same start sites, we performed RNAse protection experiments. The probe used extended from –467 to –224 (Figure 2-3D, generated by primers hTie2-UP-4 and hTie2-UP-5). Two major protected fragments of 127 and 149 nucleotides were observed (Figure 2-3C). These mapped to positions – 372 and –350 of the Tie2 5' UTR (Figure 2-3D). 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 (Figure 2-3C). 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 (Fadel *et al.*, 1998). 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 (E.P. and J.P., data not shown). Hence, the Tie2 mRNA isoforms identified here contain several features predicted to be inhibitory to a cap-dependent initiation

Figure 2-3. 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 grey. Asterisk(s) above a uORF indicates 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 adenosine residues and are encased in the gray boxes in panel 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 grey boxes and grey circles above the nucleotide, respectively.

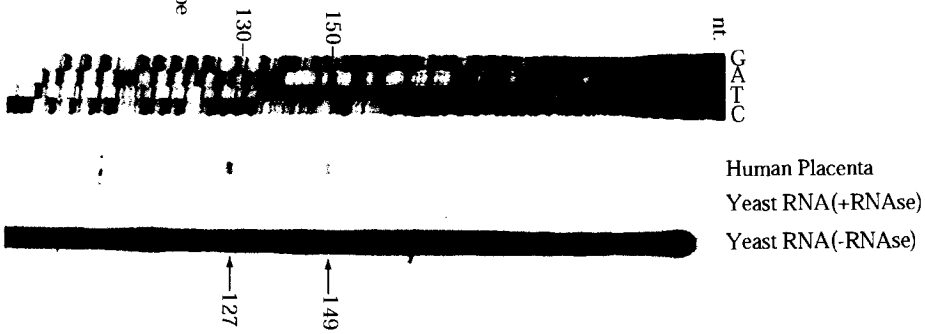
A



B



C



D

hTie2-UP-4(RPA)

-467 cataggtggagggcttgttattctcagtttcccgcctatgagaggatacccctattgtttct

-405 gaaaatgctgaccgggacccacacttcc[ ][ ]aattcctctgcccctacagcagcaa

hTie2-UP-1

-343 aagcagcagcagaagcaacagcaacagataagtgttttg**atgaattgcgagatggataggc**

hTie2-UP-5(RPA)

-281 **ttga**gtgcccccagccctgctgataccaa**atgcctttaagatacagcctttcccatcctaat**

-219 **ctacaaaggaaacaggaaaaaggaacttaa**aactccctgtgctcagacagaa**atgagactgt**

hTie2-UP-7

-157 **tacagcctgcttctgtgctgttccttcttgcctctaacttgtaa**acaagacgtagtaggacg

-95 **atgctaatggaaagtcacaaacccgctgggtttttga**aaggatccttgggacctc**atgcacat**

-33 **ttgtggaaactggatggagagatttgggggaagt**+1**ATGGACTCTTTAG**CCAGCTTAGTTCTCTG

hTie2-UP-6

+30 TGGAGTCAGCTTGCTCCTTTCTGGAACTGTGGAAGGTGCCATGGACTTGATCTTGATCAAT

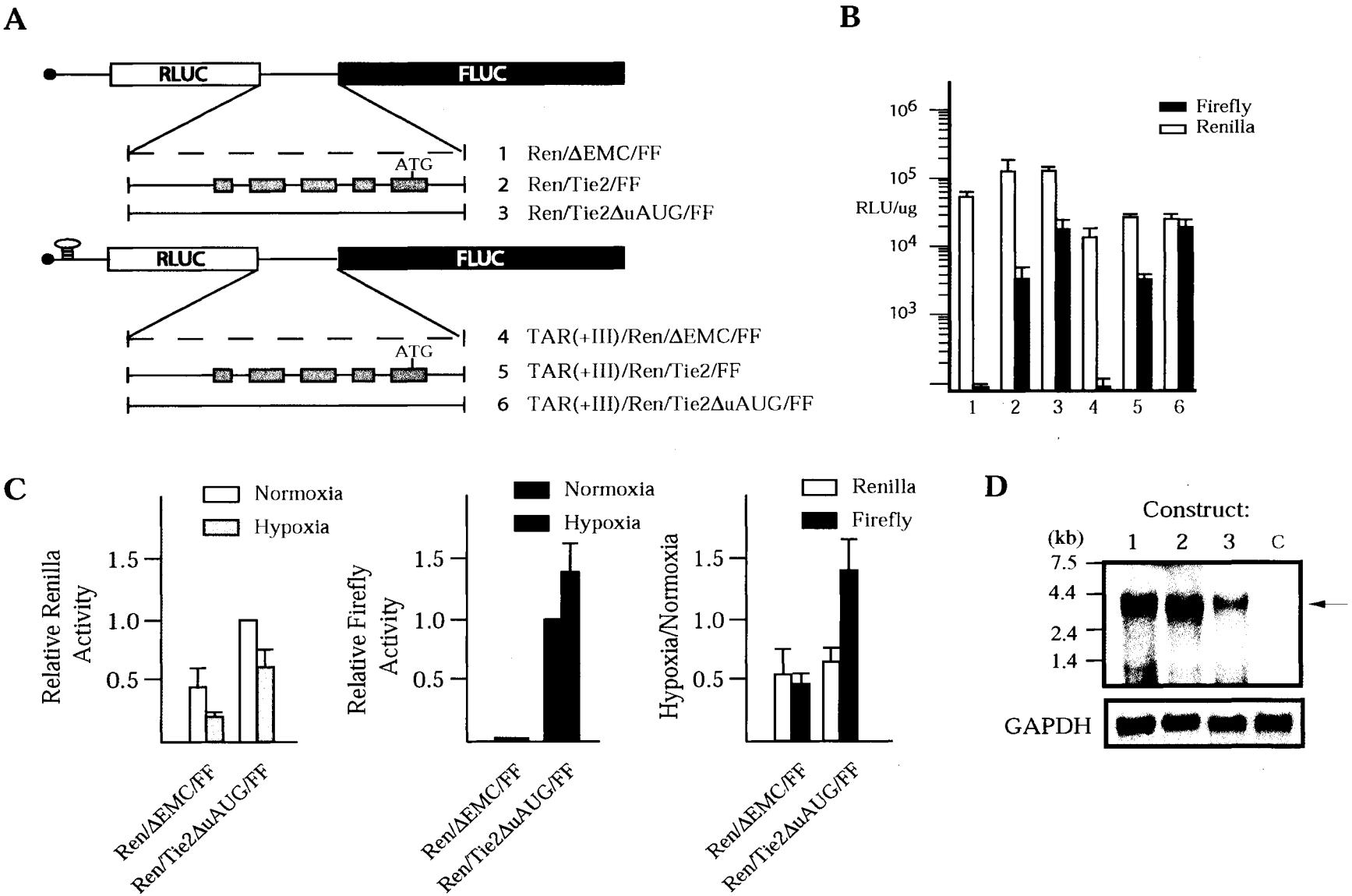
mechanism, notably a lengthy 5' UTR (372 nts), 5 uORFs, and an AUG codon embedded with the last uORF (Figure 2-3D). In this report, we further characterize the translational properties of the –372 isoform.

The Tie2 5' UTR Mediates Internal Initiation.

One mechanism by which Tie2 could be efficiently translated under hypoxic conditions (Figure 2-2B), a situation that reduces cap-dependent translation initiation (Arsham *et al.*, 2003; Johannes *et al.*, 1999), 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 (Figure 2-4A). 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 (Figure 2-4A). As a negative control for these experiments, we utilized a deletion mutant of the EMCV 5' UTR that does not support internal ribosome binding (Figure 2-4A, 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 HIV TAR element [TAR(+III)] was positioned upstream of *Renilla* luciferase. This allowed us to probe whether firefly luciferase expression was independent of *Renilla* expression and not a consequence of ribosome reinitation (Figure 2-4A). The stable secondary structure of this element (ΔG=-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 (Parkin *et al.*, 1988).

Transfection experiments with HUVECs revealed that expression vectors containing the TAR(+III) element upstream of the *Renilla* luciferase initiation codon displayed a ~5-fold reduction in *Renilla* luciferase activity relative to the constructs lacking this stem-loop barrier (Figure 2-4B; compare lanes 1, 2, and 3 to lanes 4, 5 and 6). Very low levels of firefly luciferase activity was 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 (Figure 2-4B, 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 (Figure 2-4B,

Figure 2-4. The Tie2 5'UTR mediates internal initiation *in vivo*. **A.** Schematic representation of bicistronic constructs containing the Tie2 5' UTR in the intercistronic region. The *Renilla* and firefly luciferase ORFs are denoted by a white and black box, respectively. The stem-loop structure upstream of the *Renilla* ORF in the TAR(+III) construct series represents the HIV TAR element. **B.** Quantitation of luciferase activities following DNA transfection into HUVECs. The relative light units per microgram of protein extract is denoted for both the *Renilla* and firefly luciferase proteins. The construct number is indicated below the X-axis. The results represent the average of three independent experiments performed and the error bars denote the error of the mean. **C.** Quantitation of *Renilla* luciferase activities obtained from transfected cells exposed to normoxia or hypoxia. Luciferase activities were determined, normalized to the protein content of each extract, and then set relative to the value obtained for Ren/Tie2ΔAUG/FF under normoxic conditions. Ratios of the hypoxic to normoxic values are also calculated and plotted. Values plotted are the average of 3 independent experiments with the standard error presented. **D.** Northern blot of RNA isolated from transfected HUVECs and probed for firefly luciferase. The nature of the construct is indicated above the panel and corresponds to the numbering scheme used in panel A. The arrow indicates the position of migration of the bicistronic mRNA and the bottom panel is the same blot reprobed with GAPDH, to account for possible variations in loading.


A
RLUC
FLUC
ATG
1 Ren/ΔEMC/FF
2 Ren/Tie2/FF
3 Ren/Tie2ΔuAUG/FF
RLUC
FLUC
ATG
4 TAR(+III)/Ren/ΔEMC/FF
5 TAR(+III)/Ren/Tie2/FF
6 TAR(+III)/Ren/Tie2ΔuAUG/FF
B
Firefly
Renilla
RLU/ug
10^6
10^5
10^4
10^3
1 2 3 4 5 6
C
Normoxia
Hypoxia
Relative Renilla Activity
1.5
1.0
0.5
Ren/ΔEMC/FF
Ren/Tie2ΔuAUG/FF
Normoxia
Hypoxia
Relative Firefly Activity
1.5
1.0
0.5
Ren/ΔEMC/FF
Ren/Tie2ΔuAUG/FF
Renilla
Firefly
Hypoxia/Normoxia
1.5
1.0
0.5
Ren/ΔEMC/FF
Ren/Tie2ΔuAUG/FF
D
Construct:
(kb)
1 2 3 C
7.5
4.4
2.4
1.4
GAPDH

compare lanes 2 to 5) or with Ren/Tie2ΔuAUG/FF and TAR(+III)/Ren/Tie2ΔuAUG/FF (Figure 2-4B, compare lanes 3 to 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.

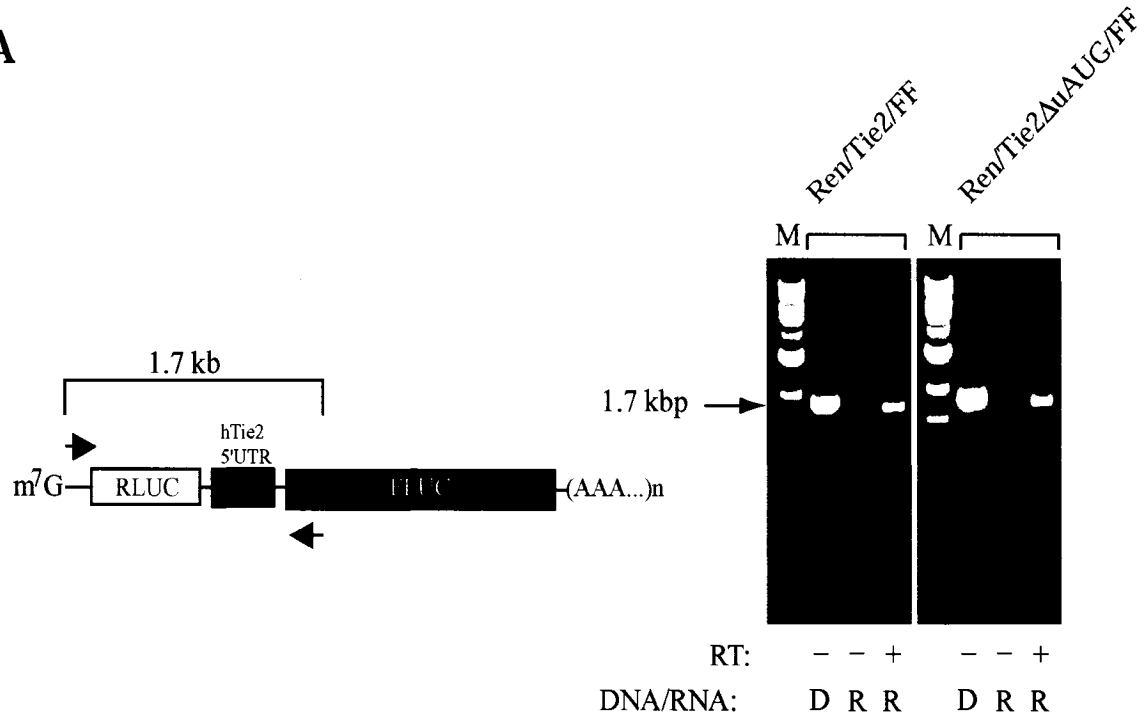
One function of the Tie2 IRES might be to maintain efficient translation during hypoxia (Figure 2-1). To assess if the putative Tie2 IRES could function in a bicistronic context under hypoxic conditions, we transfected HUVECs with Ren/ΔEMC/FF or Ren/Tie2ΔAUG/FF and placed these cells under normoxic or hypoxic conditions (Figure 2-4C). 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 (Figure 2-4C). This effect is likely an under-estimation 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/Tie2ΔuAUG/FF (Figure 2-4C). The hypoxic/normoxic ratio indicates that the potential Tie2 IRES activity was even slightly stimulated under hypoxic conditions (Figure 2-4C). 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 (Figure 2-4D). 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 (Figure 2-4B).

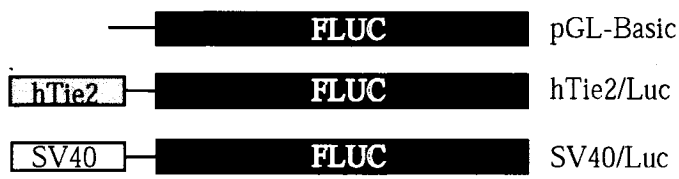
Another alternative interpretation for the observed results would be the existence of cryptic splice sites upstream of the firefly luciferase coding region that could eliminate a portion, or all of, the upstream *Renilla* sequences. Subsequent translation of a spliced transcript could then lead to increased 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/Tie2ΔuAUG/FF (Figure 2-5A). PCR primers were designed that targeted the upstream of *Renilla* and

Figure 2-5. Absence of cryptic splicing within the Tie2 5' UTR. **A.** RT-PCR analysis of total RNA isolated from transfected HUVECs. PCRs were performed from plasmid DNA (lanes labeled D) as positive controls. RT-PCRs were performed from RNA in which reverse transcriptase (RT) was added (+) or omitted (-). The expected size of the PCR products is indicated. Molecular size markers (M) are 1 kbp ladders from New England Biolabs. The locations of the PCR primers used in this amplification are schematically shown. **B.** Assessing promoter activity of the Tie2 5' UTR. Schematic representation of constructs used to test for cryptic promoter activity within the Tie2 5' UTR. The firefly luciferase ORF is denoted by a black box. The pGL-Basic vector (Promega) lacks a defined active promoter. The Tie2 5' UTR (nts –372 to +69) was placed upstream of the firefly luciferase gene. SV40/Luc contains the SV40 promoter driving transcription of the firefly luciferase gene and was used as a positive control. **C.** HUVECs were transfected with pGL-Basic, hTie2/Luc, or SV40/Luc in conjunction with the plasmid pRL-CMV (Promega). Lysates were prepared from the cells following transfection, and luciferase activities were measured. To account for variations in transfection efficiency, firefly luciferase activity was normalized to that of *Renilla* luciferase. The results represent the average of 3 independent experiments performed in duplicate and the error bars denote the error of the mean.

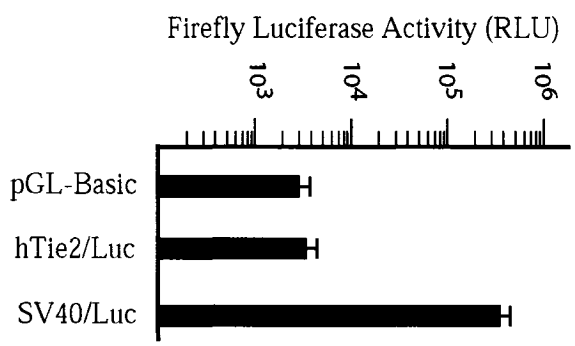
A


Ren/Tie2/FF
Ren/Tie2ΔuAUG/FF
M
M
1.7 kb
hTie2
5'UTR
m7G
RLUC
FLUC
(AAA...)n
1.7 kbp
RT: − − + − − +
DNA/RNA: D R R D R R

B


FLUC
pGL-Basic
hTie2
FLUC
hTie2/Luc
SV40
FLUC
SV40/Luc

C


Firefly Luciferase Activity (RLU)
10³
10⁴
10⁵
10⁶
pGL-Basic
hTie2/Luc
SV40/Luc

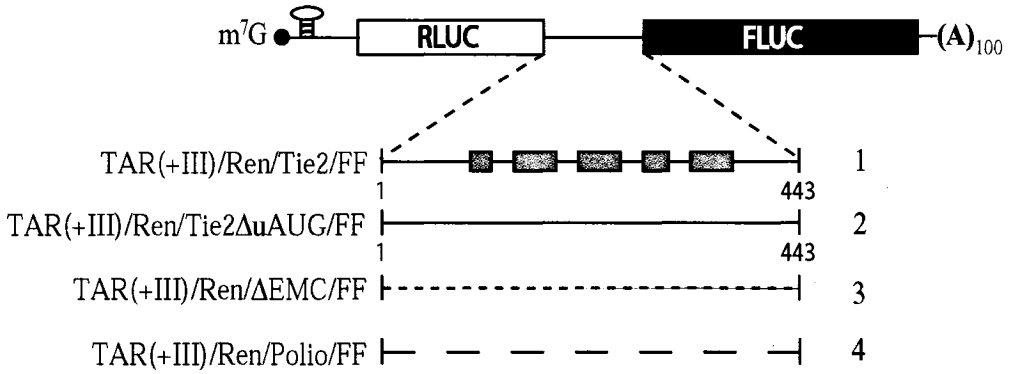
within the firefly luciferase coding region (Figure 2-5A, left panel). A single DNA product was obtained following RT-PCR of RNA from transfected cells and corresponds in size to the expected full-length PCR product encompassing the *Renilla* ORF and Tie2 5' UTR (~1.7 kbp) (Figure 2-5A). PCR products were not observed when reverse transcriptase was omitted from the RT-PCR (Figure 2-5A). 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) (Figure 2-5A).

To ensure 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 (Figure 2-5B). 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 (Figure 2-5C). On the other hand, SV40/Luc yielded over 100 times higher luciferase activity compared to hTie2/Luc and pGL-Basic (Figure 2-5C). These results indicate the absence of a detectable cryptic promoter activity within the Tie2 5'UTR.

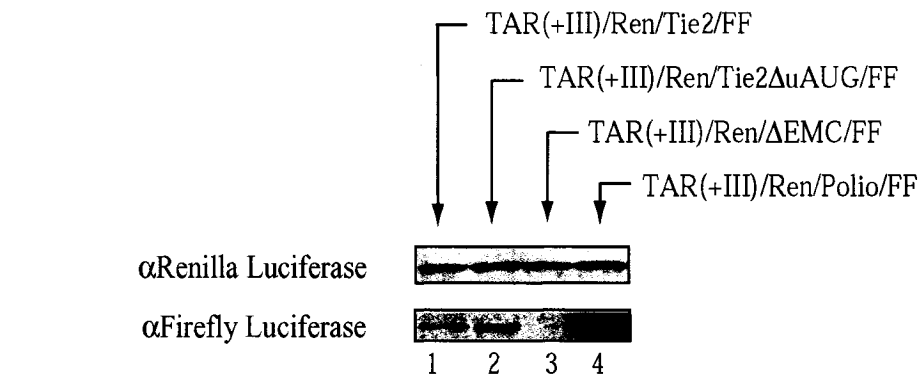
To confirm and extend these results, we performed a series of mRNA transfection experiments. We generated capped and polyadenylated mRNAs from bicistronic constructs containing: (i) the EMCV 5'UTR with the deleted IRES (negative control); (ii) the poliovirus IRES (positive control); and (iii) the Tie2 5' UTR containing or lacking all uAUGs (Figure 2-6A). Following transfection of mRNAs into HUVECs, firefly and *Renilla* production was assessed by western blotting (Figure 2-6B) or by measuring luciferase activities (Figure 2-6C). 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 (Figure 2-6B). No firefly luciferase was observed from cells transfected with TAR(+III)/Ren/ΔEMC/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

Figure 2-6. 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 panel A. Cell lysates were analysed by immunoblotting with anti-*Renilla* luciferase and anti-firefly luciferase antisera. The nature of the transfected mRNA 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/ΔEMC/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.

