Transactivation and Inhibitory Domains of Hypoxia-inducible Factor 1α

MODULATION OF TRANSCRIPTIONAL ACTIVITY BY OXYGEN TENSION*

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Hypoxia-inducible factor 1 (HIF-1) binds to cis-acting hypoxia-response elements within the erythropoietin, vascular endothelial growth factor, and other genes to activate transcription in hypoxic cells. HIF-1 is a basic helix-loop-helix transcription factor composed of HIF-1α and HIF-1β subunits. Here, we demonstrate that HIF-1α contains two transactivation domains located between amino acids 531 and 826. When expressed as GAL4 fusion proteins, the transcriptional activity of these domains increased in response to hypoxia. Fusion protein levels were unaffected by changes in cellular O2 tension. Two minimal transactivation domains were localized to amino acid residues 531–575 and 786–826. The transcriptional activation domains were separated by amino acid sequences that inhibited transactivation. Deletion analysis demonstrated that the gradual removal of inhibitory domain sequences (amino acids 576–785) was associated with progressively increased transcriptional activity of the fusion proteins, especially in cells cultured at 20% O2. Transcriptional activity of GAL4/HIF-1α fusion proteins was increased in cells exposed to 1% O2, cobalt chloride, or desferrioxamine, each of which also increased levels of endogenous HIF-1α protein but did not affect fusion protein levels. These results indicate that increased transcriptional activity mediated by HIF-1 in hypoxic cells results from both increased HIF-1α protein levels and increased activity of HIF-1α transactivation domains.

Human cells require O2 for essential metabolic processes, most notably oxidative phosphorylation. Hypoxia is a significant pathophysiologic component of many cardiovascular, hemotologic, and pulmonary disorders (reviewed in Ref. 1). Systemic hypoxia, e.g. due to anemia, evokes systemic responses such as increased synthesis of erythropoietin (EPO), the primary humoral regulator of erythropoiesis (reviewed in Ref. 2). Local hypoxia, e.g. due to vascular disease, evokes local responses such as increased synthesis of vascular endothelial growth factor (VEGF) which stimulates angiogenesis (reviewed in Ref. 3). Regardless of whether hypoxia is systemic or local in etiology, intracellular responses also occur such as the transition from oxidative phosphorylation to glycolysis (4).

These systemic, local, and intracellular responses to hypoxia all involve changes in gene expression that occur, at least in part, at the level of transcription and are coordinately regulated by hypoxia-inducible factor 1 (HIF-1), a basic helix-loop-helix transcription factor (5, 6). HIF-1 DNA binding activity is induced when mammalian cells are subjected to hypoxia (5, 7). Cis-acting hypoxia-response elements (HREs) have been identified in promoter or enhancer elements of human and mouse genes encoding EPO (8–10), VEGF (11–14), the glycolytic enzymes aldolase A, enolase 1, lactate dehydrogenase A, phosphoglycerate kinase 1 (15–18), and glucose transporter 1 (19), as well as inducible nitric oxide synthase (20) and heme oxygenase 1 (21) which control production of the vasoactive molecules NO and CO, respectively. All of these HREs are sequences of 50 base pairs or less that mediate transcriptional activation of reporter genes in response to hypoxia and contain functionally essential HIF-1-binding sites of the consensus sequence 5′-RCGTCG-3′ (18).

These studies suggest that HIF-1 plays a major role in mediating homeostatic responses to hypoxia at the transcriptional level. Therefore, to understand the molecular control of these fundamental physiologic processes, it will be necessary to determine the mechanisms by which HIF-1 transcriptional activity is regulated. In addition to hypoxia, HIF-1 DNA binding activity and expression of downstream target genes such as EPO and VEGF can be induced by exposing cells to cobalt chloride or iron chelators such as desferrioxamine, findings which are consistent with the involvement of a hemoprotein in hypoxia signal transduction (17, 22–25). HIF-1 DNA binding activity is regulated by dramatic changes in the steady-state protein levels of the HIF-1α and (to a lesser extent) HIF-1β subunits (6, 26).

