



Regulation of vascular endothelial growth factor (VEGF) expression is mediated by internal initiation of translation and alternative initiation of transcription

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Vascular Endothelial Growth Factor (VEGF) is a very potent angiogenic agent that has a central role in normal physiological angiogenesis as well as in tumor angiogenesis. VEGF expression is induced by hypoxia and hypoglycemia, and thus was suggested to promote neovascularization during tumor outgrowth. Yet, the molecular mechanism that governs VEGF expression is not fully characterized. VEGF induction is attributed in part to increased levels of transcription and RNA stability. Previously, we demonstrated that the 5' Untranslated Region (5' UTR) of VEGF has an important regulatory role in its expression. VEGF has an exceptionally long 5' UTR (1038 bp) which is highly rich in G+C nucleotides. This suggests that secondary structures in the 5' UTR might be essential for VEGF expression through transcriptional and post-transcriptional control mechanisms, as demonstrated for other growth factors. In this communication, we provide evidence that a computer predicted Internal Ribosome Entry Site (IRES) structure is biologically active and is located at the 3' end of the UTR. In addition, the results demonstrate that an alternative transcriptional initiation site for VEGF exists in the 5' UTR of VEGF. This alternative initiation site is 633 bp downstream of the main transcription start site and the resulting 5' UTR includes mainly the IRES structure. Therefore, our results suggest that VEGF is subjected to regulation at either translational level through a mechanism of ribosome internal initiation and/or transcriptional level through alternative initiation.

Keywords: vascular endothelial growth factor (VEGF); internal ribosome entry site (IRES); 5' untranslated region; translational regulation; angiogenesis

Introduction

VEGF belongs to a family of angiogenic agents with similar structural and biological activities that also include VEGFB, VEGFC, VEGFD, placenta derived growth factor (PlGF) and platelet derived growth factor (PDGF) (Keck *et al.*, 1989; Joukov *et al.*, 1996; Townson *et al.*, 1996; Ishikawa *et al.*, 1989; Tischer *et al.*, 1989; Maglione *et al.*, 1991; Yamada *et al.*, 1997).

VEGF is a very potent angiogenic agent that acts as a specific mitogen for vascular endothelial cells through specific cell surface receptors. This angiogenesis inducer is released by a variety of tumor cells and its expression in tumors is a prerequisite for successful tumor growth and metastasis. Inhibition of VEGF expression, by injection of specific antibodies or by overexpression of dominant negative VEGF receptor mutant, resulted in a dramatic inhibitory effect on tumor growth demonstrating its pivotal role in tumor angiogenesis (Kim *et al.*, 1993; Millauer *et al.*, 1994). Overexpression of VEGF induces cell transformation in cooperation with Fibroblast Growth Factor 2 (FGF2) (Guerrin *et al.*, 1997). In addition, targeted inactivation of the VEGF gene resulted in heterozygous embryonic lethality which clearly demonstrates its necessity during embryonal development (Ferrara *et al.*, 1996).

Physiological stress conditions such as hypoxia and hypoglycemia can induce VEGF expression and thus can support tumor outgrowth (Shweiki *et al.*, 1992; Goldberg and Schneider, 1994). Induced expression of VEGF was also noted in cells treated with Interleukin-1 β (IL-1 β), Transforming Growth Factor- β (TGF- β), PDGF2, Tumor Promoting Agent (TPA) and Interleukin-6 (IL-6) (Li *et al.*, 1995; Pertovaara *et al.*, 1994; Dolecki and Connolly, 1991; Stavri *et al.*, 1995; Cohen *et al.*, 1996). The promoter region of VEGF has been cloned, sequenced and found to contain numerous putative binding sites for various transcription factors such as AP1, AP2 and SP1 (Tischer *et al.*, 1991). The hypoxia and cytokine induced expression of VEGF was attributed to specific DNA motifs, located at the promoter region of VEGF, that confer response to the various stimuli. The mechanism that controls VEGF expression is not fully understood. Several lines of evidence indicate that induced VEGF expression in response to hypoxia is due to transcriptional activation as well as mRNA stabilization (Minchenko *et al.*, 1994a,b; Stein *et al.*, 1995; Ikeda *et al.*, 1995; Shima *et al.*, 1995; Levy *et al.*, 1996; Cohen *et al.*, 1996).

We have studied the effect of various cytokines on the expression of VEGF and showed that VEGF mRNA was induced by Interferon- β (IFN- β), IL-6 as well as by cobalt (Cohen *et al.*, 1996). Our results indicated that a DNA element(s) located upstream to the transcription initiation site mediates in part the response to IL-6 and cobalt, and that induction of VEGF is also mediated through a specific DNA element(s) located at the 5' UTR of the gene. The data suggested that the 5' UTR elements cooperate synergistically with DNA elements located upstream to

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the transcription initiation site. We also showed that the 5' UTR of VEGF has an important role in the expression of VEGF. Interestingly, VEGF contains a very long (1038 bp) 5' UTR which is characterized by a high G+C content (83%) upstream to the translation initiation site. A deletion of 83 bp located in the vicinity of the translation start site resulted in a significant decrease in CAT activity suggesting that it is important for maintaining proper secondary structure of the 5' UTR, which is crucial for proper translation.

