

## Activation of Hypoxia-inducible Factor-1; Definition of Regulatory Domains within the $\alpha$ Subunit\*

(Received for publication, January 6, 1997, and in revised form, February 24, 1997)

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Hypoxia-inducible factor-1 (HIF-1), a heterodimeric DNA binding complex composed of two basic-helix-loop-helix Per-AHR-ARNT-Sim proteins (HIF-1 $\alpha$  and -1 $\beta$ ), is a key component of a widely operative transcriptional response activated by hypoxia, cobaltous ions, and iron chelation. To identify regions of HIF-1 subunits responsible for oxygen-regulated activity, we constructed chimeric genes in which portions of coding sequence from HIF-1 genes were either linked to a heterologous DNA binding domain or encoded between such a DNA binding domain and a constitutive activation domain. Sequences from HIF-1 $\alpha$  but not HIF-1 $\beta$  conferred oxygen-regulated activity. Two minimal domains within HIF-1 $\alpha$  (amino acids 549–582 and amino acids 775–826) were defined by deletional analysis, each of which could act independently to convey inducible responses. Both these regions confer transcriptional activation, and in both cases adjacent sequences appeared functionally repressive in transactivation assays. The inducible operation of the first domain, but not the second, involved major changes in the level of the activator fusion protein in transfected cells, inclusion of this sequence being associated with a marked reduction of expressed protein level in normoxic cells, which was relieved by stimulation with hypoxia, cobaltous ions, or iron chelation. These results lead us to propose a dual mechanism of activation in which the operation of an inducible activation domain is amplified by regulation of transcription factor abundance, most likely occurring through changes in protein stability.

Hypoxia-inducible factor-1, a DNA binding complex first identified as a factor critical for the inducible activity of the erythropoietin 3' enhancer (1), is now recognized to be a key component of a widely operative transcriptional control system responding to physiological levels of cellular hypoxia (2–5). Deletional and mutational analysis of *cis*-acting sequences has demonstrated functionally critical HIF-1<sup>1</sup> binding sites in

many oxygen-regulated promoters and enhancers (6–12). The importance of HIF-1 in the regulation of such genes has been confirmed by the reduction or abrogation of hypoxia-inducible expression in mutant cells (13, 14) that are unable to form a functional HIF complex (15–19). Together these studies have provided strong evidence for a critical role for HIF-1 in the regulation of genes involved in a variety of important biological processes that include glucose transport and metabolism, vascular growth, vasomotor regulation, erythropoiesis, iron metabolism, and catecholamine synthesis (reviewed in Ref. 5).

As is observed for HIF-1-responsive genes (20–22), the HIF-1 complex is inducible by particular transition elements such as cobaltous ions and by iron chelating agents such as desferrioxamine (DFO) but not by inhibitors of mitochondrial respiration such as cyanide or azide (3, 8, 23). These distinctive features have led to the proposal of a specific oxygen sensing mechanism underlying these responses, most probably involving the operation of a ferroprotein sensor (20). Recent affinity purification and molecular cloning of HIF-1 (24) has revealed that the DNA binding complex consists of a heterodimer of two basic-helix-loop-helix Per-AHR-ARNT-Sim proteins HIF-1 $\alpha$  and HIF-1 $\beta$ , a molecule that is identical to the aryl hydrocarbon receptor nuclear translocator (ARNT) (25). An important goal is now to define the regions of the HIF-1 molecules that are responsible for their regulated activity and to understand the mechanism by which the complex is induced and activated in hypoxic cells.

Since in the majority of studies, HIF-1 activation does not appear to be mediated through regulation of its mRNAs (15, 17, 26), we focused our analysis on other possible mechanisms of regulation. As with other transcription factors, studies of the regulatory mechanisms are potentially complicated by the ultimate dependence of transcriptional activation on a series of interrelated events which may include nuclear accumulation, dimerization, DNA binding, co-factor recruitment, and transactivation. For HIF-1, a further difficulty in this analysis lies in the operation of the native system in all cells so far tested (2, 3, 27, 28). For these reasons we used the construction of chimeric genes to define regions of the HIF-1 genes that could confer oxygen-regulated behavior on heterologous transcription factors. Two types of chimeric gene were produced, those in which the heterologous transcription factor encoded nuclear localization and DNA binding, but lacked intrinsic transactivation potential, and others in which an activation domain was either

\* This work was supported by the Wellcome Trust and the Medical Research Council, UK. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: HIF-1, hypoxia-inducible factor-1;

DFO, desferrioxamine; ARNT, aryl hydrocarbon receptor nuclear translocator (identical to HIF-1 $\beta$ ); AHR, aryl hydrocarbon receptor; ARNT-ta, trans-activation domain from the human aryl hydrocarbon receptor nuclear translocator (amino acids 696–789); VP16, trans-activation domain from the herpes simplex virus protein 16 (amino acids 410–490); GR, the N-terminal 500 amino acids of the human glucocorticoid receptor; Gal, the N-terminal 147 amino acids of the yeast Gal4 transcription factor; MMTV, mouse mammary tumor virus promoter; EMSA, electrophoretic mobility shift assay(s).

intrinsic to the heterologous gene or added to the chimeric gene from a second heterologous gene. This allowed for both the definition of activation domains of HIF-1 genes and analysis of regulatory domains that did not necessarily contain intrinsic transactivation potential.