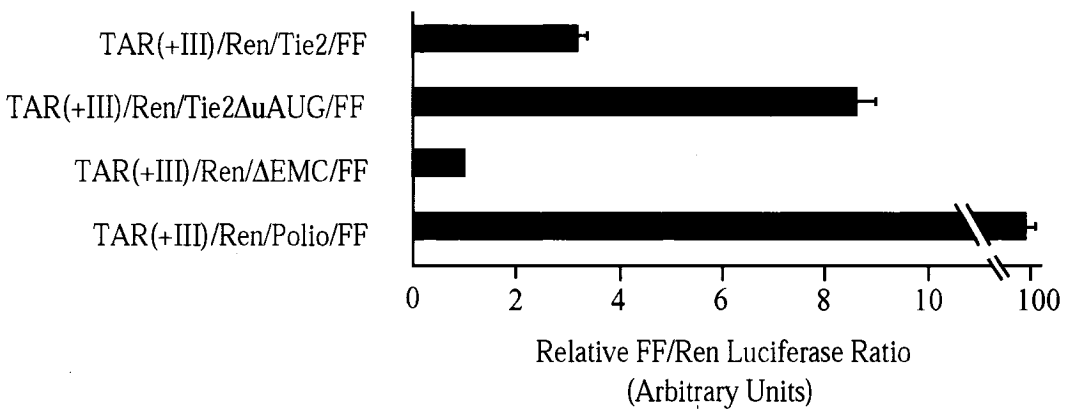
A


m7G
RLUC
FLUC
(A)100
TAR(+III)/Ren/Tie2/FF
1
443
1
TAR(+III)/Ren/Tie2ΔuAUG/FF
2
1
443
TAR(+III)/Ren/ΔEMC/FF
3
TAR(+III)/Ren/Polio/FF
4

B


TAR(+III)/Ren/Tie2/FF
TAR(+III)/Ren/Tie2ΔuAUG/FF
TAR(+III)/Ren/ΔEMC/FF
TAR(+III)/Ren/Polio/FF
αRenilla Luciferase
αFirefly Luciferase
1 2 3 4

C


TAR(+III)/Ren/Tie2/FF
TAR(+III)/Ren/Tie2ΔuAUG/FF
TAR(+III)/Ren/ΔEMC/FF
TAR(+III)/Ren/Polio/FF
0 2 4 6 8 10 100
Relative FF/Ren Luciferase Ratio
(Arbitrary Units)

TAR(+III)/Ren/Tie2/FF and TAR(+III)/Ren/Tie2ΔuAUG/FF mRNAs, with slightly more being produced from the construct lacking uAUGs (Figure 2-6B, compare lane 1 to 2). These results were confirmed by monitoring *Renilla* and firefly luciferase activities (Figure 2-6C). 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/ΔEMC/FF mRNA, consistent with the presence of an IRES in the former transcript (Figure 2-6C). The FF/Ren ratio of TAR(+III)/Ren/Tie2ΔuAUG/FF transfected cells was ~8-fold higher than that of the negative control. These results indicate that the observed Tie2 IRES activity is RNA dependent and not due to the presence of a cryptic promoter. TAR(+III)/Ren/Tie2/FF exhibited ~3-fold lower FF/Ren ratio than that detected with TAR(+III)/Ren/Tie2ΔuAUG/FF, further suggesting that the uORFs within the Tie2 5'UTR negatively affect IRES-mediated translation (See Discussion).

2.5 Discussion

The angiopoietin-Tie2 signaling pathway plays an important role in the development of normal and tumor vasculature. Tie2 expression is elevated in human cancers (breast, ovarian, hepatocellular, glioblastoma) with expression being localized in "vascular hot spots" at the leading edge of invasive tumors (Peters *et al.*, 2004). Conflicting reports have been presented regarding changes in Tie2 protein and mRNA levels during hypoxia. Increases in Tie2 protein expression have been reported to parallel increases in Tie2 mRNA levels (Christensen *et al.*, 2002; Park *et al.*, 2003; Willam *et al.*, 2000). In contrast, Mandriota and Pepper (Mandriota and Pepper, 1998) have reported decreased Tie2 mRNA levels during hypoxia and Lin *et al.* (Lin *et al.*, 2002) indicated unchanged Tie2 protein levels under hypoxia. The reasons for these discrepancies are not clear, but may reflect response differences to hypoxia among the different cell lines. In our hands, Tie2 mRNA levels decrease as a consequence of hypoxia treatment (Figure 2-2D) with protein production continuing at levels comparable to those observed during

normoxia (Figure 2-2B). Our study indicates that Tie2 protein production is secured under hypoxic conditions (Figure 2-2B), when general translation is reduced.

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 (Arsham *et al.*, 2003; Tinton and Buc-Calderon, 1999). 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 (Koumenis *et al.*, 2002). 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 (Figure 2-4C).

The Tie2 mRNA 5' UTR contains 5 uORFs, an unusually high number for a eukaryotic transcript (Figure 2-3D). The primer combination used to assess the polysome distribution of the hTie2 mRNA in Figure 2-1B spanned 5 uORFs and the first 60 nucleotides following the initiation codon (Figure 2-3D). 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 5 uORFs. We note that in both DNA and RNA transfections, expression of firefly luciferase is increased when all of the Tie2 uAUGs are deleted (Figures 2-4B and 2-6). 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 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 *cat-1* IRES, where disruption of RNA-RNA interactions between the 5' and 3' end of an IRES prevents inducible internal initiation. The *cat-1* IRES activity is restored upon inhibition of 5' end mediation initiation (Yaman *et al.*, 2003). 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* (Johannes and Sarnow, 1998; Nanbru *et al.*, 1997; Nanbru *et al.*, 2001; Stoneley *et al.*, 1998), HIF1α (Lang *et al.*, 2002), and VEGF (Akiri *et al.*, 1998; Huez *et al.*, 1998; Miller *et al.*, 1998; Stein *et al.*, 1998). The presence of IRESes 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 IRESes. Additionally, the Tie2/Ang1 signaling system has been implicated in regulating quiescence of hematopoietic stem cells in the bone marrow niche (Arai *et al.*, 2004). Tie2 expression needs to be maintained during quiescence, a physiological state associated with reduced protein synthesis (Hofmann and Hand, 1994). Hence, the Tie2 IRES described herein may serve to circumvent the translation block imposed by quiescence to keep Tie2 protein levels constant.

2.6 Acknowledgements

E.H.P. is supported by a K.M. Hunter/CIHR doctoral research award. J.P. is a Canadian Institute of Health Research (CIHR) Senior Investigator. We thank the

anonymous reviewers for their helpful comments in improving the manuscript. This work was supported by a grant from NCIC (#011040) to J.P.

Connecting Text

In order to better understand the regulation of Tie2 gene expression by the *cis*-acting elements within the 5' UTR identified by our mapping assays, we further characterized the effect of the uORFs on translation. The results of these studies are presented in Chapter 3.

CHAPTER 3

The Tie2 5' Untranslated Region is Inhibitory to 5' end-Mediated Translation Initiation

3.1 Abstract

Tie2 is an endothelium-specific receptor tyrosine kinase required for normal blood vessel maturation, remodeling, and stability. Tie2 expression is also up-regulated in various cancers implicating a role in tumor angiogenesis. Its mRNA transcript contains an unusually long (372 nucleotides) 5' UTR with five uORFs and an IRES that allows this mRNA to be translated under hypoxic conditions. This sets up an alternative initiation pathway with the potential to clash with 5' end-mediated initiation from the same template. Herein, we define experimental conditions under which the Tie2 IRES is not active, allowing us to assess the contribution of the 5' UTR to cap-dependent translation on the Tie2 transcript. We find that the Tie2 5' UTR is inhibitory to translation initiation with ribosome flow decreasing following encounters with each uORF. No single uORF was found to harbor significant *cis*-acting inhibitory activity. Our results suggest that the uORFs within the Tie2 5' UTR serve to decrease the percent of ribosomes competent for reinitiation as these traverse the mRNA 5' UTR, thus minimizing interference with the IRES.

3.2 Introduction

Angiogenesis is a process of new blood vessel formation that is the culmination of mitogenic and tissue remodeling events resulting in neovascularization. It is a physiological process that is required for normal embryonic development, female reproductive function, and wound healing. During this process, angiogenesis is tightly regulated by a balance of positive and negative factors. However, in various disease states, including diabetic retinopathy, age-related macular degeneration, rheumatoid arthritis, and several cancers, deregulation of angiogenesis contributes to disease progression (Risau, 1997). It is also well documented that tumor angiogenesis is essential for solid tumor growth and metastasis (Carmeliet, 2003). Previously published reports have indicated that angiogenesis involves the coordinated activities of at least two

families of receptor tyrosine kinases (RTKs), the vascular endothelial growth factor receptor (VEGFR) and the Tie receptor families (Mustonen and Alitalo, 1995).

The Tie receptors, Tie1 and Tie2, are endothelial-specific receptor tyrosine kinases that share a number of common structural features. Although the functional significance and signaling partners of Tie1 are not well defined, the angiopoietins Ang1 and Ang2, have been identified and characterized as ligands for the Tie2 receptor (Davis *et al.*, 1996; Maisonpierre *et al.*, 1997). Disruption of Tie2 function in mice results in embryonic lethality due to defects in vascular development, characterized by a reduction in endothelial cell number and a defect in the morphogenesis of microvessels (Dumont *et al.*, 1994; Sato *et al.*, 1995). Disrupting the function of Ang1, an agonist of the Tie2 receptor, or overproduction of the antagonist Ang2, yields a phenotype similar to disruption of the Tie2 gene, confirming the importance of the Ang/Tie2 pathway during embryonic vascular development (Davis *et al.*, 1996; Maisonpierre *et al.*, 1997). Tie2 is also expressed and phosphorylated in quiescent adult endothelial cells suggesting that it plays an active role in the maintenance of blood vessels (Wong *et al.*, 1997). Moreover, this receptor is upregulated in capillaries during the process of neovascularization, including skin wounds and tumors (Peters *et al.*, 1998; Peters *et al.*, 2004; Stratmann *et al.*, 1998; Wong *et al.*, 1997). Consistent with an essential role for Tie2 in angiogenesis, a missense mutation in the Tie2 gene has been shown to be associated with venous malformations - the most common error of vascular morphogenesis in humans, typically resulting from an imbalance of endothelial cells and smooth muscle cells (Vikkula *et al.*, 1996).

While much research effort has focused on the function and biochemistry of the Tie2 gene product, very little is known about the translational regulation of Tie2 expression. The Tie2 gene encodes an mRNA with an unusually long (372 nucleotides) 5' UTR with 5 uORFs (Park *et al.*, 2005). Upstream open reading frames (uORFs) in mRNAs are known to regulate translation in eukaryotes and are particularly common in mRNAs coding for proto-oncogenes, transcription factors, and genes involved in the control of cellular growth and differentiation (Morris and Geballe, 2000). A variety of translational control mechanisms mediated by uORFs have been documented, ranging from *cis*-acting peptide-induced ribosome stalling, position- or length-dependent

influences on downstream initiation events, regulation of IRES activity, and effects on mRNA stability (Morris and Geballe, 2000; Yaman *et al.*, 2003). When uORFs induce reinitiation of translation, this is generally an inefficient mechanism that is possible only after translation of short uORFs (Kozak, 2002; Meijer and Thomas, 2002). It is thought that the distance between the termination codon of the upstream ORF and the initiation codon at the downstream ORF affects the rate of reloading of the eIF2/GTP/Met-tRNA$_i$ ternary complex onto scanning ribosomes, with increasing distance (time) allowing for higher reinitiation efficiencies. In some cases, the frequency of reinitiation at a downstream ORF depends on the coding content of the uORF (e.g., the *Neurospora crassa arg-2* gene), the sequence context of the termination codon, or the ability of the uORF to induce shunting (Meijer and Thomas, 2002; Morris and Geballe, 2000).

Cellular IRESes have been identified in many genes involved in cell growth, proliferation, apoptosis and angiogenesis (Pickering and Willis, 2005; Stoneley and Willis, 2004). We have previously demonstrated that translation of the Tie2 mRNA is maintained during hypoxic conditions due to the presence of an IRES (Park *et al.*, 2005). The presence of an IRES on a capped mRNA raises an interesting problem to the cell – how to regulate the two initiation mechanisms (cap-dependent and IRES-mediated) in a manner that avoids non-productive interference. In this regard, it has been proposed that for the mRNA encoding the arginine/lysine transporter *cat-1*, cap-dependent initiation mechanism inhibits IRES activity. For *cat-1*, ribosomes that have initiated in a cap-dependent fashion disrupt RNA-RNA interactions between the upstream and downstream ends of an IRES and prevent inducible internal initiation (Yaman *et al.*, 2003). Physiological situations that decrease cap-dependent protein synthesis induce *cat-1* IRES activity. Interestingly, Tie2 expression does not significantly change between normoxic and hypoxic states (Park *et al.*, 2005). In this report, we investigate the contribution of 5' end-mediated initiation to Tie2 expression and find that ribosomes competent for reinitiation decrease as they traverse the 5' UTR and encounter downstream uORFs.

3.3 Materials and Methods

Generation of Monocistronic and Bicistronic Constructs

The CAT reporter plasmid, pSKII/CAT, has been previously described (Lee *et al.*, 2002). A fragment (441 bp) corresponding to the hTie2 5'UTR sequence (nucleotides – 372 to +69) was fused to the CAT-reporter gene in pSKII/CAT. To generate this construct, RT-PCR was performed by using total RNA isolated from primary human umbilical vein endothelial cells (Clonetics; Walkersville, MD). Primer sequences based on the Tie2 -5'UTR sequence are as follows: sense ($^{5'}$TTTGATATCAGATCTAAGCTTAAATTCCTCTGCCCCTACAGCAGC$^{3'}$) and antisense ($^{5'}$TTTTAGATCTGGCACCTTCCACAGTTCCAG$^{3'}$). The obtained PCR product was cloned into pSKII/CAT. Mutants of the Tie2 5'UTR were constructed using PCR and subcloned into pSKII/CAT. For generating mammalian expression vectors, fragments containing the Tie2 5'UTR and CAT reporter gene were subcloned into pcDNA3. All recombinant clones were sequenced to ensure that no additional changes had occurred. A monocistronic luciferase reporter construct was generated based on FLUC/pcDNA3. A fragment containing the Tie2 5'UTR was excised from Tie2/CAT and subcloned into FLUC/pcDNA3.

To generate expression constructs with individual Tie2 uORFs (Figure 3-5), complementary oligonucleotide pairs encompassing each uORF were annealed and cloned into the pGL-Basic plasmid (Promega; Madison, WI). DNA fragments containing individual uORFs and firefly luciferase were then transferred into pcDNA3.

Bicistronic constructs generated were based on pGEMCAT/FLUC (Lee *et al.*, 2002). The hTie2 5'UTR sequence was amplified by PCR and cloned into the intercistronic region of pGEMCAT/FLUC. CAT/EMCV/FLUC was generated by inserting the EMCV 5'UTR into the intercistronic region of plasmid pGEMCAT/FLUC. The accuracy of all constructs was confirmed by sequencing.

In vitro Transcription/ Translation Reactions

For *in vitro* translation studies, plasmids were linearized and mRNA transcripts synthesized in the presence of m^7GpppG or ApppG, as previously described (Byrd *et al.*, 2005; Pelletier and Sonenberg, 1985). Capped RNA transcripts were quantified by monitoring the incorporation of ^{3}H-CTP and the quality of each RNA preparation assessed by SYBR gold (Molecular Probes; Eugene, OR) staining following fractionation

on formaldehyde/1% agarose gels. *In vitro* translations in rabbit reticulocyte lysates and wheat germ extracts were carried out using ^{35}S-methionine as instructed by the manufacturer (Promega). Krebs translation extracts were prepared and used for *in vitro* translation reactions, as previously described (Svitkin and Sonenberg, 2004).

RNA stability

^{32}P-labeled mRNAs were incubated under standard *in vitro* translation conditions in wheat germ extracts at 25 °C for 0, 15, 30, 60, and 120 minutes, respectively. Each translation reaction (10 μL) was then treated with 50 μg of proteinase K at 37°C for 15 min, followed by phenol/chloroform extraction. After ethanol precipitation, RNA samples were resolved on formaldehyde/1% agarose gels. The gels were dried and exposed to X-ray films at –70°C.

Cell culture and transient DNA/RNA transfections

Primary human umbilical vein endothelial cells (HUVECs) were maintained in endothelial growth media-2 (EGM-2) supplemented with growth factors, 2% fetal bovine serum (FBS), 50 μg/mL gentamicin, and 50 μg/mL amphotericin according to the manufacturer's instructions.

DNA transfections into primary HUVECs were performed using Lipofectin (Invitrogen; Carlsbad, CA), as specified by the manufacturer. Briefly, 1.5-1.8x10^5 cells were seeded per 10 cm^2 plate and grown in EGM-2 medium supplemented with 2% FBS. Cells were harvested 24 hr post-transfection and CAT and *Renilla* luciferase activities were measured using the CAT ELISA (Roche Applied Science; Penzberg, Germany) and the *Renilla* luciferase reporter assay systems (Promega; Madison, WI). Transient RNA transfections were performed using the cationic lipid reagent, DMRIE-C (Invitrogen). Approximately 1.8x10^6 HUVECs were seeded per 10 cm^2 plate 24 hr prior to transfection. Cells were then transfected with 20 μg of capped and polyadenylated mRNA and harvested 8 hr post-transfection. Luciferase activities were measured using the Dual-Luciferase reporter assay system (Promega). Transfections were performed in duplicate and repeated three times. All experiments with HUVECs were performed between passages two through five.

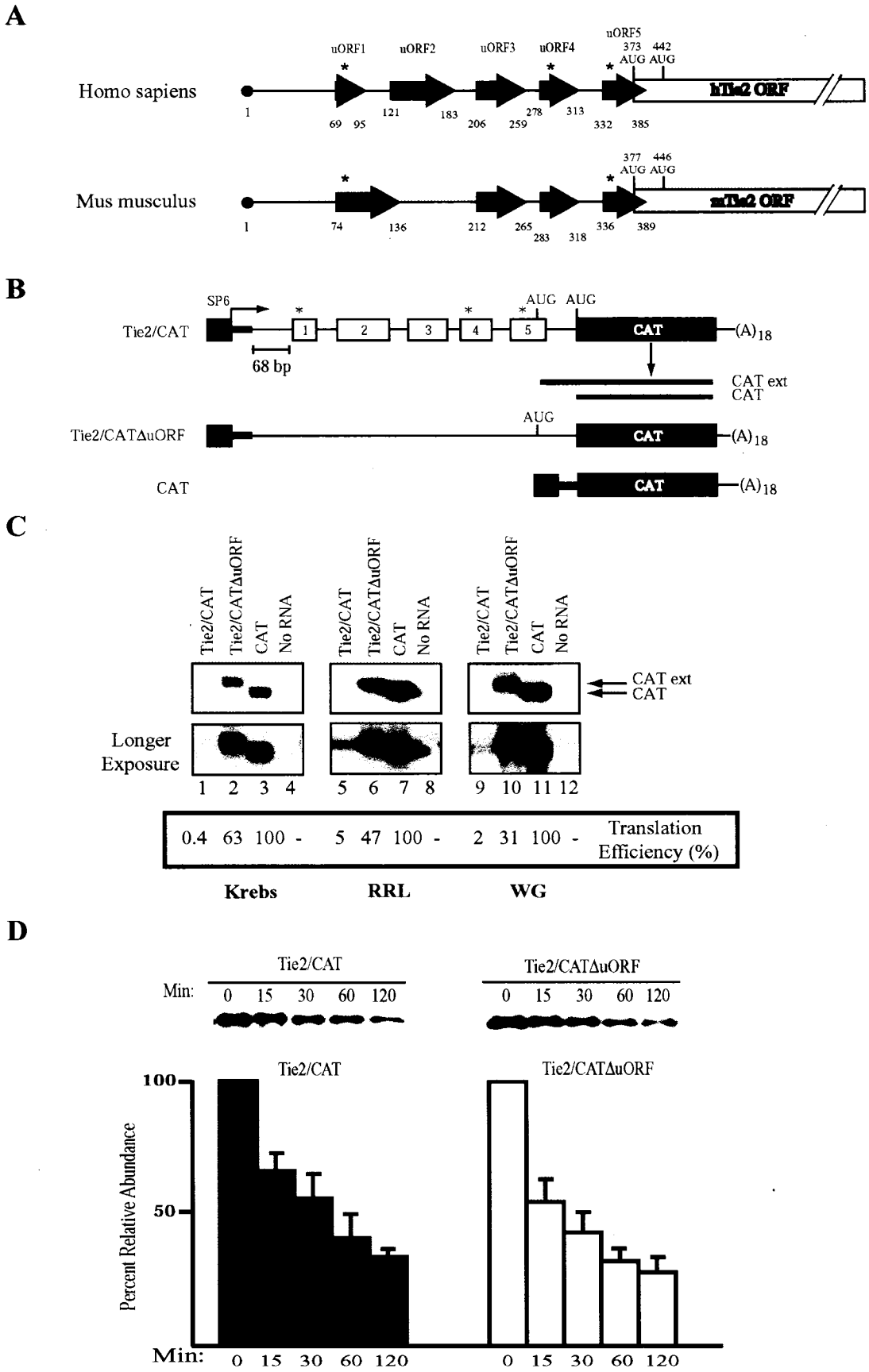
3.4 Results

The human Tie2 5'UTR contains five uORFs, the last of which overlaps with the putative Tie2 initiation codon (Figure 3-1A). Comparison of the Tie2 5'UTR sequence from the human and mouse mRNAs revealed that although the absolute number of uORFs is not conserved, the relative positions of four of the uORFs are (Figure 3-1A). Further analysis reveals that if uORF5 is translated, it should shunt ribosomes past the Tie2 initiation codon (which lies embedded within the uORF5 coding region) to a downstream AUG codon (Figure 3-1A). The presence of five uORFs, as in the Tie2 mRNA 5' UTR, is unusually rare.