The compilation of sequence information from 699 vertebrate mRNAs has indicated that more than 90% of the sequenced 5' UTRs are less than 200 bp and are devoid of AUG triplets (Kozak, 1991). However, most mRNAs coding for proto-oncogenes and other factors related to cell proliferation possess long G+C rich 5' UTRs with one or more AUGs (or alternatively CUGs) suggesting that these genes are translationally regulated. The structured 5' UTRs probably mediate regulated alternative initiation of transcription or internal initiation of translation (Prats *et al.*, 1992; Vagner *et al.*, 1995). This Internal Ribosome Entry Site (IRES) dependent translation, first noted in the picornaviruses, confers cap-independent translation (Pelletier and Sonenberg, 1988). The IRES serves to directly position the mRNA on the ribosome in the vicinity of the translation start codon in a context that does not involve upstream scanning. IRES elements were also reported for cellular mRNAs such as human immunoglobulin heavy chain binding protein (BiP) (Macejak and Sarnow, 1991). Antennapedia (Antp) mRNA of *Drosophila* (Oh *et al.*, 1992), FGF2 (Vagner

et al., 1995), Insulin like Growth Factor-II (IGF-II) (Teerink *et al.*, 1995), eukaryotic Initiation factor-4G (eIF-4G) (Gan and Rhoads, 1996) and PDGF2/*c-sis* (Bernstein *et al.*, 1997).

In this work we demonstrate that part of the 5' UTR of VEGF contains a very potent IRES element. In addition, a segment of the 5' UTR can function as a promoter which drives transcription from an alternative initiation site. Thus, the regulation of VEGF expression is controlled at multiple levels including transcription, mRNA stability and translation. Such complex regulation of VEGF expression is in accordance with its pivotal role in normal and tumor angiogenesis.

Results

The 5' UTR of VEGF augments CAT expression driven by an heterologous TK promoter

We have demonstrated previously that the 5' UTR of VEGF potentiates the expression of a reporter gene driven by the VEGF promoter (Cohen *et al.*, 1996). Since this 5' UTR is exceptionally long and is very rich in GC, it suggested that secondary structures in the 5' UTR have an important role in either transcriptional or post-transcriptional regulation of VEGF. To analyse if expression driven by an heterologous promoter will also be potentiated by the presence of the 1038 bp long 5' UTR of VEGF, we have used Chloramphenicol Acetyl Transferase (CAT) reporter plasmids that

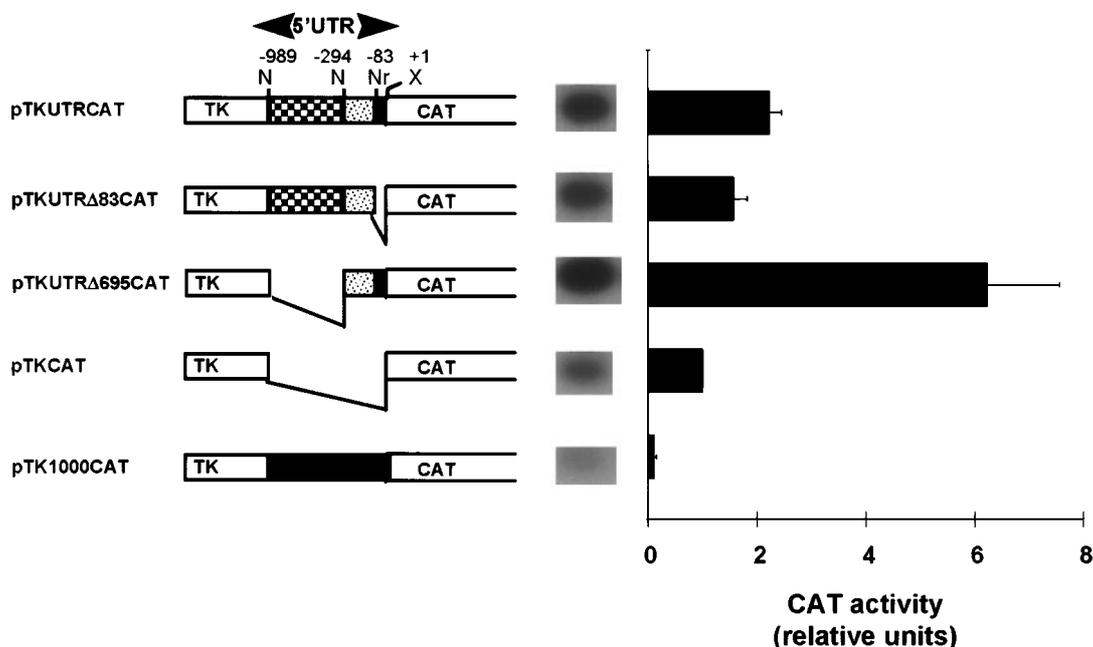


Figure 1 The 5' UTR of VEGF enhances CAT activity driven by the TK promoter. The TK promoter was cloned into CAT constructs which were already fused either to the full length VEGF-5' UTR or to 5' UTR lacking either 83 bp from the 3' end or 695 bp deletion from the 5' end to generate the plasmids pTKUTRCAT, pTKUTRΔ83CAT and pTKUTRΔ695CAT, respectively. pTKCAT does not contain any VEGF-5' UTR sequences. pTK1000CAT plasmid harbors insertion of 1000 bp 'non relevant DNA' in between the TK promoter and the CAT gene (see details under Materials and methods). 5 μg of each plasmid DNA was transfected to HeLa cells and relative CAT activity was calculated and presented in the bar graph which is the average ± s.d. of four independent experiments. The actual CAT assay spots (of a representative experiment) corresponding to the faster migrating form of monoacetylated chloramphenicol are placed to the left of the relevant bar. Abbreviations in the figure represent restriction sites Nr; *Nar*I, *Nhe*I, *X*; *Xho*I. Numbers indicate relative distance from the translation start site. The results are statistically significant as determined by the Sign test with a probability value (P) < 0.05

contained the promoter region of VEGF as well as the 5' UTR and swapped the promoter region with the basal TK promoter from the plasmid pBLCAT5 (Boshart *et al.*, 1992), generating the construct pTKUTRCAT (see illustration in Figure 1). The effect of VEGF-5' UTR, which was inserted downstream to the TK basal promoter, on CAT activity was compared with that of the original vector lacking the 5' UTR (pTKCAT). It was also compared to a vector containing 1000 bp of a non relevant DNA sequence instead of the 5' UTR (see illustrations in Figure 1 and for details see Materials and methods). HeLa cells were transiently transfected with the various constructs and relative CAT activities were determined.

