Activator-protein-1 binding potentiates the hypoxia-inducible factor-1-mediated hypoxia-induced transcriptional activation of vascular-endothelial growth factor expression in C6 glioma cells

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The endothelial cell-specific mitogen vascular-endothelial growth factor (VEGF) plays a key role in both physiological and pathological angiogenesis. The up-regulation of VEGF expression in response to reduced oxygen tension occurs through transcriptional and post-transcriptional mechanisms. To investigate the molecular mechanisms of transcriptional activation by hypoxia (1 % oxygen), fine mapping of a hypoxia-responsive region of the human VEGF promoter was carried out using luciferase reporter-gene constructs in C6 glioma cells. Here, we report that the binding site of hypoxia-inducible factor 1 (HIF1) is crucial for the hypoxic induction of VEGF gene expression. However, an enhancer subfragment containing the HIF1 binding site was not sufficient to confer full hypoxia responsiveness. Addition of upstream sequences restored the full sensitivity to hypoxia induction. This potentiating effect is due to activator protein 1 binding. The 'potentiating' sequences are unable to confer hypoxia responsiveness on their own. Our results strongly suggest that in C6 glioma cells a complex array of *trans*-acting factors facilitates full transcriptional induction of VEGF gene expression by hypoxia.

INTRODUCTION

Cells in tissues are faced with low oxygen tension under certain physiological (embryogenesis), as well as pathological (tumour growth, wound healing), conditions. The compensatory mechanisms that enable these cells to survive hypoxic conditions involve activation and repression of certain genes (for review see ref. [1]). In order to restore an adequate oxygen supply, either improved oxygen transport or new vessel formation is required. Two key factors involved in these processes are erythropoietin (EPO) and vascular-endothelial growth factor (VEGF). Experimental data have provided evidence that the genes for EPO and VEGF are regulated by low oxygen tension, using similar sensing and control mechanisms [2]. In addition to transcriptional activation, mRNA stabilization is involved in hypoxic regulation of both EPO and VEGF [3–8]. The characterization of the 3′-hypoxia enhancer of the EPO gene led to the discovery of a hypoxia-inducible protein complex, termed hypoxia-inducible factor 1 (HIF1) [9]. This transcription factor consists of HIF1α and HIF1β [arylhydrocarbon receptor (AhR) nuclear translocator; ARNT], both members of the bHLH-PAS-domain family (bHLH = basic helix-loop-helix; PAS = period-ARNT-singleminded) [10]. Similarities in the hypoxic induction of EPO and VEGF suggested that VEGF expression induced by low oxygen tension is subject to regulation by the same transcription factor. Meanwhile, HIF1 binding and transactivation was reported for the human and rat VEGF genes [5,11,12]. Results from reporter gene studies employing the rat VEGF promoter led to the conclusion that, in addition to the HIF1 binding site, other *cis*-acting elements may be involved in transcriptional control of VEGF gene expression by hypoxia [5]. Forsythe et al. have reported a residual level of hypoxic induction for human VEGF (hVEGF)–luciferase constructs lacking the HIF1 binding site [12].

In our previous study [7] we attributed the hypoxia responsiveness of the hVEGF promoter in C6 glioma cells to a 288-bp *Sac*I–*Ban*I fragment, located 1176–888 bp upstream of the transcription initiation site. In the process of fine mapping of this region it became clear that a *Pvu*II–*Ban*I subfragment, including the HIF1 binding site, is not sufficient to confer full hypoxia inducibility to the reporter gene. This finding led us to further examine the *cis*-acting elements, which may co-operate with the HIF1 binding site in hypoxic induction. The data presented here indicate that sequences upstream of the HIF1 consensus site potentiate the hypoxic induction mediated by HIF1. By employing site-directed mutagenesis, we could show that this potentiating effect is due to an activator protein 1 (AP1)-binding site similar to that found in the promoter of the anoxia-inducible VL30 retrotransposon, which has been described recently [13]. AP1 could be demonstrated to interact with this *cis*-element under both normoxic and hypoxic conditions. These results suggest that a complex cell-type-specific regulation of VEGF gene expression occurs under hypoxic conditions.