Sequences from HIF-1 $\alpha$  but not HIF-1 $\beta$ /ARNT conveyed inducible activity on heterologous transcription factors, and two regions within the C-terminal portion of the HIF-1 $\alpha$  molecule were defined, each of which possessed transactivation potential and each of which could act independently to convey inducible properties. Both domains were responsive to cobaltous ions and iron chelation as well as hypoxia. The inducible activity of one regulatory domain, but not the other, appeared to be closely connected with modulation of the level of the encoded fusion protein, most probably arising from an effect on protein stability. These studies therefore define the existence of more than one regulatory domain in the HIF-1 $\alpha$  subunit and strongly suggest the operation of more than one mechanism of regulation.

#### EXPERIMENTAL PROCEDURES

**Cell Lines, Transfection, and Experimental Conditions**—HeLa and Hep3B cells were grown in minimal essential medium with Earle's salts supplemented with 10% fetal calf serum, glutamine (2 mM), penicillin (50 IU/ml), and streptomycin sulfate (50  $\mu$ g/ml). Hepa-1 (Hepa1c1c7) cells and the ARNT (HIF-1 $\beta$ )-deficient mutant derivative, c4 cells (13, 14), were grown in minimal essential medium- $\alpha$  without nucleosides, with the same supplements.

For transactivation assays cells were transfected by electroporation using a 1-millifarad capacitor array charged at 375 V. For each transfection, approximately  $10^7$  cells were resuspended in 1 ml of RPMI 1640 containing a mixture of activator plasmid (5 or 10  $\mu$ g), reporter plasmid (10 or 50  $\mu$ g), and the transfection control plasmid pSV $\beta$ Gal (40  $\mu$ g) (Promega, Madison, WI). In experiments where amplification of the activator plasmid was desired, this was achieved by additional cotransfection with 2.5  $\mu$ g of a plasmid expressing the SV40 large T antigen, pCMV-TAg (29). After discharge of the capacitor, cells were left on ice for 10 min before being resuspended in the appropriate culture medium. Aliquots of this suspension were then used for parallel incubations. Conditions used for normoxic and hypoxic incubation were 5% CO<sub>2</sub>, balance air and 1% O<sub>2</sub>, 5% CO<sub>2</sub>, balance N<sub>2</sub>, respectively. Chemicals were used at the following final concentrations: cobaltous chloride, 100  $\mu$ M; desferrioxamine mesylate, 100  $\mu$ M; potassium cyanide, 1 mM; sodium azide, 2 mM. Unless otherwise stated experimental incubations were for 16 h. All activator plasmids were tested in at least three independent transfection experiments. Results are presented either as mean  $\pm$  S.D. or as a typical result from a set of transfections performed in parallel.

**Recombinant Plasmids**—Two different chimeric activator/reporter systems were used in transactivation assays. The first system was based on pGR, a plasmid encoding the N-terminal 500 amino acids of the human glucocorticoid receptor, and the glucocorticoid responsive reporter pMMTV-luc (Fig. 1A). pGR was constructed by cloning the 1.6-kb *Kpn*I to *Eco*RI fragment from pRShGR (30) into the corresponding sites in the polylinker of pCDNA3 (Invitrogen, San Diego, CA). MMTV-luc was made by inserting a 1.4-kb *Bam*HI fragment from pMMTV-CAT (31) containing the glucocorticoid responsive mouse mammary tumor virus promoter into the *Bgl*II site of pGL2 basic (Promega, Madison, WI). Derivative plasmids based on pGR were made by inserting the following restriction fragments 3' to the glucocorticoid receptor sequence using appropriate linkers to preserve the reading frame: pGR/AHR5–805, a 2.6-kb *Nar*I-*Xba*I fragment of murine aryl hydrocarbon receptor (AHR) from pCDNA3/ $\beta$ AHR (32); pGR/ $\beta$ 1–789, a 2.6-kb *Nco*I-*Not*I fragment of human ARNT from pBM5/Neo/hARNT (14); pGR/ $\alpha$ 28–826, a 3.1-kb *Bgl*II-*Not*I fragment of human HIF-1 $\alpha$  from pBluescript/HIF-1 $\alpha$ 3.2-3T7 (24). Further glucocorticoid receptor HIF-1 $\alpha$  fusions were all derived as restriction fragments from pBluescript/HIF-1 $\alpha$ 3.2-3T7 as follows: pGR/ $\alpha$ 166–826, *Hind*III-*Not*I; pGR/ $\alpha$ 244–826, *Sall*-*Not*I; pGR/ $\alpha$ 329–826, *Eco*RI(partial)-*Not*I; pGR/ $\alpha$ 530–826, *Eco*RI-*Not*I; pGR/ $\alpha$ 652–826, *Spe*I-*Spe*I; pGR/ $\alpha$ 28–652, *Bgl*II-*Spe*I; pGR/ $\alpha$ 28–329, *Bgl*II-*Eco*RI; pGR/ $\alpha$ 28–825, *Bgl*II-*Hpa*I.