It is clear from Figure 1 that the insertion of 1000 bp of a non relevant DNA sequences between the TK basal promoter and the CAT reporter gene resulted in a negligible CAT activity as compared with the CAT activity driven by the pTKCAT construct (at least a tenfold reduction).

On the other hand, insertion of 1038 bp corresponding to the VEGF-5' UTR downstream to the TK promoter resulted in at least doubled CAT activity as compared to the original vector pTKCAT. This implies, that insertion of 1000 bp of non relevant DNA resulted in severe inhibition of transcriptional or translational efficiency. In contrast, albeit its length, the 5' UTR of VEGF enhanced CAT activity implying that it affects the efficiency of either transcription or translation, or both. Deletion of 83 bp from the 3' end of the 5' UTR resulted in a small (30%) yet significant reduction in CAT activity as compared with that of the full length 5' UTR. This suggests that the 83 bp segment which is rich in GC nucleotides has some role in the enhancement effect conferred by the 5' UTR. Surprisingly, deletion of a 695 bp *NheI* fragment from the 5' end of the 5' UTR resulted in a marked increase (more than sixfold) in CAT activity. Therefore, the 294 bp 3'-portion of the 5' UTR contains a strong stimulatory activity which is down regulated by the 695 bp 5'-portion of the 5' UTR.

A computer predicted IRES element is found in the 5' UTR of VEGF

The data presented above indicates that the 5' UTR of VEGF potentiates the TK promoter driven CAT activity by affecting either transcriptional or translational efficiencies. We reasoned that some of this activity might be attributed to regulation at the translational level via an IRES element such as recently shown for PDGF2/*c-sis* (Bernstein *et al.*, 1997). The 5' UTRs of both genes are very long and GC rich implying that secondary structures might play a role in the activity of these regions. Secondary structures were calculated for the 5' UTR of VEGF using the EFFOLD program that computes thermodynamically favored helical stems by computing all possible alternative RNA structures based on the fluctuation of thermodynamic energy parameters within the experimental errors for these parameters (Le *et al.*, 1993). The predicted secondary structure of the 5' UTR of VEGF is illustrated in Figure 2a. Within this structure, an IRES element spanning over 198 bp upstream from the first AUG codon was observed (Figure 2b).

This element falls into the category of cellular IRES elements, which contain a Y shape structure common to IRES elements (denoted by the letters D1–D3 in Figure 2b) followed by a stem-loop structure (denoted by the letter E) just 19 bp upstream of the initiator AUG. A sequence homologous to human 18S rRNA is located 14 nucleotides upstream to the initiator AUG (Figure 2b) similar to its location in the IRES of PDGF2/*c-sis* (Bernstein *et al.*, 1997). This predicted IRES element is highly conserved in VEGF cDNA clones from bovine and murine at both the DNA and structural levels indicating phylogenetic conservation and structural significance (Figure 2c).

The activity of the 5' UTR of VEGF in bi-cistronic expression plasmids

To see if the predicted IRES element in the 5' UTR of VEGF is functional, it was tested for its ability to promote the translation of the second cistron in a bi-cistronic expression vector. We have used the bi-cistronic vector pCL, in which the CAT gene is located immediately downstream to the CMV promoter and the LUC gene is placed downstream of the CAT coding region (Figure 3a) (Bernstein *et al.*, 1997). LUC expression from this vector is low as shown previously (Bernstein *et al.*, 1997). In addition, we have used the same construct in which a hairpin structure, with calculated structural stability of $\Delta G = -40 \text{ kcal mol}^{-1}$, was inserted in front of the CAT gene (pHCL). This hairpin interferes with the efficiency of the cap-dependent translation of the CAT gene and further reduces the marginal translational activity of the LUC gene. However, this hairpin structure will not disrupt the translation of the second cistron if an IRES element is placed just upstream to its translation start site (Bernstein *et al.*, 1997; Macejak and Sarnow, 1991). Therefore, the full-length 5' UTR of VEGF was cloned between the two reporter genes in pCL and pHCL to generate the plasmids pCVL and pHCVL (Figure 3a). Similarly, a 695 bp *NheI* fragment was deleted from the 5' UTR leaving the putative IRES element and 90 nucleotides upstream of it between CAT and LUC, generating the plasmids pCAVL and pHCAVL (Figure 3a). In addition, as a positive control for IRES activity, we have used the pCEL plasmid which contains the well characterized cellular IRES element of the EMCV. As a negative control, pCPL, which harbors the PDGF2/*c-sis* 5' UTR containing a differentiation linked IRES in a bi-cistronic construct, was employed (Bernstein *et al.*, 1997). The various constructs (Figure 3a) were transfected to HeLa cells and 48 h later relative CAT and LUC activities were determined and summarized in Figure 3b. The CAT and LUC activities of the bi-cistronic vector lacking an IRES, pCL, were determined as one and the CAT and LUC activities obtained from the other vectors were calculated accordingly. It is clear from Figure 3b that CAT activities were similar for all the constructs lacking the 5' hairpin structure. As expected, the 5' hairpin structure interfered with the translation of the first cistron resulting in about a 25-fold reduction in CAT activity. The marginal LUC activity generated by the plasmid pCL is probably a result of a non specific ribosome reinitiation. This LUC activity was further decreased when the 5' hairpin structure was present in

VEGF-IRES element. Cells were transfected with the constructs indicated in Figure 3c, and subjected to either normoxia or hypoxia. The activity of the reporter genes was determined and the LUC/CAT ratio was calculated for each treatment. The results demonstrate that the ratio did not change significantly under the two conditions suggesting that hypoxia does not affect VEGF-IRES activity as determined using bi-cistronic constructs.