The Tie2 5' UTR is significantly inhibitory to translation *in vitro*.

In order to obtain insight into the putative inhibitory effects of the Tie2 5' UTR on translation initiation, we generated a series of CAT reporter constructs containing the full-length Tie2 5' UTR (Tie2/CAT) or the 5' UTR lacking all uAUGs except for the predicted initiation codon (AUG_{373}; Tie2/CATΔuORF; Figure 3-1B). Two reporter constructs were generated in which the CAT initiator AUG was placed in-frame with the Tie2 initiation codon at AUG_{373}. Tie2/CAT contains all 5 uORFs, whereas in Tie2/CATΔuORF, these have been removed by site-directed mutagenesis of the uAUGs (Figure 3-1B). Translations of Krebs-2 extracts programmed with mRNAs derived from these reporter constructs demonstrated that very little CAT protein is produced from Tie2/CAT, whereas a significant amount of CAT protein was produced from Tie2/CATΔuORF (Figure 3-1C, compare lanes 1 to lane 2). These results were also consistent with results from Tie2/CAT mRNAs translated in rabbit reticulocyte lysates (RRL; compare lanes 5 to 6) or wheat germ extracts (WG; compare lanes 9 to 10). We also observed a higher molecular weight CAT protein species (labeled CAT ext) produced when Tie2/CATΔuORF (lanes 2, 6, and 10) and Tie2/CAT (lane 5) mRNAs were translated. This product would be expected if initiation from these transcripts was predominantly from the predicted Tie2 initiation codon at AUG_{373}, as this is expected to

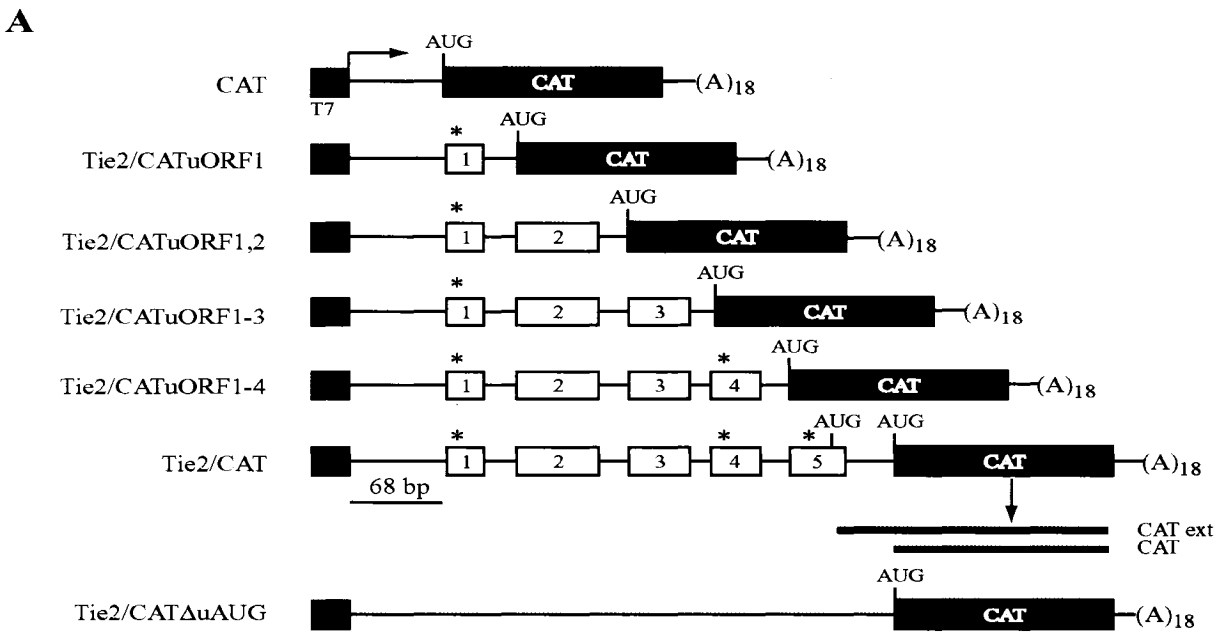
Figure 3-1. The Tie2 5'UTR is inhibitory to translation. **A.** Schematic diagram comparing the relative position and size of the uORFs in the Tie2 5'UTR of human (U53603) and mouse (NM_013690) genes. The Tie2 initiation codon is embedded within uORF5. The major Tie2 transcription start site is designated as 1 and relative positions of the uORFs from the transcription start site are indicated. The uORFs are represented by arrows. Asterisk(s) above a uORF indicates the presence of an internal AUG, in-frame with the uORF initiator AUG. **B.** Schematic representation of Tie2/CAT fusion constructs. The CAT ORF is denoted by a black box, the Tie2 uORFs are represented by white boxes, and the SP6 promoter is shown as a grey box. The thickened line represents vector-derived sequences within the 5' UTR. The asterisks above the uORFs denote the location of internal AUGs within the uORFs. **C.** Translation of the CAT reporter mRNAs in Krebs-2 extracts (Krebs), rabbit reticulocyte lysates (RRL), and wheat germ extracts (WG). Following *in vitro* translations, samples were electrophoresed into 12% SDS-polyacrylamide gels. The gels were treated with EN^{3}Hance, dried, and exposed to X-ray film (Kodak). Shown is one representative experiment from three independently performed experiments. **D.** Relative stability of Tie2/CAT and Tie2/CATΔuORF mRNA in translation extracts. Upper panel: ^{32}P-labeled Tie2/CAT and Tie2/CATΔuORF mRNAs were translated in wheat germ extracts, and at the indicated time points, an aliquot was removed from the translation reaction. Following re-isolation of the mRNAs, samples were fractionated on a 1% agarose/formaldehyde gel and quantified using a Fuji BAS2000. A representative autoradiograph from three experiments is shown. The time of isolation after start of translation and nature of the mRNA template are indicated. Lower panel: The relative stability for each mRNA at the indicated time points is calculated relative to T=0 min and plotted. The results are the average of three independent experiments with the error of the mean shown.


A
Homo sapiens
uORF1
uORF2
uORF3
uORF4
uORF5
373 442
AUG AUG
hTie2 ORF
1
69 95
121
183
206
259
278
313
332 385
Mus musculus
377 446
AUG AUG
mTie2 ORF
1
74
136
212
265
283
318
336
389
B
SP6
Tie2/CAT
AUG AUG
1
2
3
4
5
CAT
(A)18
68 bp
CAT ext
CAT
Tie2/CATΔuORF
AUG
CAT
(A)18
CAT
CAT
(A)18
C
Tie2/CAT
Tie2/CATΔuORF
CAT
No RNA
CAT ext
CAT
Longer
Exposure
1 2 3 4
5 6 7 8
9 10 11 12
0.4 63 100 -
5 47 100 -
2 31 100 -
Translation
Efficiency (%)
Krebs
RRL
WG
D
Tie2/CAT
Tie2/CATΔuORF
Min:
0 15 30 60 120
Percent Relative Abundance
100
50
Min:
0 15 30 60 120
0 15 30 60 120

generate a fusion protein with an N-terminal extension of 23 amino acids. Differences in translational efficiency are unlikely the consequence of significant differences in mRNA stability between these transcripts, as both Tie2/CAT and Tie2/CATΔuORF mRNAs show similar stabilities (Figure 3-1D). These results indicate that the Tie2 5' UTR, lacking uORFs only decreases translation efficiencies ~1.6 - 3.2 fold (depending on the extract used) when compared to CAT mRNA (Figure 3-1C, compare lanes 2, 6, and 10 to 3, 7, and 11, respectively). However, the presence of uORFs within the Tie2 5' UTR significantly reduces translational efficiency (~9 - 158 fold) (Figure 3-1C, compare lanes 1, 5, and 9 to 2, 6, and 10, respectively).

We next examined the reinitiation frequency at each uORF using CAT production as surrogate marker (Figure 3-2A). In generating these constructs, we maintained the identical intercistronic sequence and distance between the uORFs and the CAT coding region that is present in the Tie2 5' UTR. Thus, the CAT reporter was placed in the same relative context as the next expected downstream uORF and the efficiency with which CAT was produced from each construct was taken to be a reflection of the number of ribosomes competent for initiation at that position, either due to reinitiation or to ribosomes having bypassed some or all of the uORFs. *In vitro* translation of Tie2/CATuORF1 and CAT mRNAs indicated that uORF1 inhibits CAT production ~4 fold (Figure 3-2B, C; compare lanes 2 to 1). This revealed that translation at uORF2 is ~4 fold less efficient than at uORF1. On the other hand, replacing uORF3 with CAT (as in Tie2/CATuORF1,2) did not further reduce production of CAT protein (Figures 3-2B, C; compare lanes 3 to 2), indicating that uORF2 is inefficiently recognized by reinitiating ribosomes and/or ribosomes that have scanned past uORF1 and/or uORF2. Replacing uORF4 and uORF5 with the CAT ORF resulted in a further reduction in protein synthesis (~2 fold decrease for each ORF; Figures 3-2B, C; compare lanes 4 and 5 to 3 and 4, respectively). Placing the CAT ORF downstream of uORF5, but in-frame with the predicted Tie2 initiation codon (Tie2/CAT) did not further reduce protein synthesis (compare lanes 6 to 5), but did generate a polypeptide of slightly higher molecular mass when analyzed by SDS/PAGE, due to the presence of the predicted Tie2 AUG_{373} initiation codon embedded within uORF5, and as observed in Fig. 3-1C. Substituting all upstream AUGs to UUGs within the Tie2 5' UTR significantly enhanced CAT protein

Figure 3-2. Increased uORF content within the Tie2 5'UTR inhibits translation. **A.** Schematic representation of Tie2/CAT fusion constructs. The CAT ORF is denoted by a black box, the Tie2 uORFs are represented by white boxes, and the T7 promoter is shown as a grey box. The asterisks above the uORFs denote the location of internal AUGs within the uORFs. The intercistronic sequence and distance between the uORFs and the CAT coding region were maintained as present in the Tie2 5' UTR. **B.** Translation of the Tie2 uORF-containing CAT reporter transcripts in rabbit reticulocyte lysates (RRL) and wheat germ extracts (WG). Following *in vitro* translations, samples were separated on 12% SDS-polyacrylamide gels. The gels were treated with EN^{3}Hance, dried, and exposed to X-ray film. The result presented is a representative from three independently performed experiments. **C.** Quantitation from *in vitro* translation reactions programmed with mRNA from constructs shown in A. Trichloroacetic acid precipitation of translated products was used to quantify levels of protein product. Values are standardized to the efficiency obtained with Tie2/CAT. All values represent the average of at least three independent experiments and bars represent the error of the mean. **D.** Western blot analysis of extracts from cells transfected with expression vectors shown in A. Transfection efficiencies were standardized using a *Renilla* expression plasmid. Quantification of the results from three independent experiments was carried out using CAT ELISA assays (right panel). Western blots were probed for CAT (left panel). The position of migration of the CAT protein product is indicated to the right. The blot is a representative result from three experiments performed in duplicate.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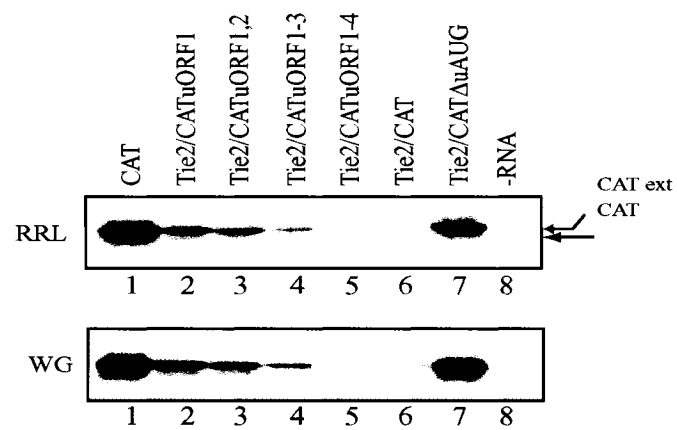
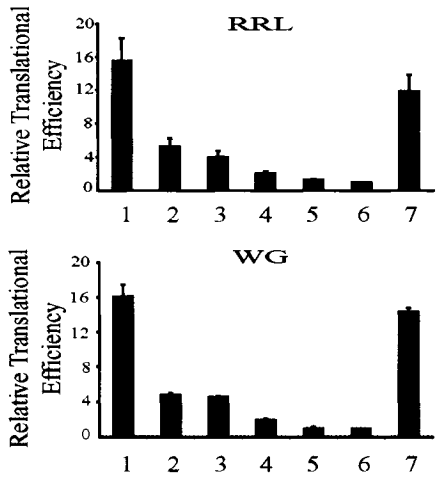
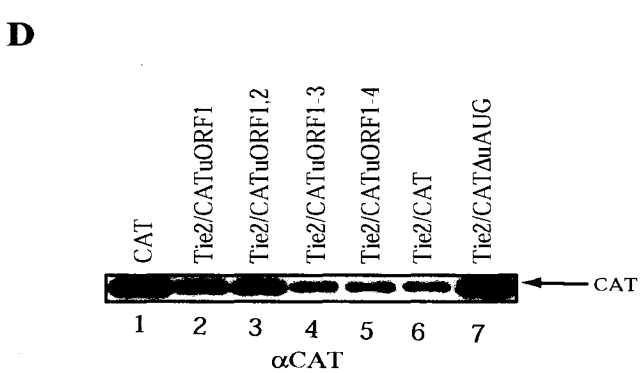
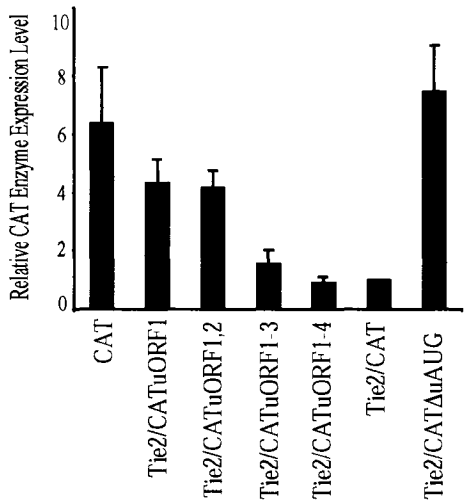
CAT
AUG
CAT
T7
(A)18
Tie2/CATuORF1
Tie2/CATuORF1,2
Tie2/CATuORF1-3
Tie2/CATuORF1-4
Tie2/CAT
68 bp
CAT ext
CAT
Tie2/CATΔuAUG
1
2
3
4
5


B
CAT
Tie2/CATuORF1
Tie2/CATuORF1,2
Tie2/CATuORF1-3
Tie2/CATuORF1-4
Tie2/CAT
Tie2/CATΔuAUG
-RNA
CAT ext
CAT
RRL
WG
1 2 3 4 5 6 7 8


C
RRL
WG
Relative Translational Efficiency
0
4
8
12
16
20
1 2 3 4 5 6 7


D
CAT
Tie2/CATuORF1
Tie2/CATuORF1,2
Tie2/CATuORF1-3
Tie2/CATuORF1-4
Tie2/CAT
Tie2/CATΔuAUG
CAT
1 2 3 4 5 6 7
αCAT


Relative CAT Enzyme Expression Level
0
2
4
6
8
10
CAT
Tie2/CATuORF1
Tie2/CATuORF1,2
Tie2/CATuORF1-3
Tie2/CATuORF1-4
Tie2/CAT
Tie2/CATΔuAUG

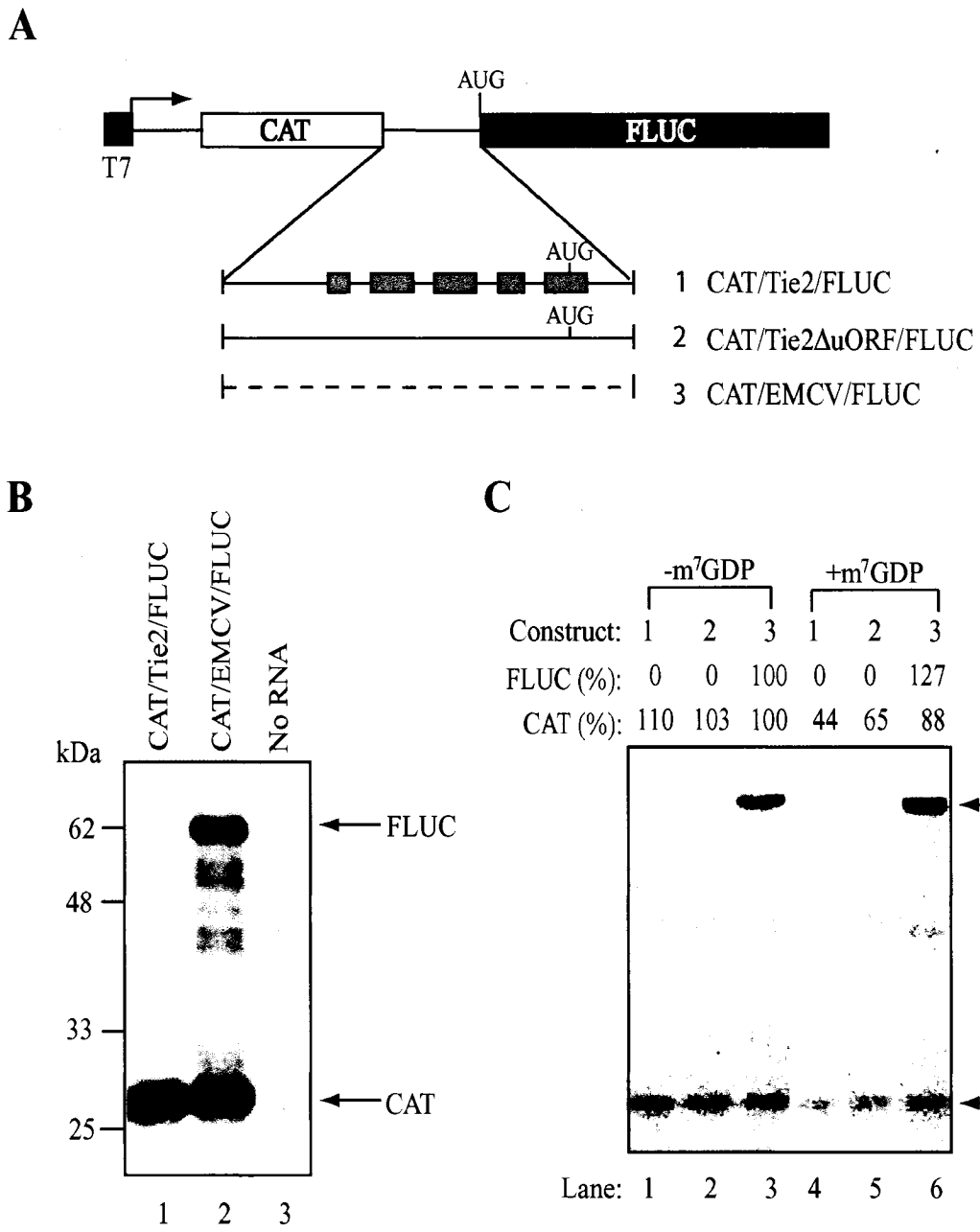
production (~15 fold) compared to translation of Tie2/CAT mRNA (Figures 3-2B, C; compare lanes 7 to 6).