HIF-1α is an 826-amino acid protein that is unique to HIF-1, whereas HIF-1β is identical to the aryl hydrocarbon nuclear translocator (ARNT) protein and is a common subunit for a family of heterodimeric transcription factors that are characterized by the presence of a 300-amino acid PAS domain immediately after the basic helix-loop-helix domain (6, 27). The basic helix-loop-helix and PAS domains comprise the amino-terminal half of HIF-1α and HIF-1β (ARNT) and are required for dimerization and DNA binding, whereas the carboxyl-terminial half of each protein is required for transactivation (28–30). Forced expression of HIF-1α was sufficient to activate transcription of reporter genes containing an HRE in non-hypoxic cells, indicating that HIF-1β (ARNT) was present in excess (18). Hypoxia and forced expression of HIF-1 had synergistic effects on reporter gene expression, indicating that in
addition to increasing the steady-state levels of HIF-1α protein, other hypoxia-induced events may be required for maximal transcription (11, 28). Deletion of the carboxyl-terminal half of HIF-1α resulted in a loss of hypoxia-induced transcription, suggesting an additional level at which HIF-1 activity may be regulated (28). In this paper we define two transcriptional domains in the carboxyl-terminal half of HIF-1α and demonstrate that their transcriptional activity is modulated by cellular O2 tension.

MATERIALS AND METHODS

Plasmid Construction—A series of GAL4 fusion protein expression plasmids were constructed using polymerase chain reaction and primers that contained a BamHI site and were designed to amplify fragments of HIF-1α cDNA. Polymerase chain reaction was performed with Pfu polymerase (Stratagene) and pBluescript/HIF-1α plasmid. Polymerase chain reaction products were ligated into the BamHI site of pGal0 (kindly provided by C. V. Dang, The Johns Hopkins University) to make in-frame fusions with the GAL4(1–147) DNA-binding domain (31). The reporter plasmid, GALAE1BLuc, containing five GAL4-binding sites upstream of a minimal E1b TATA sequence and the luciferase gene (32), was kindly provided by R. A. Maurer, Oregon Health Sciences University. The p1–390TAD was constructed by Pfu polymerase amplification of HIF-1α cDNA. Polymerase chain reaction products were ligated into the BamHI site of pGal0 at 20% O2 to determine the relative luciferase activity. Expression of HIF-1α residues 531–826 fused to the GAL4 DNA-binding domain, GalA(531–826), resulted in dramatically higher levels of luciferase activity both in Hep3B and 293 cells compared with Gal0 (our data). Compared with 20% O2, luciferase activity was 5- and 66-fold higher in GalA-expressing Hep3B and 293 cells, respectively, at 1% O2. To further localize the transcriptional activation domain(s), we deleted 46 and 122 amino acids from the NH2 terminus of the HIF-1α sequences to construct GalB(577–826) and GalC(653–826), respectively (Fig. 1A). Compared with GalA, transcriptional activation mediated by GalB(577–826) and GalC(653–826) was lower in Hep3B cells. In 293 cells, GalA, GalB, and GalC had similar activity. The lower activity of GalB(577–826) and GalC(653–826) compared with GalA(531–826) in Hep3B cells suggested that the deleted sequences included a transactivation domain. GalD(531–653) also activated reporter gene transcription in both Hep3B and 293 cells. The transcriptional activities of GalD(531–653) and GalD(653–826) were 4–10-fold higher in cells at 1% compared with 20% O2. Thus, even though deletion of amino acids 531–653 had no apparent effect on transactivation by GalB and GalC in 293 cells, this region functioned as an independent transactivation domain in 293 as well as Hep3B cells. From these results we conclude that there are two transactivation domains within the COOH-terminal half of HIF-1α which appear to have redundant effects in 293 cells and non-redundant effects in Hep3B cells.

To determine whether the effects on transcriptional activation were due to different expression levels of fusion proteins, we compared GAL4 fusion protein expression and reporter gene transcription in transiently-transfected COS cells. We used COS cells because all of the GAL4 fusion proteins were expressed at detectable levels in COS cells whereas many of these proteins were not expressed at detectable levels in Hep3B or 293 cells. Each GAL4 fusion protein was expressed at similar levels in cells cultured at 1 and 20% O2 (Fig. 2A and data not shown). Therefore, the much higher transactivation mediated by GalA(531–826), GalC(653–826), and GalD(531–653) in cells at 1% O2 relative to 20% O2 was not due to higher levels of
fusion protein at 1% O₂, but rather was due to higher specific transcriptional activity. When comparing the transcriptional activity of these three constructs in COS cells, GalD(531–653) had the lowest fusion protein levels but activated transcription as well as or better than GalA and GalC (in contrast to Hep3B and 293 cells). The transcriptional activity of Gal0 in hypoxic cells was 2 orders of magnitude lower than GalD despite higher protein levels (data not shown). Taken together, these results demonstrate that: (i) HIF-1α contains two separate and independent transactivation domains; and (ii) the transcriptional activity of these domains increased when cells were exposed to 1% O₂, an effect that was independent of protein expression levels.