The 5' UTR of VEGF promotes alternative transcriptional initiation in addition to internal initiation

To distinguish between the two possible elements embedded in the 5' UTR of VEGF, i.e. an IRES activity or an effective promoter, cells were transfected with the various bi-cistronic plasmids described above, followed by mRNA extraction and Northern blot analysis using ³²P-labeled probes corresponding to either the LUC or the CAT genes. As expected, the

LUC probe detected a single band representing the bi-cistronic mRNA which corresponds to the pCL and pCPL products (Figure 4, lanes 4 and 1 respectively). However, in an mRNA sample extracted from cells transfected with pCVL, two strong bands were observed. One band corresponded to the expected length of the bi-cistronic mRNA whereas the second was a faster migrating band (Figure 4 lane 2). When the same blot was reprobbed with the CAT probe, only one band corresponding to the bi-cistronic mRNA was detected in all cases. This implied that the 5' UTR of VEGF possesses either promoter activity, detected by Northern blot analysis as a shorter transcript that is initiated within the 5' UTR, or that the 5' UTR has both promoter and IRES activities. The fact that only a single bi-cistronic mRNA band was observed in Northern blot analysis of cells transfected with the plasmid pCAVL (Figure 4 lane 3) implied that IRES activity is located in this DNA segment. Together with the reporter gene enzymatic activity data (Figure 3)