To assess the relative translational efficiencies of these Tie2/CAT reporters *in vivo*, we transfected the various expression vectors into HUVECs and monitored the production of CAT protein by western blot and CAT ELISA assays (Figure 3-2D). Production of CAT *in vivo* from the Tie2/CATuORF1 and Tie2/CATuORF1,2 constructs was ~70 % of control CAT mRNA (Figure 3-2D, compare lanes 2 and 3 to 1) whereas the *in vitro* efficiency was observed to be ~25 % of the value obtained with CAT mRNA (Figure 3-2B, C). CAT production from Tie2/CATuORF1-3 was ~40 % of the values obtained with Tie2/CATuORF1 or Tie2/CATuORF1,2 (compare lanes 4 to 3 and 2), whereas the presence of uORF4 (Tie2/CATuORF1-4) or uORF4 and 5 (Tie2/CAT) had little additional effects on CAT production *in vivo* (compare lanes 5 and 6 to 4). Transfection of Tie2/CATΔuAUG, lacking all uAUGs, produced slightly higher levels of CAT proteins than that of the control CAT mRNA (compare lanes 7 to 1). In contrast to what we observed *in vitro*, the Tie2 AUG (present in uORF5) does not appear to be utilized *in vivo* since the CAT extended product was not obtained upon transfection of Tie2/CAT ((compare lanes 6 to 1). Hence, the same relative differences in expression among the uORF-containing reporters are observed *in vitro* and *in vivo*, although the levels relative to CAT appear higher *in vivo*. For example, *in vivo* we note a 6 fold difference in expression levels between Tie2/CAT and CAT (Figure 3-2D; compare lanes 6 to 1) compared to *in vitro* where a 16 fold difference is observed (Figures 3-2B, C; compare lanes 6 to 1).

The Tie2 IRES does not function *in vitro*.

One explanation for the differences noted *in vitro* and *in vivo* would be if the Tie2 IRES was operative *in vivo*, but not *in vitro*. To address this, we placed the Tie2 5' UTR within the intercistronic spacer between two reporter ORFs (CAT and firefly luciferase; Figure 3-3A) and tested the *in vitro* behavior of these transcripts (Figure 3-3B, C). During the course of *in vitro* translation studies using Krebs extracts, we noticed that only CAT protein was produced from CAT/Tie2/FLUC mRNAs (Figure 3-3B, lane 1). In contrast, translation of CAT/EMCV/FLUC mRNAs produced both CAT and firefly luciferase

Figure 3-3. Absence of Tie2 IRES activity *in vitro*. **A.** Schematic representation of bicistronic constructs containing the Tie2 5' UTR within the intercistronic region used to generate RNA for *in vitro* translation. The CAT and firefly luciferase (FLUC) ORFs are denoted by a white and black box, respectively. The T7 promoter and Tie2 uORFs are denoted by grey boxes. The full-length EMCV 5' UTR was used as a positive control. **B.** *In vitro* translation of bicistronic reporter transcripts in Krebs-2 extracts. Following translations, protein products were separated on 12% polyacrylamide/SDS gels. The gels were treated with EN^{3}Hance, dried, and exposed to X-ray film. The position of migration of CAT and FLUC proteins are indicated. Molecular mass markers (New England Biolabs) are shown to the left. **C.** Effect of m^{7}GDP on translation of bicistronic mRNA in Krebs extracts. Capped bicistronic mRNAs were translated in the presence or absence of 0.5 mM m^{7}GDP. Following electrophoresis and autoradiography, the bands corresponding to FLUC and CAT were quantified. The efficiency of FLUC and CAT translation is given as a percentage of control, relative to the value obtained with CAT/EMCV/FLUC mRNA in the absence of m^{7}GDP.


A
AUG
CAT
FLUC
T7
AUG
1 CAT/Tie2/FLUC
AUG
2 CAT/Tie2ΔuORF/FLUC
3 CAT/EMCV/FLUC
B
CAT/Tie2/FLUC
CAT/EMCV/FLUC
No RNA
kDa
62
48
33
25
FLUC
CAT
1 2 3
C
-m7GDP
+m7GDP
Construct: 1 2 3 1 2 3
FLUC (%): 0 0 100 0 0 127
CAT (%): 110 103 100 44 65 88
FLUC
CAT
Lane: 1 2 3 4 5 6

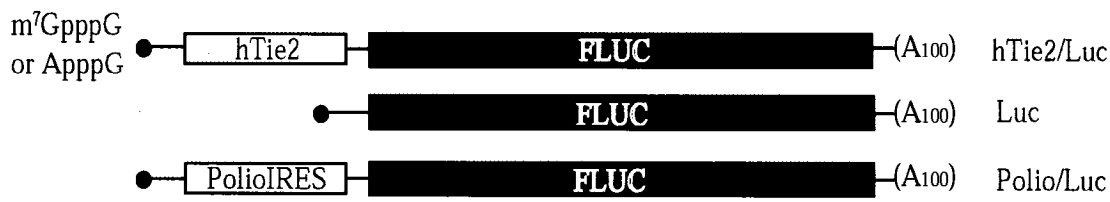
protein products as previously reported [Figure 3-3B, lane 2; (Svitkin *et al.*, 2001)]. In order to further confirm these results and document that CAT expression was cap-dependent, Krebs extracts were programmed with capped bicistronic mRNAs containing: (i) the Tie2 5' UTR (CAT/Tie2/FLUC); (ii) the Tie2 5' UTR lacking uORFs by mutating AUG to UUG (CAT/Tie2ΔuORF/FLUC); or (iii) the EMCV 5' UTR (CAT/EMCV/FLUC) within the intercistronic region. *In vitro* translation reactions were performed in the presence or absence of the cap analog m^7GDP (Figure 3-3C). m^7GDP inhibited cap-dependent translation of CAT by ~2.5-fold (Figure 3-3C; compare lanes 1-3 to lanes 4-6) and slightly stimulated EMCV IRES-dependent synthesis of firefly luciferase (Figure 3-3C; compare lanes 3 to 6). No evidence of IRES activity was observed upon translation of CAT/Tie2/FLUC (Figure 3-3C, lanes 1 and 4). Removal of the uORFs within the Tie2 5' UTR did not result in detection of firefly luciferase protein (Figure 3-3C, lanes 2 and 5). Lack of IRES activity was further confirmed when CAT/Tie2/FLUC was translated in wheat germ extracts and rabbit reticulocyte lysates (data not shown). These results indicate that the Tie2 5' UTR does not demonstrate detectable IRES activity *in vitro*.

Tie2 translation initiation occurs by internal ribosome entry and the conventional cap-dependent mechanism.

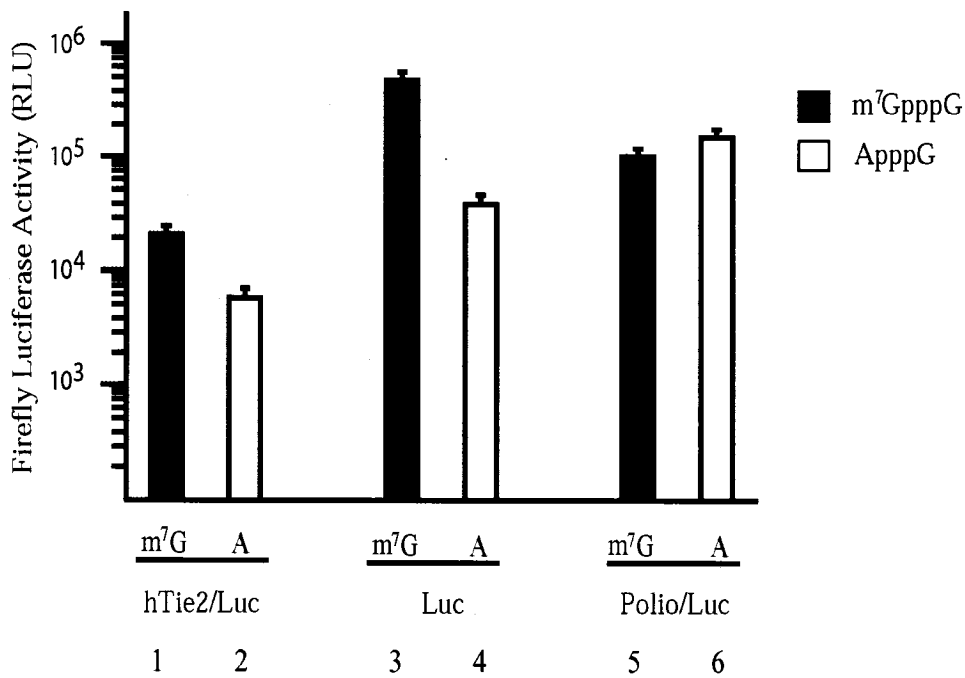
We have recently shown that the Tie2 IRES functions under conditions when cap-dependent translation is compromised. Indeed, Tie2 mRNAs remained associated with heavy polysomes under hypoxia, indicating efficient translation of Tie2 mRNA under this stress condition. However, the potential for Tie2 mRNA to also be translated in a cap-dependent mechanism was not previously addressed (Figure 3-4). We performed a series of mRNA transfections with firefly luciferase reporter mRNAs that were polyadenylated and capped with either m^7GpppG, or ApppG. We used FLUC transcripts with a short leader (Luc) or harboring the poliovirus IRES (Polio/Luc) as controls (Figure 3-4A). Following transfection of mRNA into HUVECs, firefly luciferase activity was measured and standardized to that of *Renilla* luciferase, which was used as a transfection control. The results indicate that Luc mRNA translated ~11-fold more efficiently when capped with m^7GpppG, than with ApppG (Fig. 3-4B, compare lanes 3 to 4), consistent with

Figure 3-4. Contribution of cap-dependent and IRES-mediated translation initiation of the Tie2 mRNA *in vivo*. **A.** Schematic representation of polyadenylated, monocistronic mRNAs used in mRNA transfections. **B.** HUVECs were cotransfected with *Renilla* luciferase mRNA and the m^7GpppG- or ApppG-capped FLUC mRNAs indicated in panel A. Lysates were prepared from the cells following mRNA transfection, and luciferase activities were measured. To account for variations in transfection efficiency, firefly luciferase activity was standardized to that of *Renilla* luciferase. The results represent the average of three independent experiments performed and the error bars denote the error of the mean.

A


m7GpppG
or ApppG
hTie2
FLUC
(A100)
hTie2/Luc
FLUC
(A100)
Luc
PolioIRES
FLUC
(A100)
Polio/Luc

B

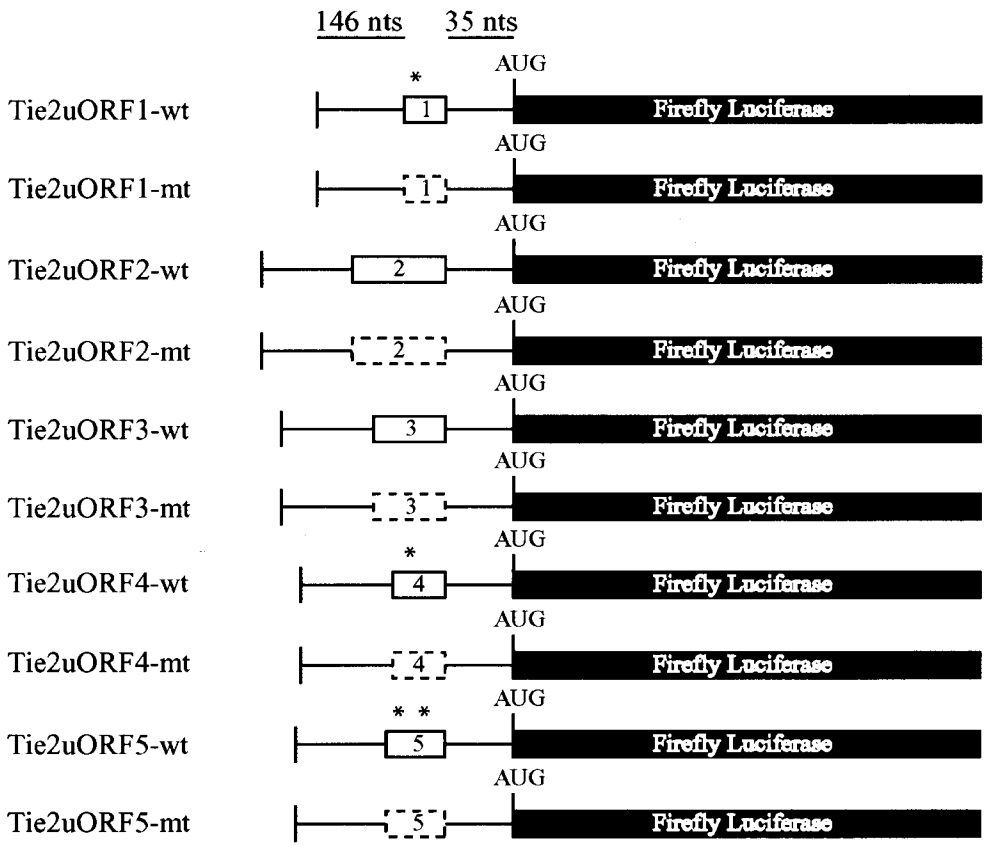

Firefly Luciferase Activity (RLU)
10^6
10^5
10^4
10^3
m7GpppG
ApppG
m7G
A
hTie2/Luc
Luc
Polio/Luc
1
2
3
4
5
6

previously published reports (Byrd *et al.*, 2005; Van Eden *et al.*, 2004). On the other hand, mRNAs containing the poliovirus IRES (Polio/Luc) produced similar levels of firefly luciferase proteins regardless of the presence of the m^7GpppG or ApppG cap structure (Figure 3-4B; compare lanes 5 to 6). Comparison of firefly luciferase activities in cells transfected with m^7GpppG-capped Luc and hTie2/Luc mRNAs demonstrated that the presence of the Tie2 5'UTR significantly inhibited firefly luciferase synthesis (Figure 3-4B; compare lanes 1 to 3), consistent with the inhibitory effect observed for the Tie2 reporter mRNAs *in vitro* (Figure 3-2B, C). HUVECs transfected with hTie2/Luc mRNAs indicated that the m^7GpppG-capped transcripts translated ~4-fold more efficiently that their ApppG-capped counterparts (Figure 3-4B; compare lanes 1 to 2). Theses results suggest that in addition to having a functional IRES, some level of Tie2 mRNA translation is cap-dependent *in vivo*.

The Tie2 uORFs do not harbor potent *cis*-inhibitory elements.

One mechanism by which uORFs can inhibit translation of downstream cistrons is by possessing inhibitory *cis*-acting elements. This possibility was experimentally addressed for the individual Tie2 uORFs by generating a series of firefly luciferase reporter constructs in which each uORF was placed upstream of the firefly luciferase coding region (Figure 3-5). The distance from the cap structure to the uORF (146 nts) and from the uORF to the firefly luciferase initiation codon (35 nts) was maintained constant in all constructs, as was the context of the uORF initiation codons ($^{5'}$cucgagAUG$^{3'}$; initiation codon is capitalized). In the absence of *cis*-inhibitory features, and by maintaining all of these parameters constant among the constructs, we expect a similar reinitiation frequency among the constructs. A parallel series of constructs, in which the initiator and internal AUGs were converted to UUGs, were generated to provide a reference set (Figure 3-5). The relative translation efficiency of these constructs was assessed in Krebs, WG and RRL. The translational efficiency obtained with the uORF containing transcript was compared to the efficiency obtained with the corresponding transcript in which the uORF had been abolished. The results indicate that in this particular reporter setup, each uORF inhibited expression of the downstream luciferase ORF ~2-fold (Figure 3-5) (with the exception of uORF1 and uORF3 which showed a 2.5-

Figure 3-5. Efficiency of reinitiation from each Tie2 uORF. Schematic representation of recombinant luciferase constructs containing the Tie2 uORFs is shown to the left. The firefly luciferase ORF is denoted by a black box, the uORFs are represented by white boxes, and uORFs in which the initiation codon and internal AUGs have been mutated to UUG are shown as dashed boxes. mRNA generated from each construct was translated *in vitro* in Krebs-2 extracts (K), wheat germ extracts (WG) and rabbit reticuolcyte lysates (RRL). The relative firefly luciferase activity is indicated as the ratio of the luciferase values obtained with the uORF-containing construct (wt) relative to values obtained with construct in which the uAUGs are mutated to UUGs (mt). The values represent the average of three independent experiments in duplicate, and are presented with the error of the mean. The sequence flanking all uORF initiation codons is: $^{5'}$cucgagAUG$^{3'}$ (initiation codon underlined).



	Relative Firefly Luciferase Activity (wt/mt)		
	K	WG	RRL
Tie2uORF1	0.5±0.1	0.4±0.1	0.6±0.2
Tie2uORF2	0.6±0.1	0.5±0.1	0.6±0.1
Tie2uORF3	0.5±0.1	0.3±0.1	0.5±0.1
Tie2uORF4	0.7±0.1	0.5±0.1	0.5±0.1
Tie2uORF5	0.7±0.1	0.7±0.2	1.0±0.2

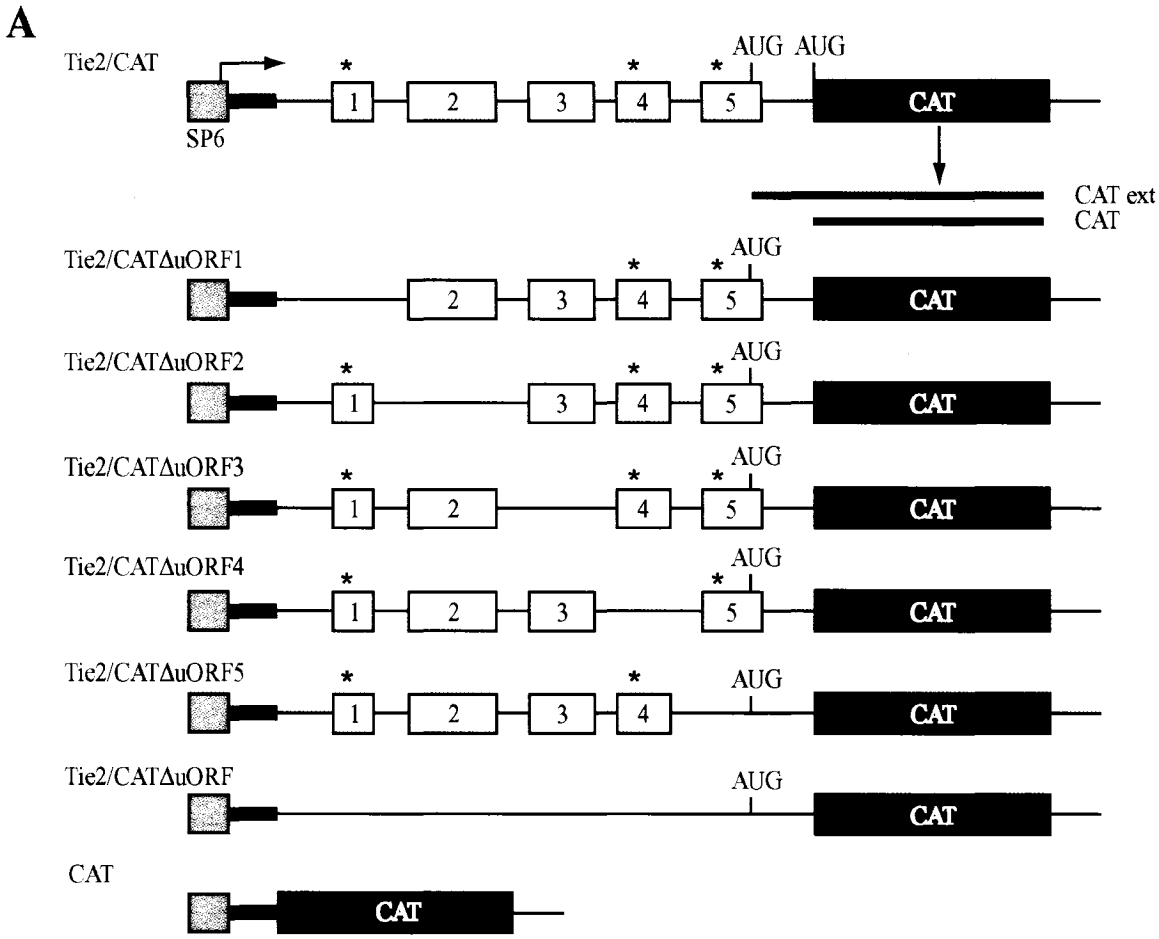
and 3-fold inhibition in WG, respectively). As the inhibitory effect of individual uORFs on downstream firefly luciferase expression is not large, we conclude that none of the individual uORFs harbor strong *cis*-inhibitory features, and that the ability of ribosomes to reinitiate following translation of any of the Tie2 uORFs is similar.