Distinct Inhibitory and Transactivation Domains—To further analyze the hypoxia-inducible transactivation domain in GalC(653–826), an additional series of deletion constructs was generated (Fig. 1B). Compared with GalC(653–826), GalE(692–826), GalF(726–826), and GalG(757–826) exhibited a progressive increase in transcriptional activity, especially at 20% O₂. Strikingly, GalH(786–826) mediated the highest level of reporter gene transcription of all the fusion constructs. Compared with Gal0 at 20% O₂, the transcriptional activity of GalH(786–826) was 90,000-fold higher in Hep3B cells and 4,000-fold higher in 293 cells (Fig. 1B). In addition, the transcriptional activity of GalH(786–826) was not increased in hypoxic Hep3B and 293 cells, but was still hypoxia-induced in COS cells (Fig. 2B). These data indicate that multiple inhibitory sequences are present within HIF-1α amino acids 653–785 which have a negative effect on transcriptional activation especially at 20% O₂. Comparison of the results for GalC(653–826) and GalH(786–826) in the three cell lines indicate that amino acids 653–785 inhibited transactivation 3–42-fold at 1% O₂ and 88–473-fold at 20% O₂. Further deletion of HIF-1α sequences in GalI(807–826) resulted in a loss of transcriptional activity in all cell types despite continued protein expression (Figs. 1B and 2B). Taken together, these results indicate that (i) a transcriptional activation domain is located between amino acids 786 and 826; (ii) amino acids 653–785 constitute an inhibitory domain which represses transactivation mediated by amino acids 786–826 especially at 20% O₂; (iii) transcriptional activity mediated by amino acids 653–826 may therefore be derepressed in cells cultured at 1% O₂; and (iv)
transactivation mediated by the fusion proteins was not a reflection of protein expression levels, but instead was indicative of the specific transcriptional activity of each construct.

Localization of the Minimal HIF-1α Amino-terminal Transactivation Domain—To further localize the transactivation domain between amino acids 531 and 653, we deleted NH₂-terminal or COOH-terminal HIF-1α sequences from GalD(531–653) to construct in-frame GAL4 fusion proteins. Deletion of amino acids 531–576 in GalJ(577–653) abolished transactivation function (Fig. 1C). GalK(531–614) and GalL(531–575) maintained or exceeded the transcriptional activity of GalD at 1 and 20% O₂ in both Hep3B and 293 cells (Fig. 1C). Transcriptional activity of GalL, in cells exposed to 1% O₂ was induced 27-fold in 293 cells and 3-fold in Hep3B cells. In COS cells, transfection of pGalD resulted in the lowest level of fusion protein expression (Fig. 2C). Therefore, the higher degree of transactivation mediated by GalK and GalL may reflect higher levels of fusion protein expression or higher specific transcriptional activity associated with the deletion mutants. GalJ(577–653) was expressed at higher levels than GalD in cells at 1 and 20% O₂, but GalJ had no transcriptional activity (Fig. 2C). These results indicate that the NH₂-terminal transactivation domain is located between amino acids 531 and 575. In contrast to the 41-amino acid GalH(786–826) transactivation domain, which was constitutively active in Hep3B and 293 cells, activity of the 45-amino acid GalL(531–575) transactivation domain was modulated by cellular O₂ concentration in all three cell types.