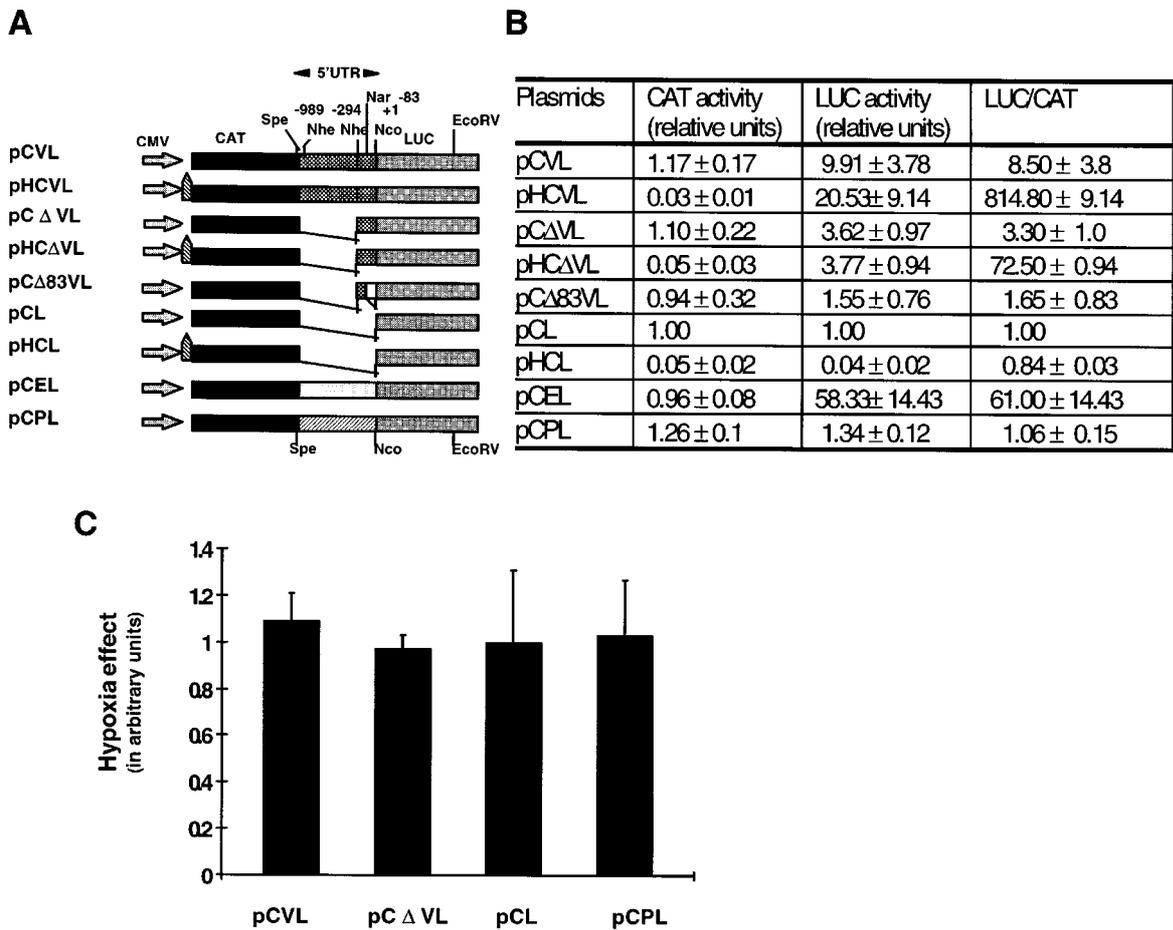


Figure 3 The presence of an IRES activity in the 5' UTR of VEGF. (a) The various bi-cistronic constructs are illustrated (for details see Materials and methods). Black boxes represent the CAT gene and gray boxes represent the LUC gene. Cone shaped boxes in front of the CAT gene represent hairpin structure and shaded boxes between the CAT and LUC genes represent the 5' UTR of either VEGF or PDGF2. Unique restriction sites and their relative distance from the first AUG codon are indicated. (b) HeLa cells were transfected with 5 μg of the various plasmids. Relative CAT and LUC activities were determined as described under Materials and methods. The LUC/CAT ratio was calculated accordingly. The results represent the average ± s.d. of five independent experiments with statistical significance as determined by the Sign test with a probability value (P) < 0.05. (c) HeLa cells were transfected in duplicates with the indicated plasmids as described above and 24 h later half was subjected to hypoxia as described under Materials and methods. Relative CAT and LUC activities were determined and the results are presented as 'hypoxia effect' which is the ratio of LUC/CAT under hypoxia versus LUC/CAT under normoxia. The data is the average ± s.d. of three independent experiments with statistical significance as determined by the Sign test with a probability value (P) < 0.05

this strongly suggests that the 5' UTR of VEGF contains both a genuine IRES element at the 3' end and an alternative promoter at its 5' end.

To further establish the existence of an IRES element and an alternative transcriptional initiation site within the 5' UTR of VEGF, the mRNA samples extracted from cells, which were transfected with the various constructs, were further analysed by Northern blot with DNA probes corresponding to the 5' end or to the 3' end of VEGF 5' UTR. As expected, the probe corresponding to the 5' end of the 5' UTR did not detect any mRNA band in cells transfected with the plasmids pCPL and pCPL since they do not harbor any sequences corresponding to the 5' UTR of VEGF (Figure 4). No band was detected in cells transfected with pCAVL since this plasmid does not contain the 5' end of the 5' UTR. However, a single slow migrating band was observed in the pCVL lane that corresponds in size to the bi-cistronic mRNA but no fast migrating band was observed in that lane (Figure 4 lane 2). A DNA probe corresponding to the 3' end of the 5' UTR generated too many bands probably due to a non specific hybridization to GC rich mRNAs since it is highly rich in GC sequences (data not shown).

To summarize, two mRNA species were transcribed from the bi-cistronic plasmid pCVL, which contains the full length 5' UTR of VEGF. A longer one, driven by the CMV promoter and corresponds in size to the bi-cistronic message, and a shorter transcript driven by the promoter sequences within the VEGF 5' UTR located between CAT and LUC genes. This shorter transcript does not include the first cistron as well as the first 700 bp sequence of the 5' UTR as

demonstrated by Northern blot analysis and possibly is a result of an alternative transcription start site within this bi-cistronic construct.

To further establish the possibility that VEGF might be subjected to regulation through alternative initiation of transcription, primer extension experiments were performed. mRNA was extracted from HeLa cells before and after 16 h of hypoxia. Two antisense primers corresponding to position -720 to -750 and -165 to -182 (relative to the translation start site) were utilized. The first (-720) enabled the identification of the major transcription start site at position -1038 (Figure 5b). This is in agreement with the reported results of Tischer and colleagues (Tischer *et al.*, 1991). A twofold increase in the band intensity was observed in primer extension results from mRNA of HeLa cells exposed to hypoxia probably as a result of transcriptional activation of VEGF mRNA as previously demonstrated (Levy *et al.*, 1995; Cohen *et al.*, 1996). The second antisense primer (-165) revealed a major start site at position -405. The sequence surrounding this transcription start site is similar to the sequence on the transcription start site around position -1038. Exposure of cells to hypoxia did not result in a significant change in the intensity of the extended band (Figure 5b). This implies that the newly identified transcription start site at position -405 is not modulated by hypoxia. The primer extension data fits with the Northern blot analysis as shown in Figure 4.

The primer extension results suggest that the upstream region to the newly identified transcription start site contains promoter sequences. Therefore, this

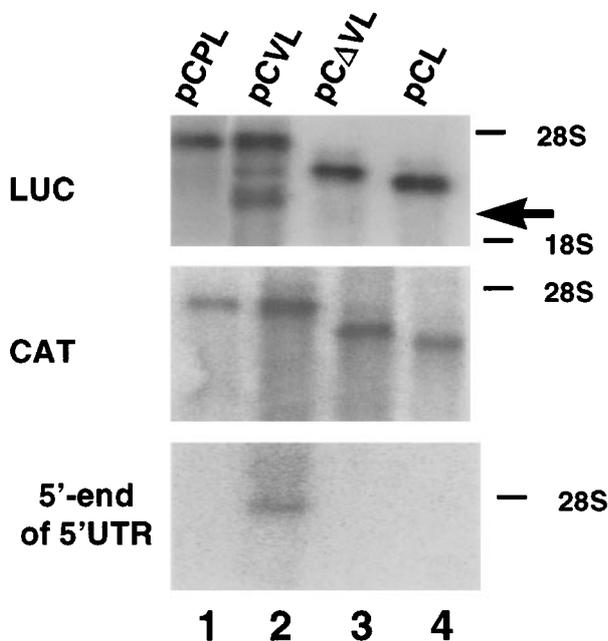


Figure 4 Northern blot analysis of cells transfected with the various bi-cistronic plasmids. HeLa cells were transfected with 15 μ g of either pCPL, pCVL, pCAVL and pCL followed by mRNA extraction and Northern blot analysis as described under Materials and methods. The blot was sequentially probed with 32 P-labeled DNA fragments corresponding to LUC, CAT and the 5'-end of the 5' UTR of VEGF as indicated. The membrane was either exposed to X-ray film or to a PhosphoImager. The 28S and 18S rRNA bands are marked and the arrow indicates the location of the fast migrating band