EXPERIMENTAL

Cell culture and transfections

C6 glioma cells (American Type Culture Collection CCL 107) were propagated in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal-calf serum (PAN-Systems). Trans-

Abbreviations used: VEGF, vascular-endothelial growth factor; hVEGF, human VEGF; EPO, erythropoietin; HIF, hypoxia-inducible factor; AP1, activator protein 1; ATF, activating transcription factor; bHLH, basic helix-loop-helix; PAS, period-ARNT-singleminded; AhR, arylhydrocarbon receptor; ARNT, AhR nuclear translocator; SV40, simian virus 40; XRE, xenobiotic response element.

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fections, hypoxic incubations and analysis of cell lysates were carried out as described previously [7]. Statistical calculations were carried out with the InStat 2.01 program.

Plasmid construction

All deletion constructs and constructs carrying mutations were derived from the hVEGF *Kpn*I–*Nhe*I–luciferase fusion construct described previously [7]. 5′-Deletions to −1176, −1015, −973 and −888 were obtained by restriction-enzyme digestion with *Sac*I, *Pvu*II, *Bsa*AI and *Ban*I respectively. Mutagenesis *in vitro*, for generation of internal mutations and deletions, was carried out using the M13 *In vitro* Mutagenesis Kit (Bio-Rad) according to the manufacturer's instructions. To test the hypoxia induction of the subfragments −1176/−888, −1168/−1015 and −1015/−888, these were subcloned in front of the minimal 40 simian virus (SV40) promoter of the pGL2 promoter vector (Promega).

Electrophoretic mobility shift assay

Nuclear extracts of normoxic and hypoxic (18 h, 1 % oxygen) C6 cells were prepared according to Semenza and Wang [9]. Annealing, purification and labelling of the oligonucleotides was performed as described previously [14]. Incubation of the double-stranded oligonucleotides hVEGF 5′-TGGCGGGTAGGTTTGAATCATCACGCAGGC-3′, hVEGF DEL 5′-TGGCGGGTAGGTCACGCAGGC-3′, AP1M1 5′-TGGCGGGTAGGTTAGAATCATCA CGCAGGC-3′ or AP1M2 5′-TGGCGGGTAGGTTTGGTTCATCACGCAGGC-3′, with 4 μg of nuclear extracts, was performed in the presence of 10 mM Hepes (pH 7.9)/200 mM NaCl/4 % (w/v) Ficoll/4 mM dithiothreitol/1 mM EDTA/BSA (0.1 mg/ml)/1 μg of poly(dI-dC) for 20 min at room temperature. The unlabelled competitor (comp) oligonucleotides AP1 comp 5′-CGCTTGATGACTCAGCCGGAA-3′, activating transcription factor (ATF) comp 5′-AGAGATTGCCTGACGTCAGAGAGCTAG-3′ or VL30 5′-TTTGAATGAGCCAATTGTA-3′ [13] were added at a 50-fold molar excess 10 min before the addition of the labelled probe. For supershift analysis 1 μg of the respective antibodies was added to the completed reaction mixture and incubation was performed for 2 h at 4 °C. Antibodies directed against Jun-family members (D), Fos-family members (K25), ATF-4 (Z5) and ATF-1 (25C10G) were obtained from Santa Cruz Biotechnology.

RESULTS

Deletional analysis of the hVEGF 5′-hypoxia enhancer

As published previously [7], the *cis*-acting elements responsible for hypoxic activation of VEGF gene transcription in C6 glioma cells reside between bp −1176 and −888 in the hVEGF 5′-flanking region. Meanwhile, the binding site for the HIF1 included in this fragment was reported to confer hypoxia activation to the hVEGF gene in endothelial [11] and Hep3B [12] cells and to the rat VEGF gene in PC12 cells [5].

To test the hypothesis that sequences other than the HIF1 consensus binding site contribute to the hypoxic response in C6 cells, further deletional analysis of sequences between −1176 and −888 was performed. Compared with the hypoxic response of the −1176 construct, hypoxia induction was significantly decreased ($P < 0.001$) by shortening to bp −1015 (*Pvu*II) (Table 1), although the HIF1 binding consensus remained intact. Induction of the reporter gene expression was completely abolished when the HIF1 site was destroyed (−973; *Bsa*AI) (Table 1).

Table 1 Potentiating sequences for hypoxia induction of the hVEGF gene are present between bp −1176 and −1015

Deletion analysis of the hVEGF promoter was performed and the constructs were tested in transient-transfection assays in C6 glioma cells. Transfection and hypoxic incubations were carried out as described in the Experimental section. The position of the hVEGF promoter fragments relative to the transcription initiation site is indicated in the left-hand column. The -fold induction by hypoxia summarized in the right-hand column represents the ratio of reporter gene activity obtained under hypoxic versus normoxic conditions. Mean values ± S.D. of at least three independent transfections are given.