The Effects of Inhibitory Sequences on the HIF-1α Amino-terminal Transactivation Domain—To test whether inhibitory sequences affect the activity of the transactivation domain present in GalL(531–575), we analyzed the transcriptional activity of fusion protein GalM(531–784) which contained both inhibitory and transactivation domains. Compared with GalL, the transcriptional activity of GalM was greatly reduced (Fig. 1D, 2D). GalN(577–784) with the deletion of both NH₂- and COOH-terminal transactivation domains had no increased transcriptional activity relative to Gal0. Immunoblot analysis demonstrated that GalA, GalL, GalM, and GalN were expressed at similar levels at both 20 and 1% O₂ (Fig. 2D). Therefore, the presence of inhibitory sequences did not decrease the expression of the fusion proteins, but rather decreased the transcriptional activity of the HIF-1α NH₂-terminal transactivation domain.

Transcriptional Activation Function of HIF-1α Is Stimulated by Other Inducers of the Hypoxia Signal-transduction Pathway—It has previously been demonstrated that EPO and VEGF mRNA, and HIF-1 DNA binding activity are induced in cells exposed to 1% O₂, CoCl₂, or desferrioxamine (7, 22–25). To determine whether CoCl₂ and desferrioxamine also modulate HIF-1α transactivation domain function, cells were cotransfected with pSVgal, GAL4E1bLuc and Gal0, GalC(653–826), or GalD(531–653). Transfected cells were cultured at 20% O₂ or exposed to 1% O₂, 75 μM CoCl₂, or 130 μM desferrioxamine. As in the case of 1% O₂ treatment with CoCl₂ or desferrioxamine resulted in much higher transcriptional activity mediated by GalC or GalD (Fig. 3A). Protein expression levels in COS cells did not correlate with transcriptional activity (Fig. 3B). These results indicate that the activity of both transcriptional domains of HIF-1α can be stimulated by exposing cells to 1% O₂, CoCl₂, or desferrioxamine, suggesting that similar hypoxia signal-transduction pathways result in the induction of HIF-1 DNA binding activity and HIF-1α transactivation domain function.

Expression of Endogenous HIF-1 and Transcriptional Activation of EPO and VEGF Reporter Genes in Response to Inducers of Hypoxia Signal Transduction—It has been well documented that HIF-1 activates human EPO and VEGF gene transcription in response to hypoxia (5, 11, 14, 28). We noted previously that the combination of forced expression of HIF-1 and exposure of cells to 1% O₂ had synergistic effects on transcriptional activation of EPO and VEGF reporters (11, 28). As
shown for Hep3B cells transfected with luciferase reporter plasmids containing HREs from the EPO (Fig. 4A) or VEGF (Fig. 4B) genes, there was a synergistic effect of forced expression of HIF-1α and exposure to CoCl2 or desferrioxamine, as previously demonstrated for 1% O2. Similar results were obtained using 293 cells (data not shown). The expression of endogenous HIF-1α and HIF-1β protein was also analyzed. HIF-1α and, to a lesser extent, HIF-1β protein levels were greatly induced in the nuclei of Hep3B and 293 cells exposed to 1% O2, CoCl2, or desferrioxamine (Fig. 4C). We have previously demonstrated that HIF-1α protein accumulates in nuclear extracts of hypoxic Hep3B cells and that HIF-1α cannot be detected in cytoplasmic extracts of hypoxic or non-hypoxic cells (6). Note that the immunoblot assays shown in Fig. 4C also demonstrate irrelevant cross-reacting bands of higher molecular weight that show no difference between the various experimental conditions and thus serve as internal controls for the specificity of the responses. The results shown in Fig. 4 thus indicate that 1% O2, CoCl2, and desferrioxamine share in common the ability to dramatically increase the steady-state level of HIF-1α protein and to increase the specific activity of the HIF-1α transactivation domains, which may account, at least in part, for the observed synergistic effects of hypoxia (or CoCl2 or desferrioxamine) and recombinant HIF-1 on reporter gene expression.