The above experimental design scored for *cis*-acting inhibitors out of context of the 5' UTR. To validate the obtained results, a series of CAT reporter constructs were generated in which the Tie2 uORFs were individually removed by mutagenesis of the initiation codons (Figure 3-6A). Whereas translation of CAT mRNA was efficient in all three extracts tested (Figure 3-6B, lane 8), fusion of the complete Tie2 5' UTR to the CAT ORF dramatically inhibited translation in RRL, Krebs, and WG extracts (Figure 3-6B; compare lanes 6 to 8). Mutagenesis of individual AUGs within the Tie2 5' UTR did not significantly alter the inhibitory potential of the Tie2 5'UTR on translation (Figure 3-6B; compare lanes 1-5 to lane 6). This suggests that no individual uORF is responsible for the poor translational efficiency observed with Tie2/CAT. Furthermore, all Tie2 chimeric mRNAs produced a protein product that migrated slightly slower than CAT (Figure 3-6B; in RRL compare lanes 1-7 to lane 8), as would be predicted if initiation was occurring at the predicted Tie2 AUG codon within uORF5. Taken together, our results suggest that the poor translational efficiency of the Tie2 5' UTR is a consequence of decreased ribosome flow as these traverse the Tie2 5' UTR.

3.5 Discussion

We have previous described the presence of an IRES within the Tie2 5' UTR (Park *et al.*, 2005). Unlike uncapped viral mRNAs which harbor IRESes (Sarnow, 2003), the presence of an IRES in a cellular mRNA establishes two mechanisms by which ribosomes can be recruited - internally and via the 5' end. Presumably in such cases, mechanisms have evolved to avoid interference between the two initiation pathways or at least this interference is used in a regulated fashion (Yaman *et al.*, 2003). The major Tie2 transcript is unusually long and contains 5 uORFs that lead us to postulate these features may be inhibitory to ribosomes that initiate at the 5' end of the mRNA. In order to test

Figure 3-6. Translational consequence of eliminating individual uORFs from the Tie2 5' UTR. **A.** Schematic representation of constructs harbouring Tie2 uORFs. The CAT ORF is indicated by a black box, the SP6 promoter by a grey box, and uORFs by white boxes. The internal AUG codons are denoted by asterisks. The production of CAT protein or the N-terminal extension of CAT (designated as CAT ext.) is depicted by thickened lines. **B.** Translation of the Tie2 uORF-containing mRNAs in rabbit reticulocyte lysates (RRL), Krebs-2 extracts (K) and wheat germ extracts (WG). Following *in vitro* translations, products were separated on 10% SDS-polyacrylamide gels and the resulting gels were treated with EN^{3}Hance, dried, and exposed to X-ray film. Experiments were carried out three independent times and a representative result is shown.

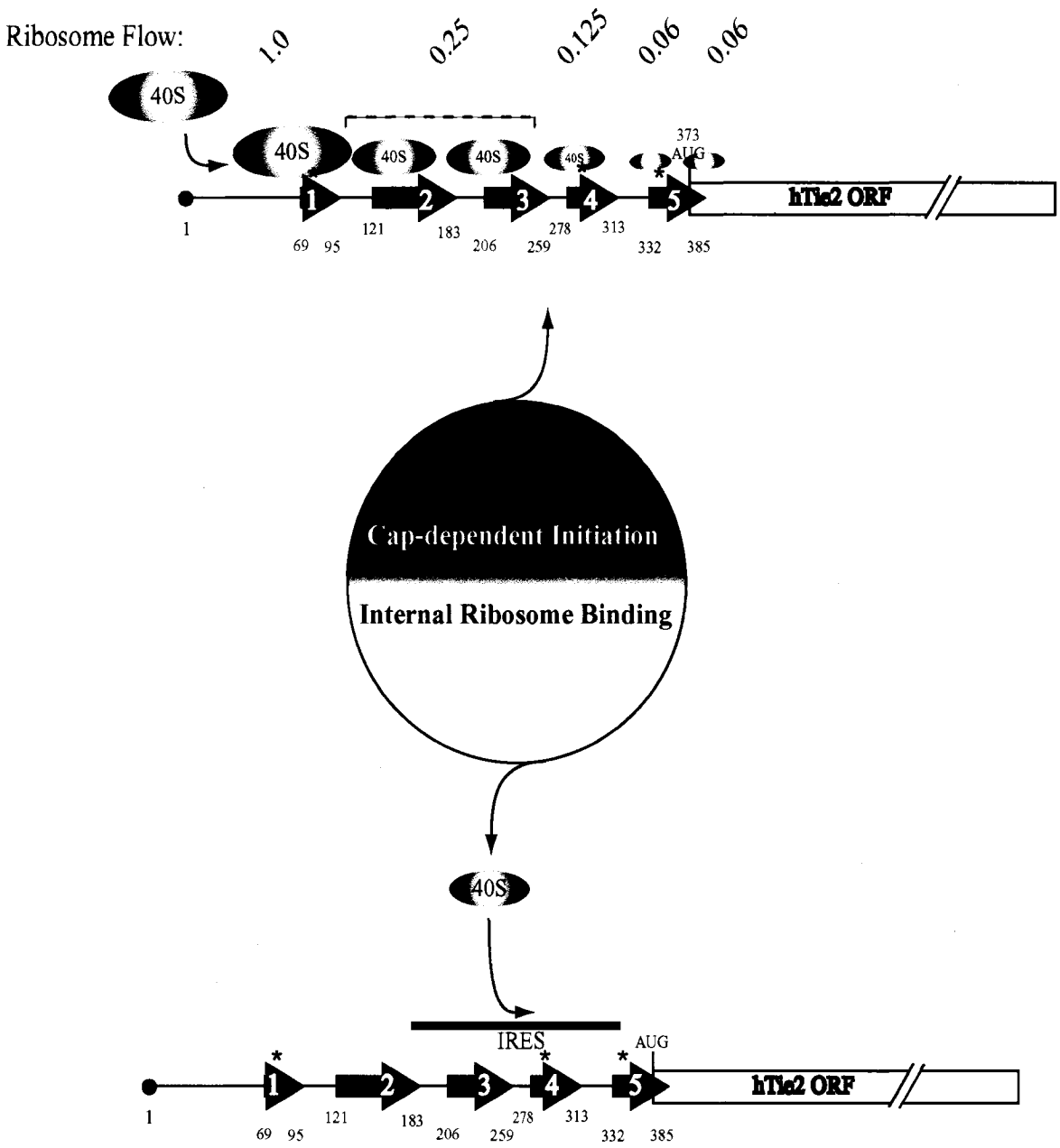

A
Tie2/CAT
SP6
AUG AUG
CAT
CAT ext
CAT
Tie2/CATΔuORF1
Tie2/CATΔuORF2
Tie2/CATΔuORF3
Tie2/CATΔuORF4
Tie2/CATΔuORF5
Tie2/CATΔuORF
CAT
B
Tie2/CATΔuORF1
Tie2/CATΔuORF2
Tie2/CATΔuORF3
Tie2/CATΔuORF4
Tie2/CATΔuORF5
Tie2/CAT
Tie2/CATΔuORF
CAT
-RNA
kDa
33–
25–
CAT ext
CAT
RRL
Krebs
WG
1 2 3 4 5 6 7 8 9

this hypothesis, we identified *in vitro* conditions that would allow us to study cap-dependent initiation in the absence of interference from internal initiation (Figure 3-3).

We have not further explored the molecular basis that could explain why the Tie2 IRES is not active *in vitro*. One possible explanation is that cell-type specific *trans*-acting factors that drive Tie2 IRES activity in HUVECs are lacking or limiting in Krebs-2 extracts, wheat germ extracts, or rabbit reticulocyte lysates. A role for *trans*-acting factors in initiation of IRESes has been well characterized (Stoneley and Willis, 2004). Cell-type specific or extract-dependent restriction of IRES activity has been previously documented for several IRESes. For example, the IRES activity of the PITSLRE protein kinase is cell-cycle regulated and is not detectable in *in vitro* translation assays in rabbit reticulocyte lysates (Cornelis *et al.*, 2000). The *c-myc* IRES does not function in reticulocyte lysates possibly due to lack of cell-type specific *trans*-acting factors and lack of a nuclear experience (Stoneley *et al.*, 2000b). Additionally, the hepatitis C virus (HCV) IRES is active in rabbit reticulocyte lysates and Krebs extracts, but not in wheat germ extracts (Pestova *et al.*, 1998b), presumably due to incompatibility between the HCV IRES and wheat germ ribosomes and/or wheat germ eIF3. The tobacco etch virus (TEV) IRES is also inactive *in vitro* in wheat germ extracts (Niepel and Gallie, 1999).

The presence of uORFs within a 5' UTR is considered a barrier to initiation as they reduce the number of downstream initiating ribosomes (Meijer *et al.*, 2000) - certainly the presence of five uORFs is unusually rare (Chappell *et al.*, 2001; Park *et al.*, 2005). Length *per se* or putative *cis*-acting elements within the Tie2 5' UTR have a minor impact on translational efficiency, as we observed only a ~1.5 - 3 fold difference in translation of CAT and Tie2/CATΔuAUG (Figure 3-1). Our results suggest a model whereby 25% of ribosomes that have initiated via a cap-dependent mechanism and have translated uORF1, will translate uORFs 2 and/or 3 (Figure 3-7). Our results also suggest that the impact of 5 sequential uORFs reduces initiation ~16-fold at the predicted Tie2 AUG codon (Figures 3-2B, C and 3-7). Of the number of ribosomes that terminate translation of uORF4, approximately 50 % appear to reinitiate at uORF5, and 50 % appear to reinitiate at ATG_{373} (presumably the result of leaky scanning past the uORF initiation codon) (Figure 3-2B, C). The mechanism responsible for the apparent loss of scanning ribosomes, or ribosomes competent for reinitiation from the Tie2 mRNA

Figure 3-7. Model indicating ribosome flow on the Tie2 5' UTR under conditions of cap-dependent or IRES-mediated translation initiation. The uORFs are indicated as arrows and the Tie2 coding region is denoted by a white box. The values indicated above the uORFs reflect the fraction of ribosomes predicted to initiate at that particular uORF, relative to the number of ribosomes initiating at uORF1. The number of ribosomes predicted to initiate at the Tie2 initiation codon is the product of these relative values, which are calculated from the results of Figure 2. The mechanism by which internally recruited ribosomes reach the Tie2 5' UTR is not known and could occur by either linear scanning or translocation to the initiator AUG codon.


Ribosome Flow:
1.0
0.25
0.125
0.06
0.06
40S
373
AUG
hTie2 ORF
1
69
95
121
183
206
259
278
313
332
385
Cap-dependent Initiation
Internal Ribosome Binding
IRES
AUG

remains unknown and we stress that we have only been able to document these events *in vitro*.

Our results also suggest that under the *in vitro* translation conditions utilized in this study, the predicted Tie2 AUG codon, embedded within uORF5 appears to be utilized (Figure 3-1). However, AUG_{373} is not utilized *in vivo* in HUVECs since we did not detect CAT protein with an N-terminal extension (Figure 3-2D). Hence *in vivo*, it would appear that the majority of ribosomes are shunted past uORF5 and initiate at the second in-frame ATG (ATG_{442}). This highlights a second difference between our *in vitro* and *in vivo* studies. Utilization of uORFs might be achieved by altering levels of ternary complex, that would change the translation efficiency of individual uORFs, as documented for GCN4 (Hinnebusch, 2000; Meijer and Thomas, 2002; Morris and Geballe, 2000) and ATF4 (Harding *et al.*, 2000). This issue deserves more investigation since the current prediction is that the hTie2 ATG_{373} is the initiation codon. Regulation of ATG selection has the potential to generate different hTie2 isoforms.

Our results suggest a model whereby both IRES-mediated and cap-dependent initiation can occur on the Tie2 5' UTR (Figure 3-7). This hypothesis is supported by the following observations. First, we previously demonstrated that Tie2 mRNAs bearing the complete 5' UTR are efficiently translated by virtue of the IRES, under conditions when overall cap-dependent translation is significantly reduced (Park *et al.*, 2005). Second, the results obtained from RNA transfections using m^7G- or A-capped monocistronic reporters indicated that internal initiation may contribute to translation of Tie2 *in vivo* to reduce its cap-dependency, relative to what is observed *in vitro* (Figure 3-4). Third, in reticulocyte lysate, wheat germ, and Krebs-2 extract systems in which the Tie2 IRES is not active, transcripts containing the Tie2 5'UTR are translated in a manner that is dependent on the cap structure (Figure 3-2). Therefore, we propose a dual mechanism for Tie2 translation initiation. Under conditions where cap-dependent protein synthesis is compromised there is expected to be a shift from a cap-dependent to an IRES-directed mechanism of translation initiation (Figure 3-7). Our findings complement previously published data describing *c-myc* protein synthesis directed by both 5'-end and IRES-mediated translation mechanisms (Stoneley *et al.*, 1998; Stoneley *et al.*, 2000b).

What is the physiological relevance of translational control of the Tie2 mRNA by its uORFs and IRES? IRES and 5' end-mediated initiation may regulate each other's activities or they may co-exist and be required under different physiological conditions. It is clear that Tie2 protein expression is maintained under conditions when global translation is compromised, such as hypoxia (Park *et al.*, 2005) and quiescence (Arai *et al.*, 2004). Thus, internal initiation may be used as a cellular "alternative" for survival under stress conditions (Komar and Hatzoglou, 2005). It should be also noted that excessive Tie2 levels would be detrimental under normal conditions of cell growth and have been shown to be associated with pathological situations, such as in breast cancer (Peters *et al.*, 1998) and vascular dysmorphogenesis (Vikkula *et al.*, 1996). Consequently, highly regulated Tie2 expression is likely important for normal vascular formation and maintenance. In an alternative model, internal initiation and cap-dependent ribosome recruitment mechanisms may occur at different times depending on the particular physiological setting, as has been reported for the *c-myc* mRNA (Stoneley *et al.*, 2000a; West *et al.*, 1998). According to this model, translation of the Tie2 mRNA via IRES activity would be a mechanism to maintain expression when cap-dependent translation is suppressed. Our results highlight the complexity of gene expression at the level of translation initiation of the Tie2 mRNA.

3.6 Acknowledgements

E.H.P. is supported by a K.M. Hunter/CIHR doctoral research award. J.P. is a Canadian Institute of Health Research (CIHR) Senior Investigator. This work was supported by a grant from CIHR (MOP-11354) to J.P.

Connecting Text

In order to gain further insight into the potential function(s) of Tie2 IRES activity in translation under different physiological conditions (i.e., quiescence), we used similar approaches to our previous studies in which we had demonstrated the presence of a functional Tie2 IRES element during hypoxia. The results of these studies are presented in Chapter 4.

CHAPTER 4

Translational Regulation of Tie2 Gene Expression during Quiescence

4.1 Abstract

Tie2 is an endothelial-restricted receptor tyrosine kinase that controls vascular assembly and endothelial quiescence. We have previously demonstrated that Tie2 protein synthesis is maintained under hypoxia due to the presence of IRES activity in the 5' UTR of Tie2 mRNA. In this report, we show that Tie2 IRES is functional during quiescence, another condition known to inhibit cap-dependent translation. We also map the location of the Tie2 IRES and show that the entire 5' UTR is required for the fully functional IRES activity. Our results suggest that the presence and maintenance of Tie2 protein expression in quiescent endothelial cells is, in part, due to the IRES activity.

4.2 Introduction

Angiogenesis is a multistep process resulting in the formation of new blood vessels from the pre-existing vasculature. This is a tightly controlled process that plays its most obvious role in early embryonic development. Although the potential for angiogenesis is maintained throughout the lifetime of an organism, once the vasculature has been established, the endothelium remains markedly quiescent in the adult, with the exceptions of female reproduction functions and wound healing (Engerman *et al.*, 1967; Hobson and Denekamp, 1984; Reynolds *et al.*, 1992; Wong *et al.*, 1997). Angiogenesis in the adult is also largely controlled by pathological situations, such as tumorigenesis and diabetic retinopathy (Folkman, 2001; Hanahan and Folkman, 1996).

Several endothelial cell specific receptor tyrosine kinases (RTKs) have been identified that are involved in blood vessel growth and differentiation, including the vascular endothelial growth factor receptors (VEGFRs) and the Tie receptors (Davis et al., 1996; Folkman and D'Amore, 1996; Maisonpierre et al., 1997; Risau, 1997; Suri et al., 1996). Tie2 receptor and its ligands, the angiopoietins (Angs) have been implicated in blood vessel remodeling, maturation, and stability (Dumont *et al.*, 1992; Jones *et al.*, 2001a; Sato *et al.*, 1995). Ang1 plays a role in vessel maturation through recruitment of mural cells such as pericytes, whereas Ang2 is involved in vessel remodeling and can

induce either angiogenesis or vessel regression depending on the context (Lemieux *et al.*, 2005; Scharpfenecker *et al.*, 2005). The Tie2 receptor is ubiquitously expressed in highly vascularized tissues, suggesting that Tie2 expression is a general feature in all endothelial cells (Dumont *et al.*, 1992; Schlaeger *et al.*, 1997; Schnurch and Risau, 1993). Targeted disruption of the Tie2 gene in mice leads to embryonic lethality, indicating an essential role of this receptor during embryonic development (Dumont *et al.*, 1994; Puri *et al.*, 1995). In cancer, Tie2 expression is upregulated in various tumors, including hepatocellular carcinoma and colorectal cancer, however, it was also reported to be heterogeneously expressed in vessels of human breast and pancreatic carcinomas (Chin *et al.*, 2003; Dales *et al.*, 2003; Durkin *et al.*, 2004; Tanaka *et al.*, 2002). Tie2 is also thought to be involved in hematopoiesis based on its expression in hematopoietic stem cells (HSCs). In fact, a recent study by Arai *et al.* has demonstrated that the Tie2/Ang1 signaling pathway plays an essential role in the maintenance of HSC quiescence in the adult bone marrow niche (Arai *et al.*, 2004). However, Tie2 function in adult bone marrow hematopoiesis appears to be different from its role in developmental hematopoiesis in that this receptor is dispensable for embryonic hematopoiesis but is specifically required during postnatal bone marrow hematopoiesis (Puri and Bernstein, 2003). In addition, the Tie2 receptor is present in quiescent endothelial cells in a variety of adult tissues (Wong *et al.*, 1997). Studies in transgenic mice expressing marker transgenes driven by the Tie2 promoter have confirmed the broad expression of Tie2 in adult vasculature (Motoike *et al.*, 2000; Schlaeger *et al.*, 1997). Moreover, Tie2 phosphorylation was detected in these normal tissues, strongly suggesting a role for Tie2 in the maintenance of quiescent adult vasculature (Wong *et al.*, 1997). Although these studies have indicated the expression of Tie2 in the quiescent vasculature, very little is known regarding how Tie2 expression is regulated at the molecular level and its function in the mature vasculature.

Cellular IRES have been identified in many genes involved in development, differentiation, cell growth, apoptosis, and angiogenesis (Carter *et al.*, 2000; Pickering and Willis, 2005; Stoneley and Willis, 2004). We have previously demonstrated that the mRNA for Tie2 contains an IRES sequence (Park *et al.*, 2005). Translation initiation from this IRES is maintained during hypoxia when global protein synthesis is decreased,

allowing Tie2 protein expression under such stress conditions. It is evident that Tie2 is expressed and activated during quiescence, another condition known to inhibit general translation, particularly cap-dependent translation (Mao *et al.*, 1992). Therefore, we hypothesized that the Tie2 IRES may serve to overcome the translation inhibition imposed by quiescence to maintain Tie2 protein expression.

In the present study, we observed that Tie2 protein is actively translated under both normal and quiescent (serum-starved) conditions as assessed by western blot analyses and metabolic labeling of human umbilical vein endothelial cells (HUVECs) followed by immunoprecipitation with anti-Tie2 antibodies. We found that the Tie2 IRES activity is maintained, but not enhanced during quiescence as observed with HIF1α, *c-myc*, and angiotensin II type 1 receptor (AT_1R) IRESes under serum deprivation (Lang *et al.*, 2002; Martin *et al.*, 2003).

4.3 Materials and Methods

Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (Walkersville, MD) and maintained in endothelial growth media-2 (EGM-2) supplemented with growth factors, 2% fetal bovine serum (FBS), 50 μg/mL gentamicin, and 50 μg/mL amphotericin according to the manufacturer's instructions. All experiments were performed on cells that had previously undergone 2 -3 passages.

Quiescence of HUVECs was induced by growth factor withdrawal. Cells at 60~70 % confluency were transferred to endothelial basal media (EBM-2) containing 0.2 % FBS and incubated under these condition for a further 24-72 hr. The viability of quiescent cells was demonstrated by transferring the serum-starved cells to rich media (EGM-2) and incubated for another 24 hrs. Cells that grew further after serum addition were then defined as quiescent and proliferating/quiescent status of the cells was confirmed using cell cycle analyses.

HUVEC cell cycle analysis

Propidium-iodide-stained samples were analyzed for cell cycle distribution as described previously (Adams *et al.*, 2005). Briefly, HUVECs from10 cm^2 plates were centrifuged at 1700 rpm for 5 min, resuspended at $1X10^6$ cells/mL in phosphate-buffered saline (PBS) containing 2 % FBS and then fixed in 70 % ice-cold ethanol overnight. The cells were recovered by centrifugation, and resuspended in 1 mL of 50 μg/mL propidium iodide containing 0.5 μg/mL of RNase A. Following the addition of propidium iodide, the samples were incubated for 3 hours at 4 °C and stained cells were used for fluorescent-activated cell scan (FACS) analysis (FACScan, Becton Dickinson, San José, CA) using the CELLQUEST software. At least 10,000 cells were evaluated per sample and each experiment was repeated at least 3 times.