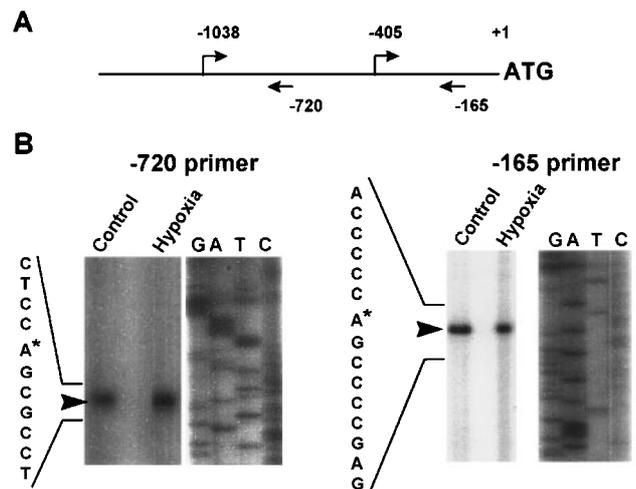


Figure 5 Mapping of the alternative transcription start site for VEGF. (a) A scheme of the 5' UTR of VEGF. The primers employed in the extension reactions are indicated by arrows and identified transcription start sites are marked by bent arrows. Numbers represent the position relative to the translation start site (ATG). (b) HeLa cells were subjected to normoxia (control) or 16 h of hypoxia. The extracted mRNA was used for primer extension assays using either primer -720 or primer -165 as detailed under Materials and methods. Genomic sequencing was performed with the same primers and separated alongside on a 6% sequencing gel and the dried gel was exposed to a X-ray film. The major extended bands are indicated by arrowheads, the sequence around it is presented and the exact transcription start site is marked by an asterisk

segment was cloned in both possible orientations upstream to the LUC reporter gene in a promoterless plasmid. Initial analysis using these constructs, clearly demonstrate that the 650 bp segment was able to promote LUC activity only in the correct orientation suggesting that this segment functions in an orientation dependent manner as expected of a promoter region segment (data not shown).

Discussion

We demonstrated in a previous publication (Cohen *et al.*, 1996) that the 5' UTR of VEGF potentiates expression by enhancing either transcription or translation. In this study, we show that this 5' UTR possesses two activities: (i) classical IRES activity that is found at its 3' end; (ii) a putative promoter activity which is located upstream to the IRES structure that drives an alternative transcription start site.

The existence of six cellular IRES structures has been reported among which are FGF2 and PDGF2/*c-sis* growth factors (Macejak and Sarnow, 1991; Oh *et al.*, 1992; Teerink *et al.*, 1995; Gan and Rhoads, 1996; Vagner *et al.*, 1995; Bernstein *et al.*, 1997). PDGF2/*c-sis* is considered a member of the VEGF gene family due to conserved protein structure motifs and interaction with the same family of receptors (Neufeld *et al.*, 1994). In addition, both factors, PDGF2/*c-sis* and VEGF, are characterized by the presence of a very long 5' UTR that affect their expression (Rao *et al.*, 1988; Tischer *et al.*, 1991). Here we show that like PDGF2/*c-sis* (Bernstein *et al.*, 1997), VEGF-5' UTR also contains an IRES element. However, the IRES activity of PDGF2/*c-sis* is linked to cellular differentiation, i.e. it is highly sensitive to cellular conditions and therefore not detectable in the present study. In contrast, the IRES element of VEGF was found active in all cell lines tested which possess angiogenic activity as reflected by constitutive expression of different levels of VEGF mRNA that is common in tumor cells and immortal cell lines.

A common RNA structural motif for the IRES elements of the cellular genes BiP, FGF2 and PDGF2 was recently reported (Le and Maizel, 1997). The main characteristics are: the existence of a Y-shape stem-loop structure with an additional stem-loop at the 3' end close to the initiator AUG codon; the existence of upstream Open Reading Frames (ORFs); a stretch of homology to human 18S rRNA that is located near the 3' stem-loop structure; and a high GC content. These characteristics of cellular IRES structures, which are similar to the core structure of the IRES elements of picornavirus (Le *et al.*, 1996), exist also in the IRES of VEGF (Figure 2b). The 5' UTR of human VEGF contains an in frame ORF which encodes for an additional 154 amino acids upstream of the reported translational initiation site (Tischer *et al.*, 1991). This ORF is initiated by a CUG codon and is followed by three additional in frame CUG codons and one out of frame AUG codon. Accordingly, the results of the present study suggest that such a deletion of 83 bp from the 3' end of VEGF-5' UTR may lead to the destruction of the Y-shape stem-loop structure of the computer predicted IRES element and consequently to a decrease in internal translation. Similar results were

observed when the same 83 bp were removed from the 5' UTR when cloned under the TK basal promoter (Figure 1). The importance of the IRES structure for VEGF expression is reflected by its phylogenetic conservation (Figure 2c).