Analysis of Transactivation Domain Function in the Context of the HIF-1 Heterodimer—The use of GAL4 fusion constructs allowed us to analyze the transcriptional activity of HIF-1α sequences in a manner that was independent of the HIF-1β transactivation domain(s), and independent of the effects of hypoxia on HIF-1α protein levels. However, it was also important to demonstrate transactivation domain function in the context of the HIF-1α/HIF-1β heterodimer. We therefore co-transfected cells with a reporter plasmid containing the VEGF HRE and cytomegalovirus promoter-based expression vectors (Fig. 5A). In the presence of empty expression vector pCEP4, reporter gene expression was induced 5-fold in hypoxic compared with non-hypoxic cells (Fig. 5B), due to induction of endogenous HIF-1 activity as demonstrated by EMSA (Fig. 5C). Cells were then transfected with p1–390, which expressed the first 390 amino acids of HIF-1α. HIF-1α(1–390) was expressed at high levels at both 20% and 1% O2 (Fig. 5C) as previously reported (28). The 2-fold-increased reporter gene transcription at 20% O2 may reflect activity of the HIF-1β transactivation domain(s) as previously reported (28). In transfected cells at 1% O2, reporter transcription was repressed (Fig. 5B), indicating that overexpression of HIF-1α(1–390) had a dominant-negative effect by competing with endogenous HIF-1α for heterodimerization with HIF-1β and binding to the VEGF HRE. Amino acid sequences 786–826, representing the COOH-terminal transactivation domain, were then fused immediately downstream of amino acid 390 (Fig. 5A). Expression of HIF-1α(1–390/TAD) resulted in 4- and 12-fold increased reporter gene transcription at 20% and 1% O2, respectively (Fig. 5B). HIF-1α(1–390) and HIF-1α(1–390/TAD) were constitutively expressed at high levels at both 20% and 1% O2 (Fig. 5C, lanes 2 and 5), but reporter transcription was significantly increased only in the presence of HIF-1α(1–390/TAD) at 20% O2 (Fig. 5B, lane 2), indicating that the COOH-terminal TADs can be constitutively expressed in the absence of hypoxia, and that overexpression of HIF-1α(1–390/TAD) can titrate the HIF-1α(1–390) heterodimers available for binding to the VEGF HRE.

FIG. 3. Induction of HIF-1α transactivation domain function by CoCl2 and desferrioxamine. A, transient transfection assay. Cells were co-transfected with pSVgal control, GAL4E1blue reporter, and pGalD(531–653) or pGalC(653–826) expression plasmids. The cells were exposed to 1 or 20% O2 either untreated or in the presence of 75 μM CoCl2 or 130 μM desferrioxamine (DFX) for 24 h. Luciferase/β-galactosidase activity was normalized to values obtained from cells transfected with pGallo and incubated at 20% O2 (relative luciferase activity). B, immunoblot analysis of GAL4 fusion proteins. Aliquots (50 μg) of nuclear extracts prepared from COS cells transiently transfected with pGalD or pGalC and treated as indicated in A were analyzed by immunoblot using a polyclonal anti-GAL4 antibody. Migration of protein standards (mass in kDa) is indicated at the right. The treatments are indicated above each lane: 20% O2 (N), 1% O2 (H), 75 μM CoCl2 (C), 130 μM desferrioxamine (D), and untransfected cells (U).

FIG. 4. Effect of CoCl2 and desferrioxamine on EPO and VEGF reporter gene transcription and endogenous HIF-1 expression. A, co-transfection of EPO HRE reporter and HIF-1α expression plasmid. Hep3B cells were co-transfected with pSVgal control, 2×WT33 reporter, and pCEP4 or pCEP4/HIF-1α expression plasmid. The cells were treated and relative luciferase activity determined as in Fig. 3. B, co-transfection of VEGF HRE reporter and HIF-1α expression plasmid. Hep3B cells were co-transfected with pSVgal control, P11w reporter, and pCEP4 or pCEP4/HIF-1α expression plasmid. C, immunoblot analysis of endogenous HIF-1α and HIF-1β protein levels in Hep3B and 293 cells. Cells were exposed for 4 h to 20% O2 (N), 1% O2 (H), 75 μM CoCl2 (C), or 130 μM desferrioxamine (D). Aliquots (15 μg) of nuclear extracts were analyzed using affinity-purified anti-HIF-1α or anti-HIF-1β antibodies. Migration of protein standards (mass in kDa) is indicated at the right.
present in the COOH-terminal half of HIF-1α here that there are two independent transactivation domains. We demonstrate directly that repression of HIF-1α function in the HIF-1 heterodimer.