Construct	Induction by hypoxia (fold)
−1176	2.7 ± 0.5
−1015	1.85 ± 0.5
−973	0.9 ± 0.05
−888	0.98 ± 0.04
−1176 ΔHIF	1.1 ± 0.2

Activation of a heterologous promoter

To test whether the sequences referred to above are able to activate a heterologous promoter in response to hypoxia, enhancer fragments comprising bp −1168 to −1015, −1015 to −888 and −1176 to −888 were subcloned and tested in front of the (non-hypoxia-responsive) SV40 promoter. In agreement with the results obtained with the hVEGF promoter the −1015/−888 fragment containing the HIF1 binding site showed a significantly ($P < 0.05$) diminished hypoxic response compared with that of the −1176/−888 fragment. Surprisingly, the fragment potentiating the HIF1-mediated effect (−1168/−1015) was not able to confer hypoxia responsiveness on the SV40 promoter on its own (Table 2).

Mutational analysis of conserved sequences

To further investigate the role of upstream sequences in hypoxia induction, the HIF1 binding site was deleted in the context of the fully responsive −1176 construct by site-directed mutagenesis. This internal deletion completely abolished hypoxia induction (Table 1, −1176 ΔHIF), thus further supporting the finding that upstream sequences by themselves are unable to confer hypoxia responsiveness. A search of the database revealed a CACACAG sequence stretch (bp −921 to −912) with high similarity to an element in the human EPO 3′-enhancer. Neither replacement

Table 2 Potentiating sequences are not able to confer hypoxia responsiveness to a heterologous promoter

Transient-transfection analysis of hVEGF enhancer fragments in combination with the SV40 promoter. Transfection and hypoxic incubations were carried out as described in the Experimental section. The position of the hVEGF promoter fragments relative to the transcription initiation site is indicated in the left-hand column. The -fold induction by hypoxia summarized in the right-hand column represents the ratio of reporter gene activity obtained under hypoxic versus normoxic conditions. Mean values ± S.D. of at least three independent transfections are given.

Construct	Induction by hypoxia (fold)
−1176/−888	2.8 ± 0.6
−1015/−888	1.7 ± 0.3
−1168/−1015	1.2 ± 0.3
SV40	1.0 ± 0.2

Table 3 Potentiating effect of sequences between −1176 and −1015 on hypoxia induction is due to an AP1 consensus binding site

Site-directed mutagenesis was performed on a luciferase fusion construct containing −1176 bp of the hVEGF 5′-flanking sequence to create internal deletions and point mutations of putative binding sites for the AhR complex (XRE) or AP1-transcription factors. The resulting expression vectors were tested in transient-transfection assays in C6 glioma cells. Levels of induction by hypoxia are given relative to that obtained for the wild-type (−1176) construct (a). A sequence comparison for both the XRE and the AP1 binding sites, as well as the point mutations introduced, are shown below (b). The AP1 binding concensus is underlined. Transfection and hypoxic incubations were carried out as described in the Experimental section. Mean values ± S.E.M. of at least three independent transfections are given.

(a)

Construct	Wild-type induction (%)
−1176	100 ± 4.5
ΔXRE	106.9 ± 17.9
ΔAP1	81.6 ± 1.0
AP1M1	79.5 ± 1.8
AP1M2	74.6 ± 5.4
−1015	68.7 ± 10.3

(b)

Binding site	Oligonucleotide	Sequence
XRE	hVEGF	TCACGC
	Human EPO	TCACGC
	Mouse EPO	TCACGC
AP1	hVEGF	TTTGAATCA
	Mouse VEGF	TTTGAATCA
	VL30	TTTGAATCA
	AP1M1	TTaGAATCA
	AP1M2	TTTGgtTCA

with the mouse EPO sequence nor internal deletion of the motif had any effect on the hypoxia induction of the hVEGF–luciferase fusion construct (results not shown).