DNA binding sites to AP1, AP2, SP1, IL-6 response element, and positive regulatory domain I exist not only in the promoter region but also in the 5' UTR (Tischer *et al.*, 1991; Cohen *et al.*, 1996). All these DNA binding motifs are present in the 650 bp segment upstream to the putative IRES element suggesting their involvement in transcriptional regulation. Our previous study (Cohen *et al.*, 1996) clearly demonstrated that DNA elements in the 5' UTR cooperate synergistically with DNA elements located upstream to the transcription initiation site. The results of this study imply that this cooperative effect was probably due to an alternative initiation site (as demonstrated by the primer extension analysis) that enabled part of the 5' UTR to act as promoter. This assumption is further supported by our initial analysis of the ability of this 650 bp fragment to drive the expression of the LUC gene in an orientation dependent manner. Further analysis of this region is being performed to characterize this putative alternative promoter site. The two transcription start sites for VEGF are about 650 bp apart. In accordance, different cell types contain various lengths of VEGF transcripts. Human vascular smooth muscle cells contain VEGF transcripts of 5.5, 4.4 and 3.7 kb mRNA (Tischer *et al.*, 1991) and bovine pituitary folliculo stellate contain 4.2, 3.7 and 3.4 kb transcripts (Tischer *et al.*, 1989). In hypoxia induced HeLa cells, three mRNA species with observed sizes of 4.4, 3.7, and 2.0 kb are induced (Akiri *et al.*, unpublished data). The difference in mRNA sizes may be attributed in part to the observed alternative initiation site. It is possible that an alternative initiation site for VEGF was not detected previously since the identification of the transcription start site was performed in U937 cells that exhibited only one prominent message of 3.8 kb (Tischer *et al.*, 1991). In addition, the primer extension analysis was performed with primers corresponding only to the 5' end of the 5' UTR (Tischer *et al.*, 1991). Current research using primer extension analysis, as described here, is targeted to characterize alternative promoter usage in cell lines representing various differentiation states and tissues.

Regulation of expression due to alternative promoter usage, i.e. alternative transcription start, was demonstrated for various growth factors and regulatory genes. This regulatory mechanism was implicated to be essential for tissue specific expression as well as stimulatory specific expression (Furbass *et al.*, 1997; Ghazi *et al.*, 1996; Adamo *et al.*, 1991). The presence of alternative transcription initiation start sites was also reported for the closely related factor PDGF2 (Fen and Daniel, 1991; Dirks *et al.*, 1995). The most prominent alternative transcription start site extends only 18 nucleotides upstream of the translation start site thus enabling escape from both the translational inhibition mediated by the 5' UTR and the translational modulation mediated by the D-IRES within the 5' UTR. Similarly, this study points to the existence of an alternative transcription initiation site that might have a role in VEGF expression. Since the alternative initiation start site is 650 nucleotides downstream from

the major initiation site, it will generate a transcript that contains only the IRES 'consensus' features, subjected mainly to translation through a ribosome internal initiation mechanism. Therefore, it seems that the overall combined consequence of the transcription/translation control mechanisms finely modulate the expression of VEGF. Strong IRES activity might be advantageous in cases when cap-dependent translation is inhibited such as under stress and during mitosis. VEGF is considered to be a stress induced protein in cases such as hypoxia and hypoglycemia (Shweiki *et al.*, 1995). Under stress the level of active translation initiation factor eIF-4E, which is considered to be the factor limiting recruitment of ribosomes, is further reduced (Sonenberg, 1996; Vagner *et al.*, 1995). It is expected that under such circumstances internal initiation of translation is advantageous and essential. Our results demonstrate that VEGF-IRES activity is not further enhanced during hypoxia. However, severe inhibition of protein synthesis occurs under extreme hypoxia (Kraggerud *et al.*, 1995). Unlike many proteins, induction of VEGF under this condition is well documented. Therefore, this unique induction of VEGF may be attributed to internal initiation of translation which is cap-independent.

Materials and methods

Cell culture

HeLa and N-tera cells were obtained from ATCC (Rockville, Maryland) and L8 cells, were kindly obtained from Dr E Keshet (The Hebrew University, Israel). The cell lines were all grown in Dulbecco's Modified Eagle Medium supplemented with gentamicin, glutamine, and 10% fetal calf serum (FCS).

Plasmids

To generate the plasmids in which the whole 5' UTR of VEGF or segments of it are cloned in between a TK promoter and the CAT gene, the plasmids p1.8CAT, p1.8CAT Δ NrX and p1.8CAT Δ NheCAT (Cohen *et al.*, 1996) were completely digested with *Hind*III and partially digested with *Nhe*I. This led to the removal of the promoter region which was replaced with TK basal promoter. This promoter segment was PCR amplified from the plasmid pBLCAT5 (Boshart *et al.*, 1992) with corresponding primers to which compatible restriction sites were added. The resulting plasmids (Figure 1) were designated pTKUTRCAT, pTK Δ 83CAT, and pTK Δ 695CAT respectively. To generate a similar plasmid in which the 5' UTR of VEGF was replaced with a non relevant DNA segment, a 1000 bp *Xho*I–*Sal*I fragment from the Baculovirus expression plasmid pAcYMI (Matsuura *et al.*, 1987) was used to replace the 5' UTR (pTK1000CAT illustrated in Figure 1).