Northern Blot analysis

Total RNA was isolated using TRIzol reagent (Invitrogen), and Northern blot analysis was performed as described previously (Park *et al.*, 2005). Total RNA (10 μg) 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; Palo Alto, CA) at 68°C with a ~339-nt human Tie2 mRNA-specific ^{32}P-labeled probe. All signals were measured by densitometry (BAS-2000II, Fuji Film, Tokyo) and arbitrarily normalized relative to the 18S rRNA level. Autoradiography of the membrane was performed at -80°C with film (Kodak X-OMAT) to visualize the radioactive signal.

Western blot analysis

HUVECs were lysed in buffer containing 25 mM HEPES (pH 7.4), 137 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, and 2 μg/mL PepstatinA. Proteins were separated by 7-12 % SDS-polyacrylamide gel electrophoresis, transferred to PVDF membranes (Millipore Corporation; Bedford, MA), incubated with appropriate primary antibodies followed by horseradish peroxidase anti-immunoglobulin conjugate, and developed with the Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Science, Inc.; Boston, MA).

Metabolic Labeling with ^{35}S-Methionine

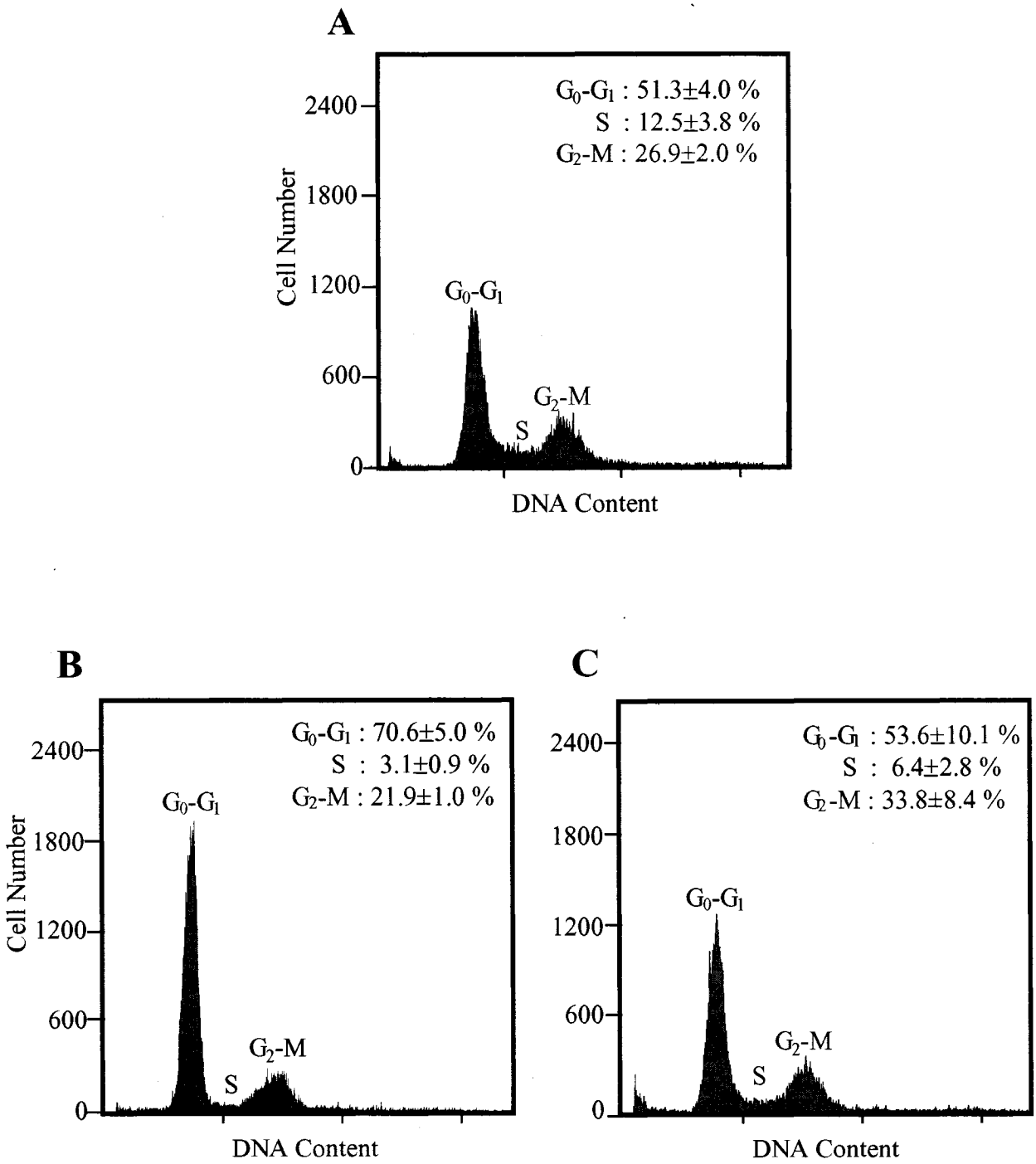
HUVECs were plated at $2.7x10^5$ cells per 150 mm plate and incubated for 24 hr, followed by a further 24 hr incubation under either normal or serum starvation conditions. Cells were then washed with PBS and incubated in methionine-free DMEM medium (Sigma; St. Louis, MO) supplemented with 0.2 or 10 % fetal calf serum and ^{35}S-methionine (New England Nuclear; Boston, MA) 4 hr prior to harvesting. Cells were washed three times with PBS and harvested in lysis buffer [25 mM HEPES (pH7.4), 137 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 PepstatinA]. The cell lysates (500 μg of total protein) were pre-cleared with Protein G-Sepharose and immunoprecipitations performed with anti-Tie2 (Santa Cruz Biotechnology, Inc.; Santa cruz, CA). The immunoprecipitates were then separated by SDS-PAGE, followed by fluorography. Experiments were performed in triplicate at least twice.

4.4 Results

Cell cycle analysis of proliferating and quiescent HUVECs

In order to induce quiescence, HUVECs were cultured in EBM-2 containing 0.2 % FBS for 24-72 hours and the cell cycle status of the endothelial cells was evaluated by FACS analysis. Serum-starved cells grown in the EBM-2 supplemented with 0.2 % FBS for 24 hr showed a reduction in cell cycle progression in comparison to the corresponding proliferating cells grown in EGM-2 (Figure 4-1, compare B to A). In serum-deprived HUVECs, the relative percentage of cells that were arrested in G_0-G_1 phase increased compared to the percentage in proliferating cells. In addition, the proportion of serum starved cells in the S phase of the cell cycle was significantly lower than that of the corresponding proliferating cells, indicating that cells subjected to 24 hr growth factor deprivation were prevented from progressing from G_0-G_1 to S phase. These results were consistent with cell cycle profiles previously observed in quiescent HUVECs

Figure 4-1. Flow cytometry for cell cycle parameters of propidium-iodide-stained HUVECs under various conditions. Histograms illustrate cell cycle profiles of (A) proliferating, (B) serum-starved, and (C) serum-starved and then -restimulated HUVECs. Proportions of cells in the G_0-G_1, S, and G_2-M phases of the cell cycle represent the means from three experiments $\pm$ standard errors of the mean.


A
G_0-G_1 : 51.3±4.0 %
S : 12.5±3.8 %
G_2-M : 26.9±2.0 %
Cell Number
2400
1800
1200
600
0
G_0-G_1
S
G_2-M
DNA Content
B
G_0-G_1 : 70.6±5.0 %
S : 3.1±0.9 %
G_2-M : 21.9±1.0 %
Cell Number
2400
1800
1200
600
0
G_0-G_1
S
G_2-M
DNA Content
C
G_0-G_1 : 53.6±10.1 %
S : 6.4±2.8 %
G_2-M : 33.8±8.4 %
2400
1800
1200
600
0
G_0-G_1
S
G_2-M
DNA Content

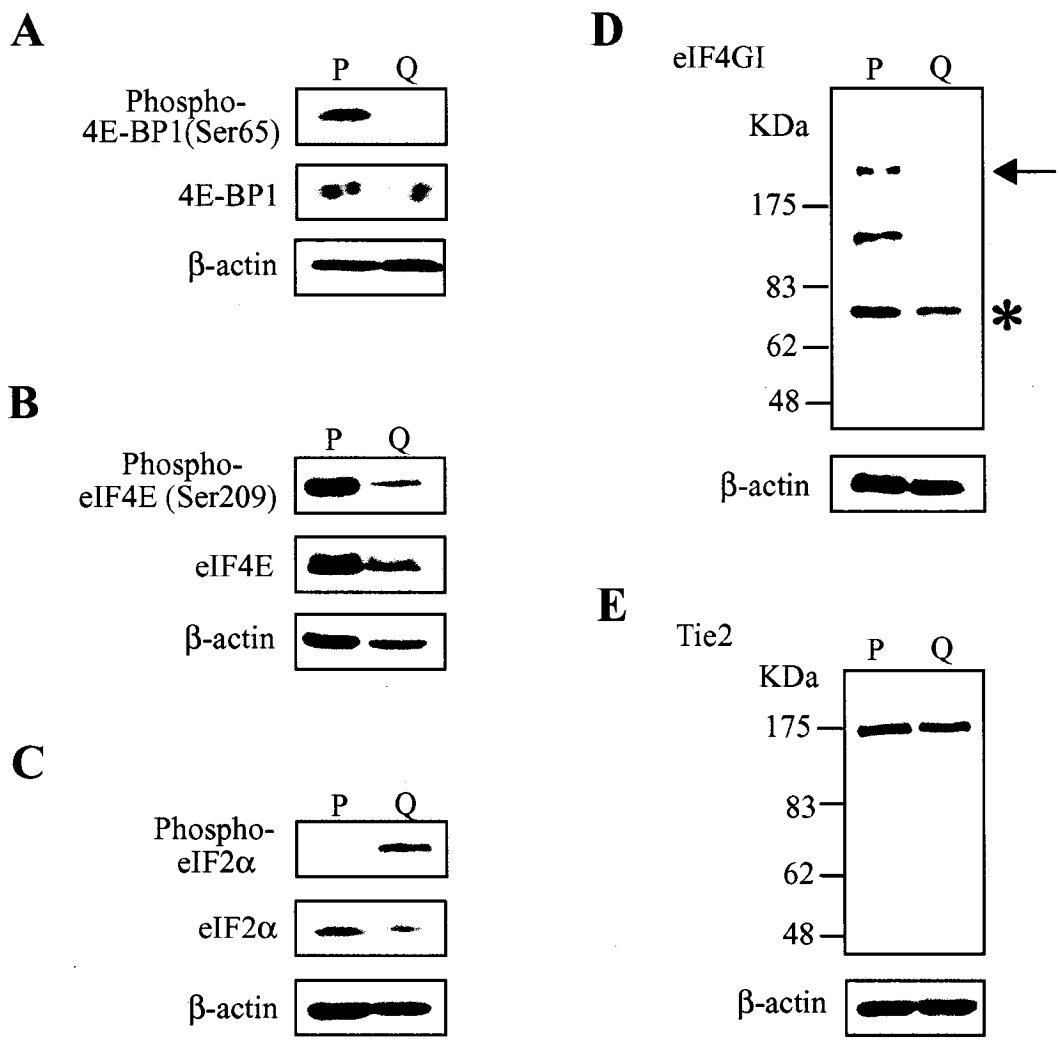
(Adams *et al.*, 2005). We observed similar a cell cycle distribution when cells were exposed to serum starvation up to ~72 hr (data not shown).

To assess whether the serum-starved cells were in the quiescent state (G_0) of the cell cycle, starved cells were further incubated after replacing the medium with EGM-2 containing growth factors and 2 % FBS, and cell cycle distribution was assessed. As shown in Figure 4-1C, following serum addition, the proportion of cells distributed in the G_0-G_1 phase decreased by ~17 %, similar levels to that in proliferating cells (compare C to A), and the proportion of the S and the G_2-M phases increased (compare C to B). These data indicate that cell cycle arrest in HUVECs by serum deprivation is reversible and these serum starved cells were in quiescent state.

Tie2 protein levels remain unchanged during quiescence when global protein synthesis is inhibited.

Quiescence has been shown to be triggered by serum deprivation, resulting in a significant reduction in general translation (Chang *et al.*, 2002; Mao *et al.*, 1992). This inhibition of protein synthesis correlates with the decrease of translation initiation factors (by transcriptional/posttranscriptional mechanisms) or posttranslational modifications of these factors including proteolytic cleavage of the initiation factors (Clemens *et al.*, 1998; Mao *et al.*, 1992; Marissen and Lloyd, 1998; Wang *et al.*, 1999). Therefore, it appears that limitation of one or more functional eIFs is responsible for the low levels of protein synthesis observed in the quiescence phase. To further confirm translational repression in quiescent HUVECs, we analyzed the levels and phosphorylation status of several initiation factors by western blot analysis. As shown in Figure 4-2A, decreased phosphorylated isoforms (top panel) and increased dephosphorylated isoforms of 4E-BP protein (middle panel) were observed in quiescent HUVECs (Q), compared to proliferating cells (P). The levels of total eIF4E protein and phosphorylatd eIF4E were significantly reduced under serum starvation (Figure 4-2B). We have also investigated the phosphorylation of eIF2α during quiescence. In many cell systems, relatively small changes in the phosphorylation status of eIF2α are associated with the inhibition of global protein synthesis (Morley *et al.*, 2005; Morley *et al.*, 2000; Srivastava *et al.*, 1998). Using antisera recognizing either total eIF2α or eIF2α in the phosphorylated form,

Figure 4-2. Western blot analysis of translation initiation factors and Tie2 protein levels during quiescence. Cytoplasmic lysates from proliferating and quiescent HUVECs were prepared as described under "Materials and Methods." Equal amounts of total protein were separated by SDS-PAGE, transferred to Hybond-C membranes, and probed with appropriate antisera. The position of full-length eIF4GI is indicated by the arrow and the position of eIF4GI-specific cleavage product by the asterisk. The approximate locations of protein molecular size markers are shown at the left. P: proliferating HUVECs; Q: quiescent HUVECs


A
P Q
Phospho-
4E-BP1(Ser65)
4E-BP1
β-actin
B
P Q
Phospho-
eIF4E (Ser209)
eIF4E
β-actin
C
P Q
Phospho-
eIF2α
eIF2α
β-actin
D
eIF4GI
P Q
KDa
175
83
62
48
β-actin
E
Tie2
P Q
KDa
175
83
62
48
β-actin

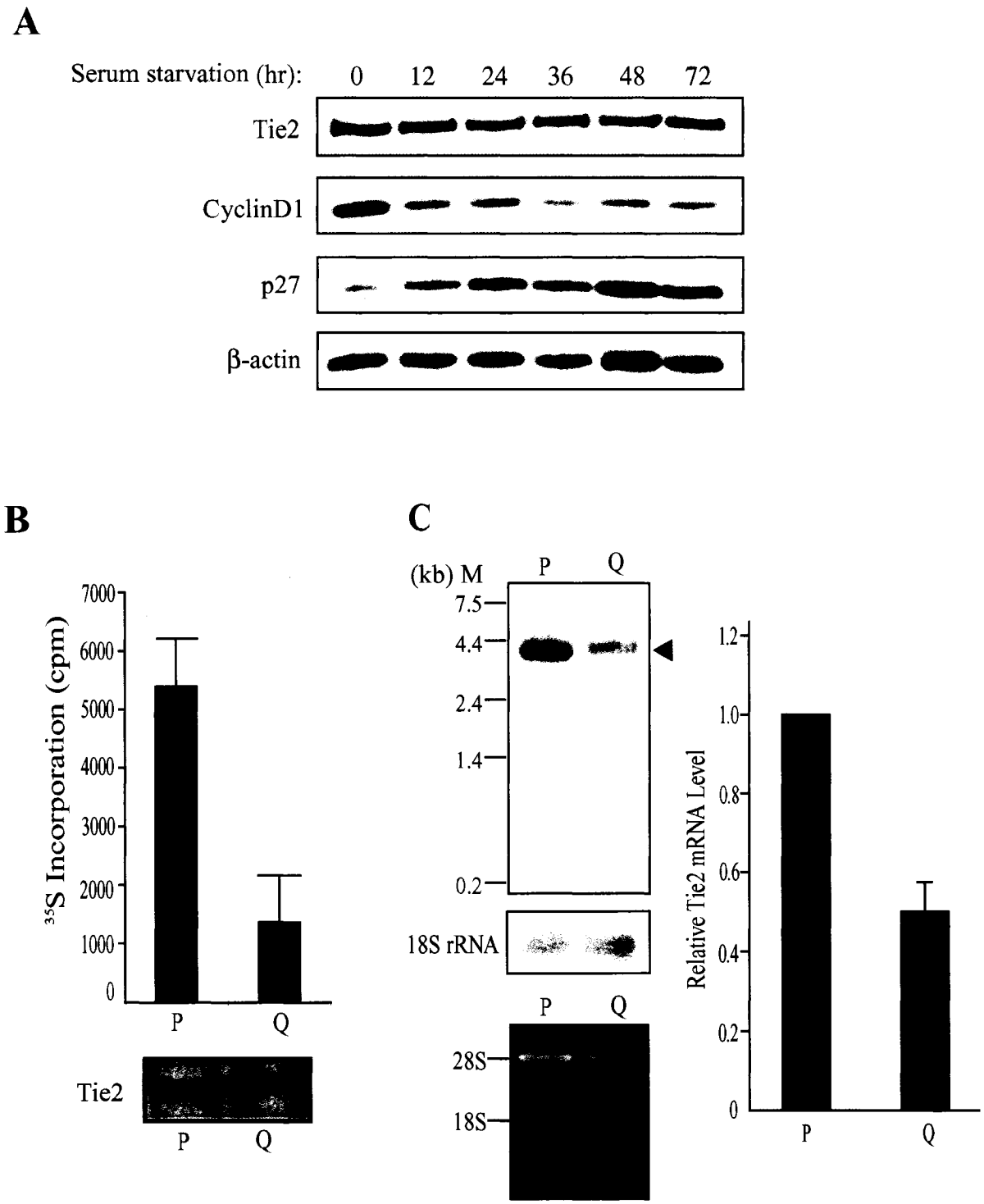
we found that the level of phosphorylated eIF2α was significantly increased in quiescent HUVECs (Figure 4-2C), consistent with previously published reports (Datta *et al.*, 2004; Duncan and Hershey, 1985; Gupta *et al.*, 1997). Furthermore, previous studies have shown proteolytic cleavage of eIF4G protein under serum starvation conditions (Clemens *et al.*, 1998; Marissen and Lloyd, 1998). We also observed a reduction in the amount of full-length eIF4GI and the appearance of the 76-kDa cleavage product in quiescent HUVECs (Figure 4-2D). Similar results were observed with eIF4GII under serum starvation (data not shown).

To characterize Tie2 protein levels under quiescent conditions, we performed Western blot analysis on total protein. Interestingly, Tie2 protein was present in both proliferating and quiescent cells at similar levels up to ~72 hr exposure to serum deprivation (Figures 4-2E and 4-3A). The levels of CyclinD1 significantly decreased within 12~24 hr in response to serum-starvation, with a concomitant increase in the levels of p27. These results are consistent with previously described effects of quiescence on CyclinD1 and p27 expression levels (Rivard *et al.*, 1996).

To measure the extent to which translation in endothelial cells is reduced in quiescent state, we measured the rate of [^{35}S]methionine incorporation into newly synthesized proteins in HUVECs under control proliferating and serum-starved conditions (Figure 4-3B, Upper panel). Analysis of metabolic labeling revealed a significant reduction in [^{35}S]methionine incorporation by 75 % in quiescent cells compared to proliferating cells, supporting the idea that overall protein synthesis is inhibited during quiescence. However, the synthesis rate of Tie2 protein remained unchanged in cells under quiescent conditions as assessed by metabolic labeling of HUVECs followed by immunoprecipitation with anti-Tie2 antibodies (Figure 4-3B, lower panel). Taken together, theses results indicate that translation of Tie2 mRNA is maintained when overall protein synthesis is reduced upon serum starvation.