FIG. 5. Analysis of COOH-terminal transactivation domain function in the HIF-1 heterodimer. A, expression plasmid constructs. pCEP4, parental expression plasmid containing cytomegalovirus promoter with no insert. pCEP4/1–390, insert encoding HIF-1α amino acids 1–390. pCEP4/1–390TAD, insert encoding HIF-1α amino acids 1–390 fused to amino acids 786–826 (COOH-terminal transactivation domain present in GalH). B, co-transfection with VEGF reporter plasmid. 293 cells were co-transfected with pSVgal control, P11w reporter, and the indicated expression plasmid and exposed to 20 or 1% O2 for 24 h. Relative luciferase activity represents mean and S.E. values from three independent plates normalized to results for pCEP4 in cells at 20%. C, analysis of HIF-1 DNA binding activity in transfected cells. Nuclear extracts were prepared from cells transfected with pCEP4 (1), p1–390 (2), or p1–390TAD (3) and exposed to 20 or 1% O2 for 4 h. Aliquots (5 μg) were analyzed by EMSA using an 18-base pair oligonucleotide probe containing the HIF-1-binding site from the EPO HRE. Arrows indicate HIF-1 DNA binding activity, a constitutive DNA binding activity (C), and HIF-1 activity containing truncated (1–390 or 1–390TAD) forms of HIF-1α (HIF-1α).

We identified an inhibitory domain (ID; amino acids 576–625) and two minimal transactivation domains (TAD-N and TAD-C, respectively). The activities of these transactivation domains within the context of the intact COOH-terminal region (amino acids 531–826) were repressed in cells at 20% O2 and increased in response to hypoxia.

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Functional Analysis of the Carboxyl-terminal Half of HIF-1α—We demonstrated previously that COOH-terminal deletions of HIF-1α markedly decreased transactivation of an EPO reporter gene in hypoxic cells (28). We demonstrate directly here that there are two independent transactivation domains present in the COOH-terminal half of HIF-1α which we designate as the NH2-terminal (amino acids 531–575) and COOH-terminal (amino acids 786–826) transactivation domains (TAD-N and TAD-C, respectively). The activities of these transactivation domains within the context of the intact COOH-terminal region (amino acids 531–826) were repressed in cells at 20% O2 and increased in response to hypoxia.

We identified an inhibitory domain (ID; amino acids 576–785) between the two transactivation domains which repressed the transcriptional activity of TAD-N and TAD-C particularly in cells at 20% O2. In cells at 20% O2, the activity of TAD-N present in GalL and TAD-C present in GalH was more than 1 and 2 orders of magnitude higher, respectively, than the activity of ID/TAD-N and ID/TAD-C sequences present in GalM (Fig. 1D) and GalC (Fig. 1B). Both TAD-C and TAD-N are rich in acidic residues (17 and 27%, respectively) and hydrophobic residues (27% in each), which are present in many previously described transactivation domains (reviewed in Ref. 37). There is, however, no specific sequence similarity between these two regions.

Deletion analysis suggested that hypoxia-inducible transcriptional activity of GalC may result from derepression of TAD-C by ID in response to 1% O2. Negative regulatory domains have also been identified in c-Myb (38–40), C/EBPβ (41, 42), and heat shock factor 1 (43). In the case of C/EBPβ, interaction between the inhibitory and transactivation domains was demonstrated in a yeast two-hybrid system and repression could be eliminated either by deletion or phosphorylation of the inhibitory domain (41, 42). We were unable to demonstrate a functional interaction between the ID and TAD-C sequences of HIF-1α in the yeast two-hybrid system (data not shown). Our data indicated that transcriptional activation of HIF-1α was repressed in cells at 20% O2, and removal of ID sequences or exposure to hypoxia (1% O2) activated the TAD-C present in GalH. The regulation of HIF-1α transcriptional activity may involve changes in protein phosphorylation, conformation, and/or induction of co-activator(s) in response to hypoxia.

While this work was in progress, Li et al. (44) reported that (i) a single mouse HIF-1α transactivation domain was located within the COOH-terminal 83 amino acids; (ii) the transactivation and hypoxia-regulatory domains were not separable; and (iii) the COOH-terminal 45 amino acids (778–822) did not contain a functional transactivation domain. Our results differ from those reported in several important respects: (i) we have identified two hypoxia-inducible transactivation domains in the COOH-terminal half of human HIF-1α. (ii) Deletion analysis of HIF-1α revealed that removal of ID sequences resulted in a potent transactivation domain that was constitutively active in Hep3B and 293 cells, establishing that ID and TAD-C are distinct domains. (iii) Two minimal transactivation domains were localized to residues 531–575 and residues 786–826, and shown to be highly potent activators in Hep3B, 293, and COS cells. The amino acid sequences of TAD-N and TAD-C are identical in human and mouse HIF-1α proteins (6, 44) (Fig. 6). (iv) We demonstrated that all of the GAL4 fusion constructs were expressed as proteins of the predicted molecular weight in
HIF-1α Transactivation Domains