To address the question of which *cis*-acting element(s) are involved in the potentiation of HIF1-mediated hypoxic induction, internal deletions and point mutations were introduced between bp −1176 and −1015. A putative xenobiotic response element (XRE; −1122/−1116), also present in the human and mouse EPO promoters, was removed without any effect on hypoxia induction (Table 3, ΔXRE). Comparison of analogous sequences of the human and mouse VEGF promoters revealed several conserved stretches of nucleotides. One of these (−1131/−1123) revealed an 8-out-of-9 bp similarity to one of the recently postulated basic leucine zipper/ATF binding sites in the promoter of the anoxia-inducible VL30 retrotransposon [13]. Deletion of this element in the −1176 construct (Table 3, ΔAP1) significantly diminished ($P = 0.001$) the hypoxia induction to the level observed with the −1015 deletion mutant (Table 3). The same observation was made using point mutations of the consensus site (Table 3, AP1M1 and AP1M2) described previously to prevent AP1 binding [15]. These results suggest that the AP1 consensus binding site is indeed the element conferring a 'potentiating' effect.

Transcription factor binding to the 'potentiating' element

To determine which transcription factor(s) mediate the 'potentiating' effect through binding to the *cis*-element identified, electrophoretic mobility shift assays were performed. Incubation of a double-stranded oligonucleotide spanning the putative AP1 binding site with nuclear extracts prepared from normoxic or

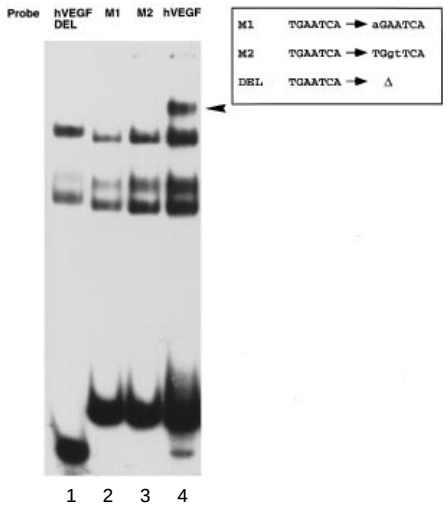

Figure 1 Mutations interfering with the 'potentiating' function prevent formation of a distinct DNA–protein complex

Electrophoretic mobility shift assay was performed as described in the Experimental section. Nuclear extracts were prepared from hypoxic C6 cells. The AP1 complex is indicated by an arrowhead. The point mutations introduced are shown on the right.

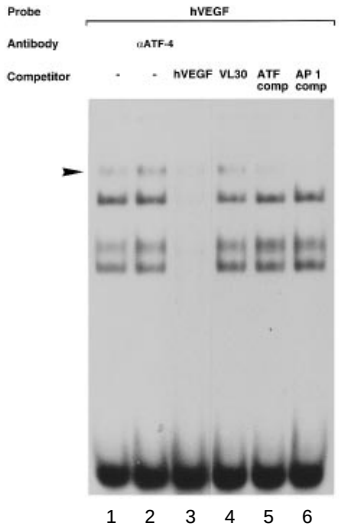

Figure 2 AP1 consensus binding site competes for DNA–protein complex formation at the 'potentiating' sequences

Competition analysis of the hVEGF oligonucleotide was performed as described in the Experimental section. Nuclear extracts were prepared from hypoxic C6 cells. The AP1 complex is indicated by an arrowhead.

hypoxic C6 cells did not reveal any differences in the binding pattern and affinity of the DNA–protein complexes (Figure 3a, lanes 1 and 2). Point mutations (Figure 1, lanes 2 and 3) or an internal deletion (Figure 1, lane 1) introduced into the oligonucleotide prevented the formation of the slowest migrating complex. In combination with the functional data this finding suggests that this complex mediates the potentiating effect. Three lines of evidence indicate that the proteins constituting the slowest-migrating complex formed with hypoxic nuclear extracts belong to the Fos and Jun families of transcription factors. (1)