To investigate the status of endogenous Tie2 mRNA in HUVECs under quiescent conditions, we performed Northern blot analysis (Figure 4-3C). Tie2 mRNA levels decreased 2-fold in response to serum starvation, while there was no change in the Tie2 protein levels as observed above (Figure 4-3, compare C to A and B). There was no change in Tie2 mRNA stability during quiescence (data not shown). These results

Figure 4-3. Levels of endogenous Tie2 protein, but not its mRNA, are maintained in quiescent HUVECs. **A.** HUVECs were exposed to serum starvation for 12, 24, 36, 48, and 72 hrs. Equal amounts of total protein were separated by SDS-PAGE, transferred to Hybond-C membranes, and probed with Tie2, cyclinD1 and p27 antibodies. **B.** Upper panel: Global protein synthesis determined by metabolic labeling of proliferating (P) or quiescent HUVECs (Q). Cells were preincubated for 24 hr in control (proliferating) or serum-starved conditions, incubated in the presence of [^{35}S]methionine for 1 hr, and immediately harvested. The incorporation of [^{35}S]methionine into protein was measured as described previously (Park *et al*, 2005). Lower panel: Metabolic labeling of Tie2 under serum starvation. HUVECs were metabolically labeled with [^{35}S]methionine under control and serum-starved conditions and immunoprecipitated with anti-Tie2 antibodies. Immunoprecipitates were resolved by SDS-PAGE and visualized by fluorography. **C.** Northern blot analyses of RNAs isolated from proliferating and quiescent HUVECs and hybridized with a Tie2 specific ^{32}P-labeled probe (top panel). The same blot was reprobed with 18S rRNA (middle panel), to account for possible variations in loading. RNA integrity was indicated by SYBR gold (Molecular Probes; Eugene, OR)-stained 28S and 18S rRNAs as shown in the bottom panel. The arrowhead indicates the position of migration of Tie2 mRNA.


A
Serum starvation (hr): 0 12 24 36 48 72
Tie2
CyclinD1
p27
β-actin
B
^{35}S Incorporation (cpm)
7000
6000
5000
4000
3000
2000
1000
0
P
Q
Tie2
P
Q
C
(kb) M
P
Q
7.5
4.4
2.4
1.4
0.2
18S rRNA
P
Q
28S
18S
Relative Tie2 mRNA Level
1.2
1.0
0.8
0.6
0.4
0.2
0
P
Q

suggest that endogenous Tie2 protein levels are most likely modulated at the translational/ posttranslational level during quiescence.

The Tie2 IRES is functional during quiescence.

Cellular stresses such as amino acid starvation, glucose starvation, growth factor withdrawal, heat shock, oxygen deprivation, apoptosis, and mitosis are known to inhibit cap-dependent, but not cap-independent, translation (Hellen and Sarnow, 2001). We have previously demonstrated that Tie2 IRES is functional under hypoxic conditions (Park *et al.*, 2005). To asses if the Tie2 IRES is also active during quiescence, HUVECs were transfected with a bicistronic reporter construct containing the Tie2 5' UTR in the intercistronic space between the firefly and *Renilla* luciferase coding regions. The transfected cells were subjected to serum starvation for 24 hr and luciferase activities were measured (Figure 4-4). As a negative control for these experiments, we utilized a deletion mutant of the EMCV 5' UTR that does not support internal ribosome binding (Figure 4-4A, construct Ren/ΔEMC/FF). Although serum starvation reduced the expression of both *Renilla* and firefly luciferases, the *Renilla* luciferase activity was affected to a much greater extent, consistent with previously reported observation with HIF1α, VEGF, and *c-myc* IRESes during serum starvation [Figure 4-4B; (Lang et al., 2002)]. As a result, the firefly luciferase/*Renilla* luciferase ratio of Ren/Tie2/FF construct doubled in serum starved HUVECs when compared with HUVECs grown in normal conditions (Figure 4-4B). These results indicate that the Tie2 IRES is functional during quiescence.

The entire Tie2 5' UTR appears to be required for fully functional IRES activity.

To provide some context to understanding how these results could impact on internal initiation of Tie2, we undertook a deletion mapping strategy of the previously described Tie2 IRES (Park *et al.*, 2005). Using the bicistronic plasmid setting, a number of deletions were generated in the Tie2 5' UTR and positioned between the *Renilla* and firefly luciferase ORFs (Figure 4-5A). Bicistronic constructs were generated in which the uAUGs were ablated to remove uORFs that could inhibit downstream reporter activity and generate a false negative signal (by decreasing reinitiation at, or shunting ribosomes

Figure 4-4. The Tie2 IRES activity is maintained during quiescence. **A.** Schematic representation of bicistronic constructs containing the Tie2 5' UTR in the intercistronic region. The *Renilla* and firefly luciferase ORFs are denoted by a white and black box, respectively. **B.** Bicistronic constructs were transfected to HUVECs and 5 hr post-transfection, cells were subjected to serum starvation (0.2 % FBS) or allowed to remain under control conditions (2 % FBS + growth factors) for an additional 24 hr before harvesting. Luciferase activities were determined, normalized to the protein content of each extract, and then set relative to the value obtained for Ren/Tie2/FF under control conditions. The ratios of firefly to *Renilla* luciferase activities are also calculated and plotted. The results represent the average of three independent experiments performed and the error bars denote the error of the mean.

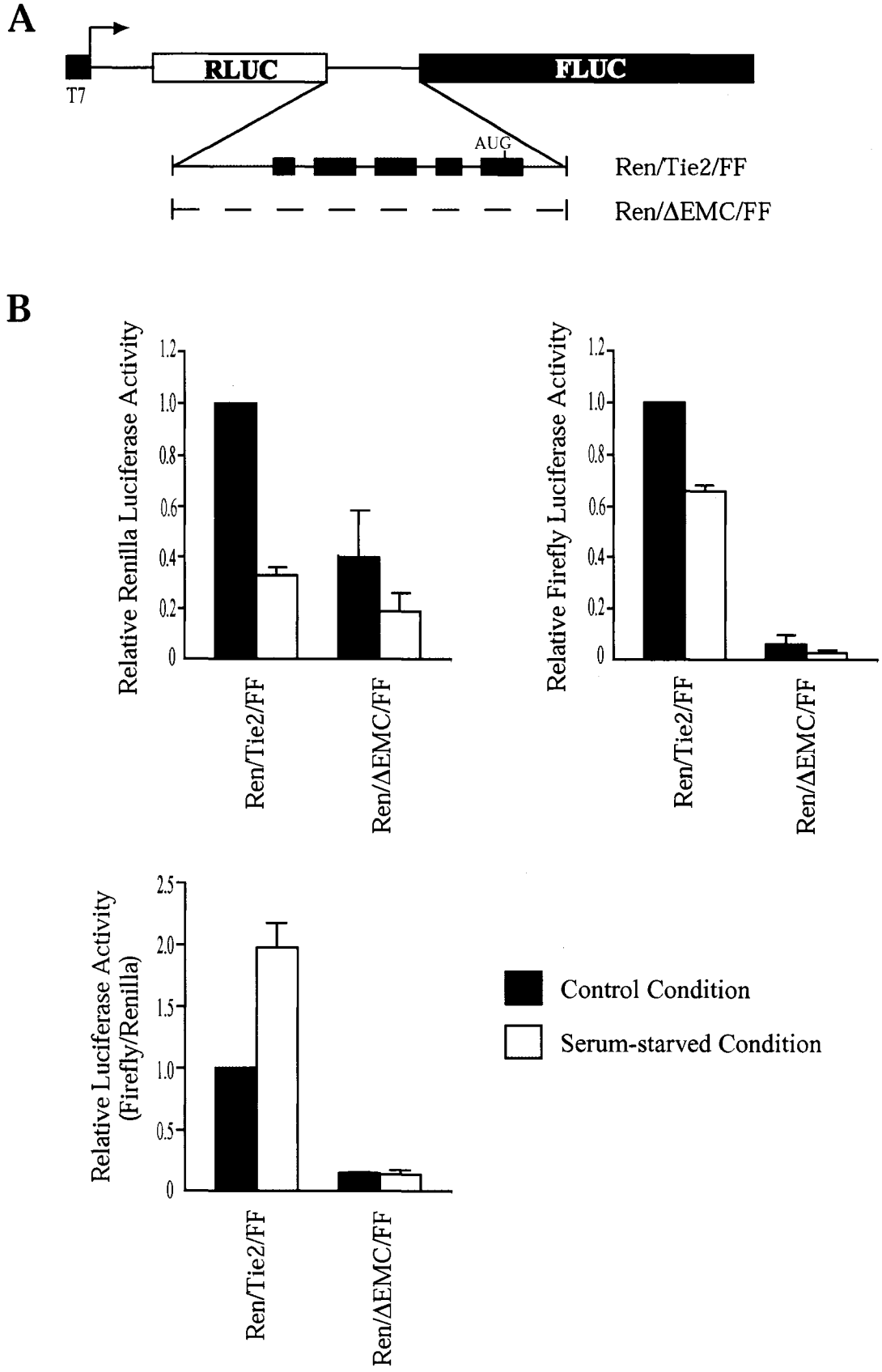
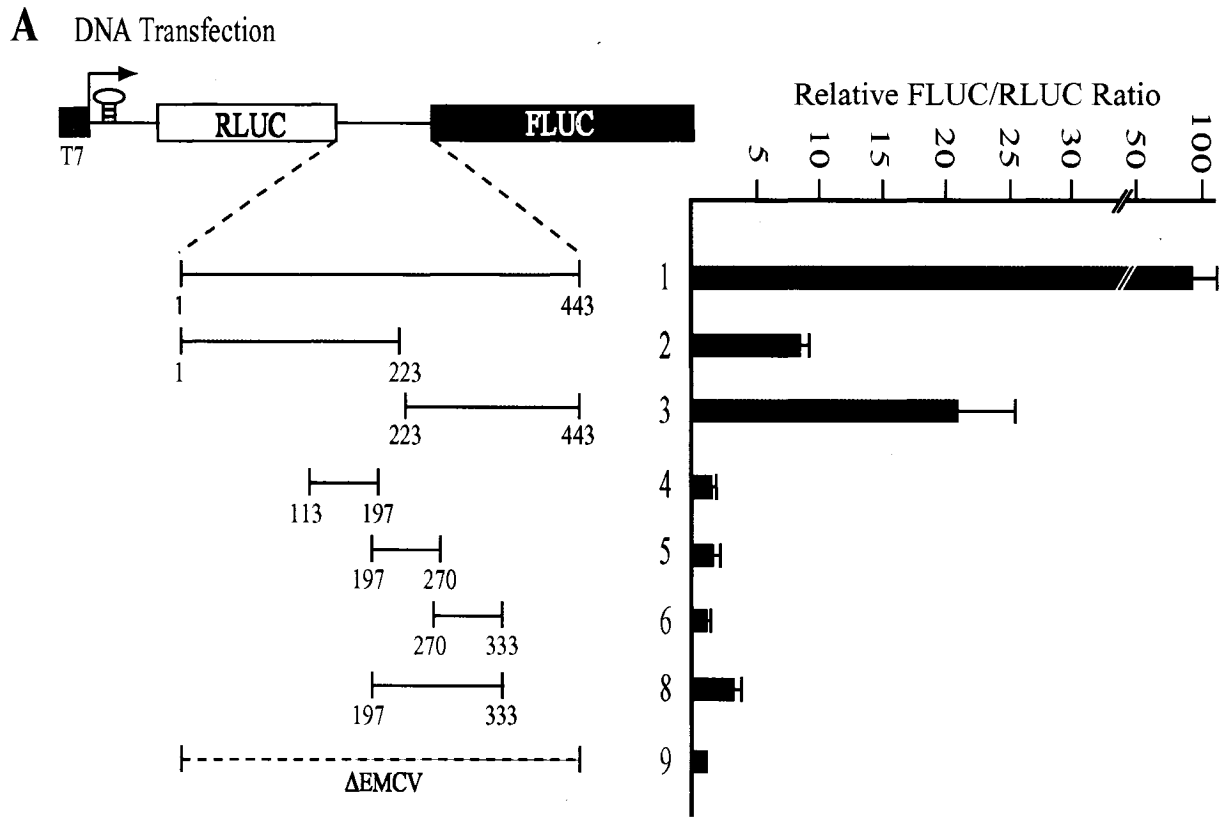
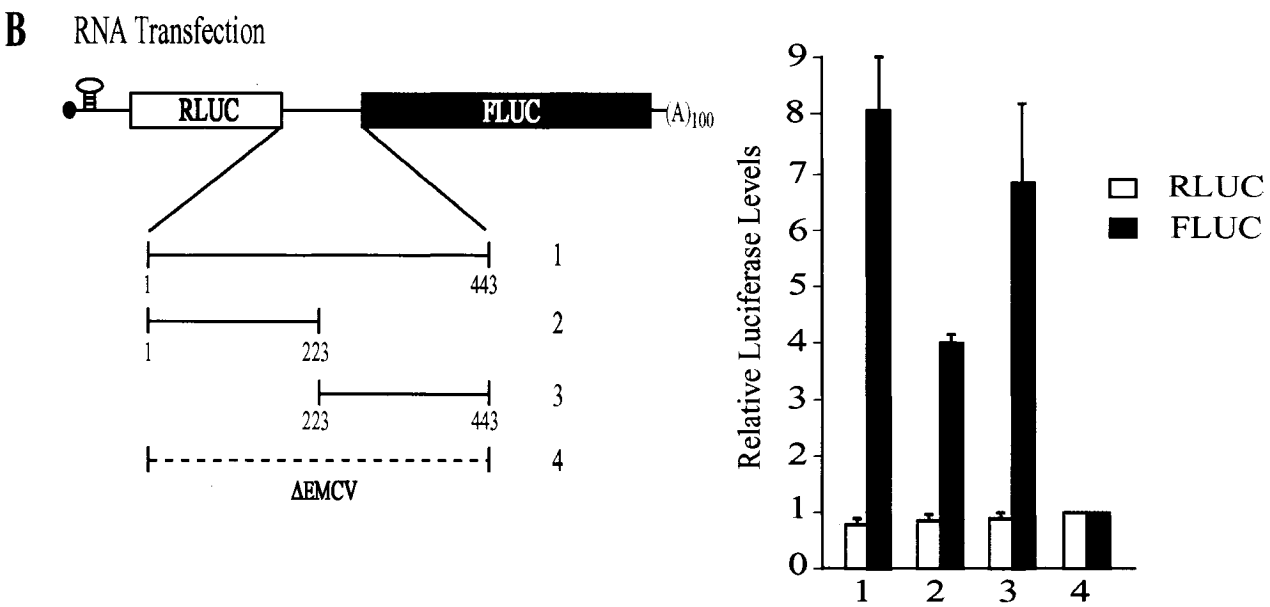

A
RLUC
FLUC
T7
AUG
Ren/Tie2/FF
Ren/ΔEMC/FF
B
Relative Renilla Luciferase Activity
Relative Firefly Luciferase Activity
Relative Luciferase Activity (Firefly/Renilla)
Ren/Tie2/FF
Ren/ΔEMC/FF
Control Condition
Serum-starved Condition

Figure 4-5. Deletion mapping analyses of the Tie2 IRES element. **A.** Left Panel: Schematic representation of the bicistronic reporter constructs containing truncated versions of the Tie2 5'UTR in the intercistronic region between the coding regions for *Renilla* and firefly luciferases. The 5'end of the Tie2 transcription start site is designated as 1 and nucleotide numbers indicate relative positions from this 5' end. Right panel: Luciferase activities were measured from HUVEC lysates following DNA transfection. The relative firefly luciferase/*Renilla* luciferase ratio (FLUC/RLUC) obtained from each construct is shown. The ratio of the negative control (lane 9) was arbitrarily set to 1. The results represent the average of three independent experiments performed and the error bars denote the error of the mean. **B.** Quantification of luciferase activities following RNA transfection into HUVECs. The relative firefly and *Renilla* luciferase values obtained from each bicistronic mRNA in the transfection are shown. Normalizations to protein content were performed for each extract. The results represent the average of three independent experiments.


A
DNA Transfection
RLUC
FLUC
T7
1
443
1
223
223
443
113
197
197
270
270
333
197
333
ΔEMCV
Relative FLUC/RLUC Ratio
5
10
15
20
25
30
50
100
1
2
3
4
5
6
8
9


B
RNA Transfection
RLUC
FLUC
(A)100
1
443
1
223
223
443
ΔEMCV
1
2
3
4
Relative Luciferase Levels
0
1
2
3
4
5
6
7
8
9
RLUC
FLUC
1
2
3
4

past the firefly initiation codon). As a negative control for these experiments, the bicistronic construct containing the EMCV 5'UTR with the deleted IRES was used (Figure 4-5A, lane 9; Figure 4-5B, lane 4). As expected, DNA transfection assays showed that IRES activity is detectable from the bicistronic construct harboring the full-length Tie2 5' UTR (Figure 4-5A, lane 1). We have previously reported that this is not due to the presence of a cryptic promoter, splicing, or preferential mRNA degradation (Park *et al.*, 2005). Deletion studies indicated that truncating either half of the 5' UTR retained IRES activity. Deletion mutants encoding nts 1-233 or nts 223-443 were capable of mediating internal initiation, although nts 223-443 appeared to have stronger activity than nts 1-223 (Figure 4-5A, compare lanes 2 to 3). Further deletions within both these regions failed to narrow the region responsible for IRES activity.

In order to confirm these results, we performed mRNA transfections into HUVECs. The configuration of the transfected mRNAs is shown in Figure 4-5B. Whereas the mRNA containing a deletion within the EMCV IRES showed no IRES activity, the bicistronic mRNAs containing the full-length Tie2 5' UTR, nts 1-233, or nts 233-443 showed IRES activity (Figure 4-5B, compare lanes 1-3 to 4). The results confirmed those obtained with DNA transfections, illustrating that two regions within the Tie2 5' UTR are capable of independently mediating IRES activity although fully functional IRES activity most likely requires the entire 5' UTR (Figure 4-5B). In addition, they indicate that the observed Tie2 IRES activity is unlikely due to a cryptic promoter or alternative splicing event.

4.5 Discussion

Much evidence, based on targeted gene disruption studies, has shown the importance of Tie2 receptor during the development of the embryonic vasculature. However, the role of this receptor in the adult vasculature has been relatively unexplored. Angiogenesis is a tightly regulated process which is maximally active in the developing embryo and downregulated in the healthy adult. Therefore, vascular endothelial cells in the adult are normally quiescent, dividing every 2-5 years; in a normal adult vessel, only 1 in every 10,000 endothelial cells is in the cell division cycle at any given time

(Engerman *et al.*, 1967; Hanahan and Folkman, 1996; Hobson and Denekamp, 1984; Woltering *et al.*, 1997). However, in response to proangiogenic factors and cytokines from inflammatory cells or tumors, the quiescent vasculature can become activated to grow new capillaries (Fox *et al.*, 2001; Hanahan and Folkman, 1996).

In our present study, we observed that Tie2 protein production is maintained during quiescence, when global protein synthesis is greatly reduced (Figures 4-2E and 4-3A). Our data support the previous observations indicating Tie2 expression in a wide range of adult rat tissues (Wong *et al.*, 1997). More recently, Arai *et al* have demonstrated that the Tie2/Angl signaling plays a critical role in the maintenance of HSC quiescence in the bone marrow microenvironment (Arai *et al.*, 2004). However, there are currently no reports indicating how Tie2 expression is regulated at the molecular level during this translation block. We hypothesized that the Tie2 IRES may serve to circumvent the translation inhibition imposed by quiescence to maintain constant Tie2 protein levels.

During quiescence, the rate of protein synthesis is partly modulated by the phosphorylation state of eIFs. Consistent with previously published reports, increased phosphorylation of eIF2α was observed in quiescent HUVECs [Figure 4-2C, top panel; (Gupta *et al.*, 1997)]. In addition, a decrease in eIF4E and a increase in the unphosphorylated (eIF4E-sequestering) form of 4E-BP1 were detected in these cells, suggesting that a loss of cap-dependent translation occurs during quiescence (Figure 4-2B, middle panel and 4-2A). Association of 4E-BP1 with eIF4E has also been shown to block phosphorylation of eIF4E, consistent with our observations of decreased eIF4E phosphorylation under serum starvation [Figure 4-2B, top panel; (Wang et al., 1998a)]. However, there have been conflicting data regarding whether phosphorylation of eIF4E modulates cap-binding or control of translation (Kleijn et al., 1995; McKendrick et al., 2001; Minich et al., 1994; Scheper et al., 2002; Zuberek et al., 2003). Proteolytic cleavage of eIF4G associated with serum-starvation also contributes to decreased general protein synthetic rates [Figure 4-2D; (Clemens *et al.*, 1998; Marissen and Lloyd, 1998)].

Cellular IRESes are considered likely to have an important function in maintaining synthesis of gene products when cap-dependent translation is inhibited, such as during hypoxia, apoptosis, and amino acid starvation (Carter *et al.*, 2000). Recently,

we have demonstrated that the Tie2 mRNA contains an IRES element located in the 5' UTR (Park *et al.*, 2005). Translation initiation from this IRES was maintained, and even stimulated during hypoxia, securing Tie2 protein production when global protein synthesis is decreased. Similarly, Lang *et al* have reported that the 5' UTR of HIF1α mRNA contains an IRES that was not affected by hypoxic stress even though there was a significant reduction in cap-dependent translation (Lang *et al.*, 2002). Furthermore, these investigators demonstrated that HIF1α, VEGF and *c-myc* IRES activities were less affected by serum starvation than through cap-dependent translation. Since Tie2 expression is maintained under quiescent conditions, we decided to investigate whether the Tie2 IRES could also play a role to circumvent the translation inhibition imposed by quiescence. Our results demonstrated that serum starvation affects the Tie2 IRES activity much less than cap-dependent translation, similar to the observation with HIF1α, VEGF, and *c-myc* IRESes during serum deprivation [Figure 4-4; (Lang *et al.*, 2002)]. Our data also indicated that the entire Tie2 5' UTR is required for maximal IRES activity, suggesting the possibility that the full length 5' UTR is needed for the IRES element to be properly folded and thus to effectively recruit *trans*-acting factors critical for its activity (Figure 4-5). It is also possible that the Tie2 IRES contains several noncontiguous sequence elements that show IRES activity on its own (i.e., the IRES is modular in nature) and that these modules cooperate to direct efficient internal initiation, as observed in the Bip and PDGF IRESes (Sella *et al.*, 1999; Yang and Sarnow, 1997). Transcriptional mechanisms do not appear to participate in maintenance of the protein levels during quiescence as a significant reduction of Tie2 mRNA levels were observed in response to serum starvation compared to those in normal conditions (Figure 4-3C). Taken together, these results indicate that the presence of IRES in the Tie2 5' UTR allows translation of the mRNA to be maintained under conditions that are inhibitory to cap-dependent translation (e.g., hypoxia and quiescence).