COs. Although some of the differences between studies may be due to the use of different cell types, it is also possible that the negative results reported elsewhere were due to lack of expression of GAL4 fusion constructs, since fusion protein expression levels were not determined (44). We have demonstrated that TAD-C (residues 786–826) functioned as a potent transcriptional activation domain in Hep3B, 293, and COS cells. When fused to the GAL4 DNA-binding domain, TAD-C also activated reporter gene transcription in Saccharomyces cerevisiae (data not shown), indicating that amino acids 786–826 of HIF-1α constitute a transactivation domain that is active in both mammalian and yeast cells. In contrast, a GAL4 fusion construct containing mouse HIF-1α amino acids 778–822, which are identical to human HIF-1α amino acids 782–826, was reported to lack transcriptional activity, but its protein expression was not verified (44).

Regulation of HIF-1α—When Hep3B cells were subjected to hypoxia, the steady-state levels of HIF-1α and HIF-1β protein and HIF-1 DNA binding activity increased dramatically (5, 6, 26). We have previously shown that forced expression of full-length HIF-1α resulted in much lower protein levels at 20% O2 than at 1% O2 (28), whereas COOH-terminal-truncated HIF-1α protein (amino acids 1–390) was expressed at equally high levels in transfected cells at 20% or 1% O2 (28), suggesting that sequences COOH-terminal to amino acid 390 were required for regulation of HIF-1α protein levels by cellular O2 tension. In contrast, we have demonstrated in this study that all the GAL4 fusion proteins containing COOH-terminal HIF-1α sequences were expressed at similar levels in cells at 20% and 1% O2 (Fig. 2), suggesting that HIF-1α sequences NH2-terminal to amino acid 531 are required for the regulation of HIF-1α protein levels by cellular O2 tension. Whether sequences COOH-terminal to amino acid 531 are also necessary, but not sufficient, remains to be established.

We demonstrated previously that forced expression of HIF-1 and exposure to 1% O2 had synergistic effects on EPO and VEGF reporter gene transcription (11, 28). In this study, we provide evidence that the synergistic effects are due at least in part to increased activity of the HIF-1α transactivation domains in response to hypoxia. HIF-1α transactivation domains were also activated by exposure of cells to CoCl2 and desferrioxamine independently of protein expression levels. In the same experiment, endogenous HIF-1α protein levels were induced in cells exposed to 1% O2, CoCl2, or desferrioxamine, similar to the effects of these agents on EPO and VEGF mRNA expression that have previously been reported (22–25). These data suggest that hypoxia signal-transduction pathways are involved in cells exposed to 1% O2, CoCl2, or desferrioxamine, independent of protein expression levels. In the inhibitory domain, ID (amino acids 576–785), and derepressed in response to hypoxia. However, we were unable to directly demonstrate repression of TAD-C by ID in trans (data not shown) and whether ID participates in the physiological regulation of TAD-C function remains to be determined. TAD-N also appears to be repressed by ID, but in addition, when isolated from ID, the activity of TAD-N is still hypoxia-inducible in all three cell types, suggesting the existence of a second mechanism by which hypoxia induces HIF-1α transcriptional activity. Several additional observations provide further evidence for considerable complexity in the regulation of HIF-1α transcriptional activity. First, deletion of ID sequences resulted in a graded, rather than single-step, increase in transcriptional activity. Second, differences were observed between cell types with respect to whether TAD-C function was hypoxia-inducible or constitutively active. These results suggest that cell types may differ with respect to the array of expressed kinases and/or co-activators that can modulate HIF-1α transcriptional activity. Nevertheless, it is clear from studies involving all three cell types that transcriptional mediated by HIF-1α is dramatically increased in response to hypoxia. The modulation of both HIF-1α protein expression and transactivation domain function may ensure that the biological activity of HIF-1α is very tightly regulated by O2 tension in mammalian cells.

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