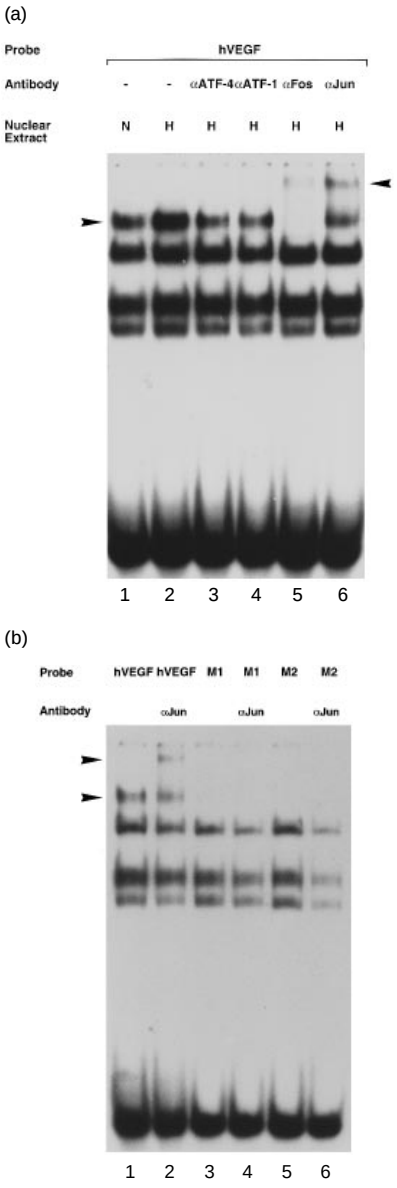

Figure 3 Protein complex at the 'potentiating' element consists of members of the Fos and Jun families of transcription factors

(**a**) Supershift analysis of the hVEGF oligonucleotide was performed as described in the Experimental section. Nuclear extracts were prepared from normoxic (N) or hypoxic (H) C6 cells. The AP1 complex and supershifts are indicated by arrowheads. Use of oligonucleotides point-mutated in the AP1 binding site (for mutations see Figure 1) demonstrates the specificity of the Jun-supershift for the slowest-migrating complex (**b**).

Specific competition for this complex was observed with a 50-fold molar excess of an unlabelled oligonucleotide containing an AP1 consensus binding site (Figure 2, lane 6) and less efficient competition using an ATF consensus site (Figure 2, lane 5; complete competition at 100-fold molar excess, results not shown). (2) No complex formation was observed with point mutations known to prevent AP1 binding [15] (Figure 1, lanes 2 and 3). (3) Supershift analysis revealed immunoreactivity for Fos- and Jun-family members in this complex (Figure 3a, lanes 5 and 6). The specificity of the partial Jun supershift for this complex was proven by using the mutated oligonucleotides in the analysis: prevention of formation of the slowest-migrating complex did equally prevent formation of the supershift. The faster-migrating complexes are unaffected (Figure 3b). Similar results were obtained by using nuclear extracts prepared from normoxic cells (results not shown). Finally, no competition was observed for the VL30 oligonucleotide known to bind ATF-4 in anoxic fibroblasts (Figure 2, lane 4) and antibodies against ATF-4 and ATF-1 did not supershift the complex (Figure 3a, lanes 3 and 4).

DISCUSSION

In a previous study we have shown that VEGF responds to reduced oxygen tension by means of both transcriptional activation and mRNA stabilization [7]. The major determinant of the transcriptional activation in response to hypoxia is the HIF1 [5,11,12], a bHLH-PAS-domain protein [10]. This finding is further supported by our data obtained from transient transfections of hVEGF promoter–luciferase fusion constructs into C6 glioma cells. Internal deletion of the HIF1 binding site in a full response vector described previously (1176 bp of 5′-flanking region [7]) completely abolished hypoxia induction.

On the basis of our earlier study we hypothesized the involvement of a CACACAGC (bp −921 to −912) sequence stretch, highly similar to the human EPO 3′-hypoxia enhancer, in hypoxia-induced up-regulation of VEGF gene expression. Replacement of this element in the human EPO enhancer by the mouse sequence completely abolished the hypoxia response [9]. Conversely, in the hVEGF 5′-flanking region, neither replacement of the motif by the mouse EPO sequence, nor internal deletion, had any effect on hypoxic induction, indicating that this sequence stretch is not involved in hypoxic up-regulation of VEGF gene expression.