It has been speculated that Tie2 receptor may play a pivotal role in quiescent adult vasculature in addition to embryonic development. Therefore, it is important to understand the events that regulate Tie2 expression and how it is altered in disease states. In this study, we demonstrated that Tie2 protein levels are maintained during quiescence, in part, due to the presence of functional IRES in the 5' UTR. Nonetheless, the IRES does

not fully explain how Tie2 protein levels remain during quiescence since the IRES-mediated translation also decreased under quiescent conditions even though the extent is much less than cap-dependent translation (Figure 4-4B). Further investigations are required to assess whether other molecular mechanisms (e.g., posttranslational modifications such as protein stability) are involved in the maintenance of Tie2 protein levels during quiescence.

4.6 Acknowledgements

E.H.P. is supported by a K.M. Hunter/CIHR doctoral research award. J.P. is a Canadian Institute of Health Research (CIHR) Senior Investigator.

CHAPTER 5

General Discussion

Many studies have recently demonstrated a crucial role of Tie2 in angiogenesis since it was identified as a receptor tyrosine kinase expressed predominantly in vascular endothelium. Abolished Tie2 function in mice resulted in embryonic lethality due to vascular abnormalities, suggesting a role in blood vessel maturation and maintenance. In the adult, in addition to the endothelium of ovary, Tie2 is expressed and activated in quiescent endothelial cells (Wong *et al.*, 1997). Furthermore, this receptor is upregulated in capillaries during the neovascularization processes such as skin wounds and various tumors [e.g., hepatocellular carcinoma, colorectal cancer, and breast carcinoma; (Chin *et al.*, 2003; Dales *et al.*, 2003; Durkin *et al.*, 2004; Tanaka *et al.*, 2002)]. Consistent with an essential role for Tie2 in angiogenesis, a constitutively activating missense mutation in the Tie2 gene has been identified in humans, resulting in venous malformations. Despite the significant contribution of Tie2 in new blood vessel formation, most of the studies have focused on the function and biology of the Tie2 protein. The mechanisms underlying translational regulation of Tie2 gene expression are not well defined. Using a series of mutants and biochemical assays, we have shown that Tie2 translation is regulated by the coordinated action of *cis*-acting elements such as an IRES and uORFs. Furthermore, we have provided evidence to support the idea that the Tie2 IRES plays a role in maintaining its expression under conditions when cap-dependent translation is unfavorable (e.g., hypoxia and quiescence).

Translation of the vast majority of mammalian mRNAs occurs by a scanning mechanism. However, the 5' UTR of human Tie2 mRNA has features that are incompatible with efficient ribosomal scanning. It is unusually long (~372 nucleotides), contains 8 uAUGs and five uORFs, and can potentially form complex, stable secondary structures (Park *et al.*, 2005; Park *et al.*, 2006). These characteristics are often found in IRES-containing mRNAs whose gene products are involved in controlling cellular growth, differentiation, stress, development, and programmed cell death. Interestingly, several gene products of IRES-containing mRNAs, such as VEGF, FGF2, PDGF, and Cyr61 have been shown to play roles in angiogenesis, suggesting that IRES-mediated translation may be used during angiogenesis in situations such as wound healing,

cardiovascular and cerebral ischemia, and many types of cancers (Bernstein *et al.*, 1997; Huez *et al.*, 1998; Johannes *et al.*, 1999; Stein *et al.*, 1998; Vagner *et al.*, 1995).

We have observed that Tie2 mRNA is efficiently translated in contrast to the prediction based on the complex structure of its 5'UTR. This was shown by the fact that the majority of Tie2 mRNA expressed in HUVECs is associated with polyribosomes (Figure 2-1). Importantly, translation of Tie2 was not impaired under hypoxic conditions when overall translation was reduced (Figures 2-1 and 2-2). We have identified an IRES element within the Tie2 5' UTR and anticipated that the presence of this *cis*-acting element would explain, at least in part, how Tie2 mRNA is able to be efficiently translated under hypoxia.

Global protein synthesis via the conventional cap-dependent scanning mechanism is a tightly regulated process and it is controlled by changes in the phosphorylation status of eIFs (e.g., 4E-BP and eIF2α). We have observed a decrease in the phosphorylation of 4E-BP and an increase in the phosphorylation of eIF2α in response to hypoxic stress (data not shown); as a result, global translation is compromised. These results were consistent with previously published reports (Arsham *et al.*, 2003; Koumenis *et al.*, 2002; Tinton and Buc-Calderon, 1999). Moreover, using ^{35}S-methionine metabolic labeling of cells, we and others have demonstrated that hypoxia contributes to an overall decrease in protein synthesis in different cell types by 50~85 %, and in particular, cap-dependent translation [Figures 2-2C and 2-4C; (Lang *et al.*, 2002)].

However, protein synthesis of a subset of mRNAs is well maintained or even induced during hypoxia (Lang et al., 2002; Stein et al., 1998). The idea that the Tie2 IRES allows an escape from the global downregulation of protein synthesis under hypoxia was supported by our observations in which IRES activity was maintained, and even slightly stimulated, under hypoxic conditions as previously described with the functional IRESes in the HIF1α and VEGF mRNAs (Lang *et al.*, 2002; Stein *et al.*, 1998). Our data provides evidence for understanding how Tie2 mRNAs are translated despite a cumbersome structured 5' UTR and how its production is secured under unfavorable environmental conditions. Nevertheless, the IRES does not completely explain how Tie2 protein levels are sustained under hypoxic stress as the Tie2 IRES activity is moderate compared to the poliovirus IRES activity (Figure 2-6C; note that

cellular IRESes are, in general, much weaker than viral IRESes). It is likely that Tie2 protein expression is regulated at multiple levels by complex mechanisms, including posttranslational regulation, such as protein stability, during hypoxia.

Similarly, we have observed that Tie2 IRES element is functional during quiescence when global protein synthesis is dramatically reduced as indicated by ^{35}S-methionine metabolic labeling (Figures 4-4 and 4-3B). The Tie2 IRES activity was less affected than cap-dependent translation during quiescence induced by serum starvation (Figure 4-4). Consistent with our results, serum starvation affected the activities of some cellular IRESes, such as the HIF1α, *c-myc*, and AT_1R IRESes less than cap-dependent-translation [discussed below; (Lang *et al.*, 2002; Martin *et al.*, 2003)].

Transcription start site mapping of Tie2 mRNA indicated the existence of several mRNA isoforms containing unusually long 5' UTRs (>350 nucleotides) with five uORFs (Park *et al.*, 2005). Upstream open reading frames in mRNAs are known to regulate translation in eukaryotes (Morris and Geballe, 2000). In human, uAUGs and uORFs are found in a significant proportion of mRNAs, perhaps as much as ~48 %, and are particularly common in mRNAs coding proto-oncogenes, transcription factors, and genes involved in the control of cellular growth and differentiation (Geballe and Sachs, 2000; Meijer and Thomas, 2002; Pesole *et al.*, 2000). A variety of translational control mechanisms mediated by uORFs have been demonstrated as described in section 1.7 of my thesis.

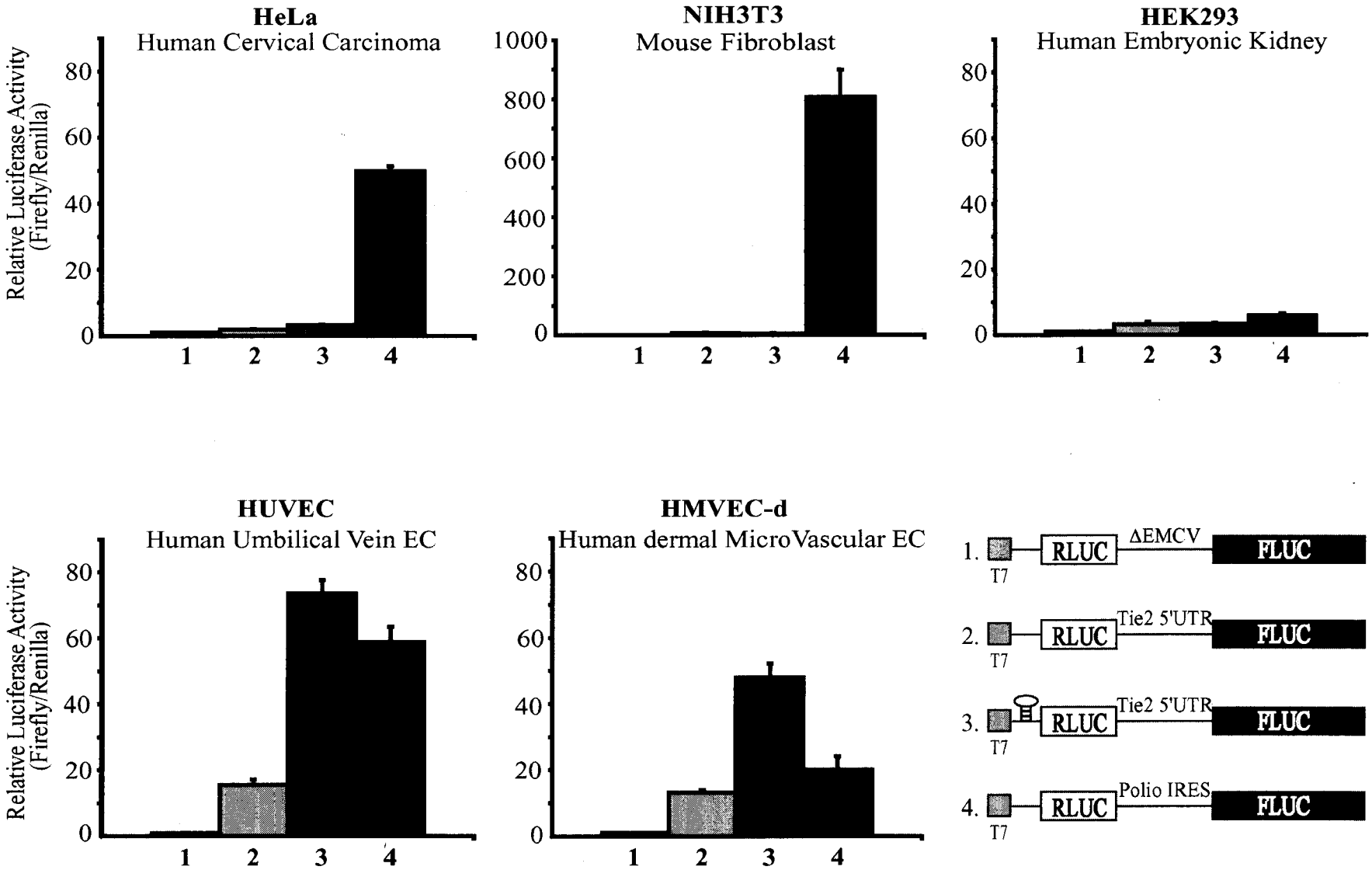
As mentioned earlier, the Tie2 5' UTR harbors an IRES that allows this mRNA to be translated under hypoxic conditions. Unlike uncapped viral mRNAs containing IRESes, cellular mRNAs harboring IRESes can be translated by both cap-dependent and IRES-mediated initiation mechanisms (Stoneley *et al.*, 2000b). Using both m^7GpppG- and ApppG-capped monocistronic mRNAs containing the Tie2 5' UTR, we confirmed that both mechanisms can contribute to Tie2 protein synthesis (Figure 3-4). The interplay between these two mechanisms may provide control over Tie2 expression, which would be responsive to various cellular translation conditions. When cellular cap-dependent translation is ongoing, translation of Tie2 could occur partly via cap-dependent scanning, but it would be limited by the secondary structure of the 5' UTR and presence of the regulatory uORFs. On the other hand, when cap-dependent scanning is downregulated

during stress conditions (e.g., hypoxia), the Tie2 IRES would allow continuity of Tie2 protein synthesis. Interestingly, several cellular mRNAs containing IRES elements in their 5' UTR also contain small uORFs located within the 5' UTR sequence. In most cases, the significance of these uORFs for translation is not clear. Recently, it has been demonstrated that translation of a uORF is required for increased *Cat-1* IRES activity during amino acid starvation. On the other hand, the presence of uORFs in the Tie2 5' UTR appeared to exert an inhibitory effect on the IRES activity as shown in Figures 2-4B and 2-6B and C.

We were unable to detect Tie2 IRES activity in *in vitro* translation assays in the presence of rabbit reticulocyte lysate, wheat germ extract, and Krebs extract (Figure 3-3). In addition, our preliminary data obtained from bicistronic assays using different cell lines indicated that the efficiency of Tie2 IRES-driven translation varies among the cell lines tested (Figure 5-1). In these experiments, the Tie2 IRES was significantly more active in endothelial cells (HUVEC and HMVEC-d), compared to non-endothelial cells (HeLa) and it was almost inactive in NIH3T3 and HEK293. These observations imply that the function of the IRES element could be modulated by cell-type specific *trans*-acting factors, which might be lacking or limiting in these lysates or non-endothelial cells. However, we can not rule out the possibility that the cell-type specificity was due to transcriptional effects as bicistronic DNA transfections were performed to assess Tie2 IRES activity. Further analyses (e.g., bicistronic mRNA transfections) will answer possible cell-type specificity of the Tie2 IRES activity. Cell-type specific or extract-dependent IRES activity has been previously demonstrated for a number of IRESes (discussed in sections 1.6.2.5 and 3.5). The molecular mechanism of IRES/ITAF interaction has recently been explored for some cellular IRESes such as Apaf-1 and Bag-1 (discussed in section 1.6.2.6). Further studies are required to identify and characterize potential *trans*-acting factors for Tie2 IRES activity, if the IRES activity is cell-type specific. This could explain why the IRES is not functional in certain environments. The effect of these factors, then, can be rigorously tested in cell-free extracts.

To further investigate the impact of the Tie2 uORFs on translational regulation without interference from internal initiation, we utilized *in vitro* translation assay

Figure 5-1. A comparison of the Tie2 IRES efficiency to drive translation in five different cell lines. Luciferase activities were measured from cell lysates following DNA transfection. The relative firefly luciferase/*Renilla* luciferase ratio obtained from each construct is shown and the construct number is indicated below the X-axis. The ratio of the negative control (construct No.1) was arbitrarily set to 1. The results represent the average of three independent experiments performed and the error bars denote the error of the mean. **Right lower panel:** Schematic representation of the bicistronic reporter plasmids containing the Tie2 5' UTR in the intercistronic region. The *Renilla* and firefly luciferase ORFs are denoted by a white and black box, respectively. The stem-loop structure upstream of the *Renilla* ORF represents the HIV TAR element.


HeLa
Human Cervical Carcinoma
NIH3T3
Mouse Fibroblast
HEK293
Human Embryonic Kidney
HUVEC
Human Umbilical Vein EC
HMVEC-d
Human dermal MicroVascular EC
Relative Luciferase Activity
(Firefly/Renilla)
0
20
40
60
80
200
400
600
800
1000
1
2
3
4
1. T7 RLUC ΔEMCV FLUC
2. T7 RLUC Tie2 5'UTR FLUC
3. T7 RLUC Tie2 5'UTR FLUC
4. T7 RLUC Polio IRES FLUC

systems. These conditions can not support internal ribosome entry on the Tie2 5' UTR as described earlier; therefore the contribution of the cap-dependent translation can be directly assessed. Using deletion mutagenesis analyses, we found that the Tie2 5' UTR is inhibitory to translation initiation with ribosome flow decreasing as they encounter with each uORF (Figure 3-2B and C). Moreover, we did not find any strong *cis*-acting elements in the 5' UTR inhibitory to the downstream translation (Figure 3-5). Our results suggest that the uORFs within the Tie2 5' UTR serve to decrease the amount of ribosomes competent for translation reinitiation, as they scan the mRNA.

We have mapped the location of the Tie2 IRES to identify the boundaries of the Tie2 IRES and showed two regions capable of exerting IRES activity – one encompassing uORFs 1-3 and the other encompassing uORFs 3-5 (Figure 4-5). However, the sequence throughout the entire 5' UTR appears to be required for the fully functional IRES activity as observed in other cellular IRESes [e.g., L-*myc* IRES (Jopling *et al.*, 2004)]. It is possible that the full length 5' UTR is needed for the IRES element to be properly folded and thus to effectively recruit *trans*-acting factors critical for its activity. Another possibility is that the Tie2 IRES contains several noncontiguous sequence elements that display IRES activity on its own (i.e., the IRES is modular in nature) and that these modules cooperate to direct efficient internal initiation. In fact, several other cellular IRESes have been shown to be modular. For instance, the Bip and PDGF IRESes can be separated into nonoverlapping fragments which show partial IRES activity (Sella *et al.*, 1999; Yang and Sarnow, 1997). The hypothesis that cellular IRESes can be modular is supported by the observation that a short segment of the *Gtx* IRES shows enhanced activity when present in multiple copies (Chappell *et al.*, 2000a). The complementarity to rRNA observed in the *Gtx* IRES module appears to be a specific mechanism that is not used by the Tie2 gene, and it is possible that the modular nature of Tie2 IRES could be determined by the cooperation of structural domains. Although we have not further characterized the Tie2 IRES element, it would be interesting to identify modules that could contribute to internal initiation if the IRES is modular and investigate how precisely these motifs regulate the IRES activity.

A large body of evidence has shown that angiogenesis is a crucial event for normal embryonic development. However, except for normal physiological processes

and pathological conditions, the adult vasculature is notably quiescent; with only ~0.01 % of cells actively proliferating (Hanahan and Folkman, 1996; Hobson and Denekamp, 1984). In quiescent/serum-deprived cells, the overall translation rate is declined and some evidence has suggested that this repression is due to a lack of function of one or more eukaryotic translation initiation factors (Duncan and Hershey, 1985; Mao et al., 1992; Wang et al., 1999). Interestingly, we and others have observed that Tie2 protein levels are maintained during quiescence, suggesting that it escapes the general repression of translation initiation [Figures 4-2E and 4-3A and B; (Wong *et al.*, 1997)] Recently, the Tie2/Ang1 signaling axis has been implicated in regulating quiescence of hematopoietic stem cells in the bone marrow niche (Arai *et al.*, 2004). We investigated if the Tie2 IRES activity contributes to overcoming the overall inhibition in protein synthesis under quiescent conditions. Our results from bicistronic assays indicated that Tie2 IRES is functional in quiescent endothelial cells and IRES-mediated translation is less affected by serum starvation than cap-dependent translation (Figure 4-4B). Similar results were observed for HIF1α and *c-myc* IRES activities in serum-deprived cells (Lang *et al.*, 2002). However, the IRES does not fully explain how Tie2 protein levels remain during quiescence since the IRES-mediated translation also decreased under quiescent conditions even though the extent is much less than cap-dependent translation (Figure 4-4B). Therefore it is possible that other posttranslational mechanisms (e.g., protein stability) are involved in maintaining Tie2 protein levels constant during quiescence.

What is the physiological relevance of translational control of the Tie2 mRNA by its *cis*-acting elements? It is apparent that Tie2 protein expression is maintained under conditions when global protein synthesis is greatly reduced, such as hypoxia and quiescence. It has been indicated that excessive activation/expression of Tie2 is associated with pathological conditions, such as vascular dysmorphogenesis and various cancers, suggesting that highly regulated Tie2 expression is probably important for normal vascular formation and maintenance. Therefore, under stress conditions when cap-dependent translation is unfavorable, Tie2 IRES element may play an important role to maintain the level of this essential protein for survival. Under normal conditions of cell growth, the Tie2 uORFs, as regulatory elements, most likely limit production of Tie2 protein that would be deleterious if produced in excess. It is possible that the IRES- and

5′ end-mediated initiations may regulate each other's activities or they may co-exist and be required under different physiological conditions.

Taken together, it is becoming more evident that cellular processes, such as angiogenesis, are highly coordinated events that are regulated, both spatially and temporally, and at multiple levels, including the translational level. Our results underscore the complexity of Tie2 gene expression at the level of translation initiation. By studying the molecular mechanism of Tie2 gene expression, we should gain a better understanding of the events that regulate the expression of this important gene, and how it is altered in disease states.

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