In the first step towards the construction of bi-cistronic vectors containing the 5' UTR of VEGF or segments of it in between the CAT and the LUC genes, the 5' UTR of VEGF was amplified by PCR with 5' primer (5' UTRVEGF AGAACTAGTCGCGGAGGCTTGGG) containing a *Spe*I restriction site and 3' primer (3' UTRVEGF CTTGC-CATGGTTTCGGAGGCCCG) that starts with an *Nco*I site overlapping the first ATG codon of VEGF and additional 14 bp upstream. This segment was cloned into the plasmid pBluescript SK II (Stratagene) containing the LUC gene with *Nco*I site around its first ATG codon and an upstream *Spe*I site. The resulting plasmid pSKVLUC, in

which the 5' UTR of VEGF was fused to the first ATG of the LUC gene as in the case of the native VEGF, was digested with *Spe*I, which is located at the 5' end of the UTR, and *Eco*RV, which digest in the coding region of the LUC gene. In the second step, the UTR-LUC fragment was isolated and used to replace the 5' UTR of PDGF in the bi-cistronic plasmid pCPL (Bernstein *et al.*, 1997). The isolated UTR-LUC fragment was cloned into the bi-cistronic plasmid pCPL digested with the same restriction enzymes to release the PDGF UTR (Figure 3) which was swapped with the 5' UTR of VEGF. The resulting plasmid, pCVL, (Figure 3) contains the whole 5' UTR of VEGF downstream to the CAT gene fused to the ATG of the LUC gene. Similarly, to insert a hairpin structure in front of the CAT gene in the plasmid pCVL, the same *Spe*I–*Eco*RV segment replaced the corresponding PDGF segment in the plasmid pHCPL (Bernstein *et al.*, 1997) generating the plasmid pHCVL illustrated in Figure 3. To generate a 695 bp deletion in the 5' UTR of VEGF, the plasmids pCVL and pHCVL were digested with *Nhe*I and subjected to self ligation so that the *Nhe*I 695 bp segment was missing resulting with the plasmids pC Δ VL and pHC Δ VL respectively. To obtain the construct pC Δ 83VL in which 83 bp from the 3' end of the 5' UTR from the plasmid pC Δ VL were removed, the plasmid was digested with *Nar*I and *Nco*I, and subjected to self ligation following Klenow fill-in reaction. The Encephalomyocarditis Virus (EMCV)-IRES element was PCR amplified using a 5' primer containing a *Spe*I restriction site (5' EMCV-IRES AGAACTAGTGCCTCTCCCTCCCC) and a 3' primer containing a *Nco*I site (3' EMCV-IRES GCCGAATTCC-CATGGTATTATC) and cloned into pCPL to generate the bi-cistronic plasmid pCEL, as described above for the 5' UTR of VEGF.

DNA transfections and reporter genes analysis

HeLa cells were transfected by the calcium phosphate–DNA coprecipitation method as described previously (Chen and Okayama, 1987). Cells were transfected with 5 μ g of the various plasmids DNA, 3 μ g of pRSV β GAL, and 7 μ g of pUC19 serving as carrier DNA and the cells were harvested 48 h later using the lysis buffer of the Luciferase assay kit (Promega). CAT assays were performed as described (Weisz *et al.*, 1992) and LUC assays were performed according to the manufacturer's instructions using TD-20/20 luminometer (Turner Design, Promega). Reporter genes activities were normalized for protein concentration and transfection efficiencies as described (Weisz *et al.*, 1992). Each set of transfection experiments was repeated at least three times generating similar results.

Northern blot analysis

2×10^6 cells/plate (57 cm²) were transfected as described above with 15 μ g of each plasmid without carrier DNA. Forty-eight hours later, the cells were harvested and total RNA was extracted using Tri-Reagent (MRC, Cincinnati, OH) according to the manufacturer's instructions. 500 μ g of total RNA was digested with 50 units of DNaseI RNase free (Boehringer Mannheim) and following phenol/chloroform extraction and ethanol precipitation, mRNA was isolated using mRNA isolation kit (Boehringer Mannheim). 2 μ g of purified mRNA samples were loaded on 1.2% agarose gel and Northern blot analyses were carried out as described (Weisz *et al.*, 1992). DNA segments corresponding to the whole coding regions of CAT and LUC genes were generated by PCR with the appropriate primers and were used as probes. In addition, a 300 bp *Spe*I–*Sac*I DNA fragment corresponding to the 5' end and a 300 bp *Nco*I–*Nhe*I fragment corresponding to the 3' end of the 5' UTR of VEGF were excised from the plasmid

pCVL and were also used as probes. The blots were either exposed to X-ray films that were scanned and analysed by Scanalytics densitometry system using RFLPscan computer analysis program (Billerica, MA) or analysed by a Fuji PhosphorImager.

Primer extension analysis

Two synthetic oligonucleotides, -720 and -165, corresponding to their positions on the 5' UTR of VEGF were labeled with γ -³²P-ATP using polynucleotide kinase (Promega): (i) -720 to -750 (5'-CCTCTT-TCTGCTGTTTCCAAAATCCACAG-3') identical to primer 5117 as in (Tischer *et al.*, 1991), and (ii) -165 to -182 (5'-ACACCGCCGCCTCACCCG-3'). Approximately 5×10^5 c.p.m. of each oligonucleotide were annealed with 2 μ g of HeLa cells extracted mRNA in a 30 μ l volume containing 100 mM KCl and 50 mM Tris-HCl pH=8.3 for 5 min at 95°C and cooled slowly to room temperature over 2 h. The hybridized primer-RNA

complex was extended in a 50 μ l volume containing 50 mM Tris-HCl pH=8.3, 50 mM KCl, 10 mM MgCl₂, 0.5 mM Spermidine, 10 mM DTT, 1 mM dNTPs, 40 U of RNase inhibitor (RNA Guard, Pharmacia) and 15 U of AMV reverse transcriptase (Promega) for 60 min at 50°C followed by, 30 min at 55°C, and 30 min at 60°C. Following ethanol precipitation the samples were separated on a sequencing gel (6% polyacrylamide, 8 M urea) alongside with dideoxy sequencing reactions, size markers, and the dried gel was exposed to a X-ray film. The dideoxy sequence reactions were generated with the same primer as was used for the primer extension with VEGF genomic subclone (the plasmid p1.8CAT, Cohen *et al.*, 1996) containing the 5' UTR of the VEGF gene.

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