In transient transfection assays in Hep3B cells, Forsythe et al. found a similar inducibility of an enhancer subfragment containing the HIF1 consensus binding site (bp −1005 to −906) compared with the full-length vector [12]. However, further dissection of the hypoxia-responsive region (−1176/−888) described for C6 glioma cells [7] led us to the unexpected finding that the subfragment (−1015/−888) containing the HIF1 binding consensus is not sufficient to confer hypoxia responsiveness comparable with that of the full-length construct in this cell line. This observation was made regardless of whether the VEGF or the SV40 promoter was used. Addition of upstream sequences between −1176 and −1015 restored full inducibility. We therefore named this region the 'potentiating' sequences. Database searches revealed a 6 bp element similar to bp −77 to −72 of the human EPO promoter (EMBL/GenBank/DDBJ accession no. M11319) in the region investigated. With the sequence TCACGC, this sequence stretch resembles a consensus XRE reported to bind the AhR/ARNT/2,3,7,8,-tetrachlorodibenzo-*p*-dioxin complex [16]. As the AhR and HIF1α share structural features, such as the PAS domains and the dimerization partner ARNT [10], it appeared possible that another PAS-domain protein complex plays an accessory role in hypoxia activation. Competition of the two PAS domain proteins, AhR and HIF1α, for ARNT recruitment has recently been reported, although activation of the AhR pathway was shown to have no influence on HIF1-mediated hypoxic induction [17]. In agreement with this finding, our transfection studies with a reporter gene construct lacking the XRE revealed no change in hypoxic induction, suggesting that the AhR complex is not involved in transcriptional activation by hypoxia.

Comparing human and mouse VEGF sequences in the 'potentiating' region revealed stretches of 10–15 bp that were highly conserved between the species, among them a potential

AP1 binding site (bp −1129/−1123). A similar site has been reported in the promoter of the anoxia-responsive VL30 retrotransposon investigated recently. The anoxia-inducible binding of ATF-4 to a neighbouring variant basic-leucine-zipper site was shown by Estes et al. [13]. Protein binding to an oligonucleotide containing both sites was competed by an AP1 consensus oligonucleotide, although an antibody directed against c-*jun* did not supershift the complex competed for by the AP1 consensus binding site [13].

Deletion of the VL30 similarity in the fully inducible −1176 hVEGF construct diminished induction levels to that obtained with the −1015 construct, indicating that this *cis*-acting element mediates the potentiation of the HIF1-binding-site-based effect. Employing point mutations shown previously to prevent AP1 binding [15] yielded the same result, indicating that altered spacing of transcription-factor binding sites is not responsible for the effect observed. Using competition and supershift analyses we could show that AP1 binds to this site independently of the oxygen partial pressure. However, with the broadly reactive antibodies used, we cannot exclude that the composition of the AP1 complex differs under normoxic and hypoxic conditions. In our assays immunoreactivity was detected for both Fos- and Jun-family members, indicating that the transcription factor is composed of a Fos–Jun heterodimer. Unexpectedly, the αJun antibody supershifted only parts of the complex. A lower affinity of the αJun antibody to the rat-Jun protein present in the complex, when compared with the human c-*jun* it was raised against, would be one explanation for this observation. A more attractive model would be the steric hindrance of antibody binding to Jun by a third protein. As the epitope recognized by the antibody (amino acids 247–263) and the binding interface for the co-activator p300 (up to amino acid 246 for maximum binding [18]) are immediately adjacent, steric hindrance by the presence of p300 appears possible. The co-activator p300 is known to provide physical links between enhancer-bound transcription factors and the RNA–polymerase II complex [19]. Recently, it has been reported that p300 is part of the hypoxia-inducible HIF1 complex [20]. To date, the partner to which p300 bridges in this complex is unknown. Jun, constitutively bound to an upstream sequence, would be an attractive partner.

Constitutive binding of the AP1 complex to the 'potentiating' *cis*-element under both normoxic and hypoxic conditions is consistent with the observation that the −1176/−1015 fragment is unable to confer hypoxia responsiveness on its own in reporter assays. Although AP1 has been shown to be hypoxia-inducible in both its DNA-binding and transactivation capacities in, for example, HeLa [21] and Hep3B [22] cells, no alterations in AP1 function could be shown in other cell lines [22]. Thus AP1-mediated modulation of gene expression in response to hypoxia may occur in a cell-type-specific manner.

Taken together, our results have shown that: (i) the binding consensus for HIF1 is crucial for hypoxia-induced transcriptional activation of the VEGF gene in C6 glioma cells. The complete loss of inducibility after internal deletion of this *cis*-element, however, is in contrast with the results published by Levy et al. [5] and Forsythe et al. [12] for rat and hVEGF genes in PC12 and Hep3B cells respectively. Using mutations or deletions of the HIF1 binding consensus, both authors found residual inducibility. (ii) Upstream sequences have a potentiating effect on hypoxia induction, which can be attributed to an AP1 binding site. This effect may be part of a cell-type-specific modulation pathway, allowing the fine-tuning of the transcriptional response to hypoxia.

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