The second system was based on pCOTG (a plasmid containing an SV40 origin of replication and a cytomegalovirus-promoted truncated Gal4 gene encoding amino acids 1–147), and the Gal4-responsive luciferase reporter pUAS-tk-luc (consisting of two copies of a 17-base pair

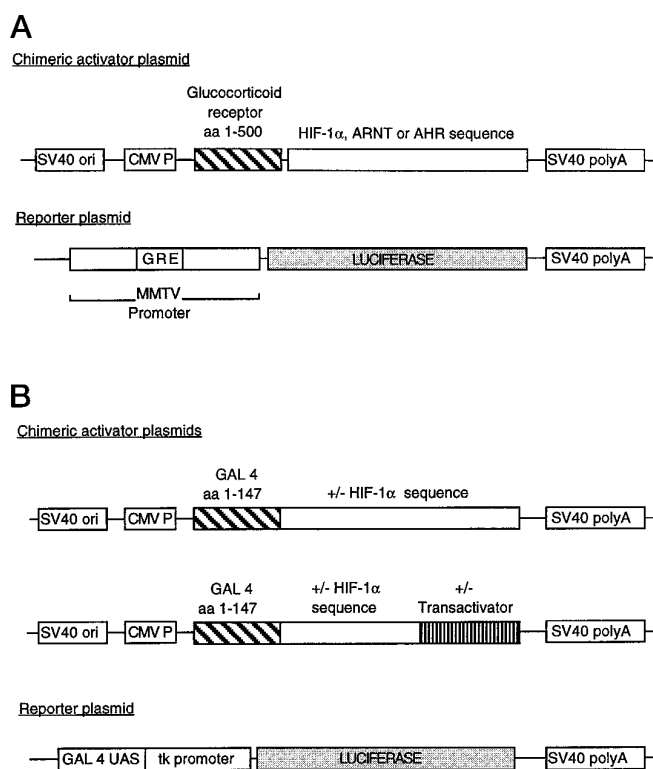


FIG. 1. Schematic representation of the different types of chimeric activator plasmid and reporter plasmid used. See "Experimental Procedures" for a full description.

Gal4 upstream activating site and the tk promoter, -105 to +50 (33), inserted into the *Hind*III site of pA3LUC (34)) (Fig. 1B). To analyze the function of sequences from the C-terminal region of HIF-1 $\alpha$ , derivative plasmids based on pCOTG were made by inserting, 3' to the Gal4 sequence, the following restriction fragments from pBluescript/HIF-1 $\alpha$ 3.2-3T7 using appropriate linkers to preserve the reading frame: pGal/ $\alpha$ 530–826, a 1.6-kb *Eco*RI-*Xba*I fragment; pGal/ $\alpha$ 652–826, a 1-kb *Spe*I-*Spe*I fragment; pGal/ $\alpha$ 530–652, a 369-base pair *Eco*RI-*Spe*I fragment.

To analyze the regulatory function of HIF-1 $\alpha$  amino acids 530–652 on the operation of heterologous activation domains, sequences coding for the human aryl hydrocarbon receptor nuclear translocator (amino acids 696–789) (ARNT-ta) and herpes simplex virus protein 16 (amino acids 410–490) (VP16) were generated by PCR using *Pfu* polymerase with priming oligonucleotides incorporating in frame *Spe*I and *Xba*I restriction sites and inserted 3' to the HIF-1 $\alpha$  sequence in pGal/ $\alpha$ 530–652 to generate pGal/ $\alpha$ 530–652/ARNT-ta and pGal/ $\alpha$ 530–652/VP16, respectively. Control plasmids pGal/ARNT-ta and pGal/VP16 were derived by deletion of the HIF-1 $\alpha$  sequence from these plasmids and insertion of appropriate linkers to preserve the reading frame. Further derivatives of pGal/ARNT-ta, containing subsequences of HIF-1 $\alpha$ , were generated by PCR amplification of pBluescript/HIF-1 $\alpha$ 3.2-3T7 using *Pfu* polymerase and priming oligonucleotides incorporating *Eco*RI and/or *Spe*I sites to permit in frame insertion into pGal/ARNT-ta. These priming oligonucleotides were designed to amplify the appropriate codons to generate pGal/ $\alpha$ 530–634/ARNT-ta, pGal/ $\alpha$ 530–611/ARNT-ta, pGal/ $\alpha$ 530–582/ARNT-ta, pGal/ $\alpha$ 549–652/ARNT-ta, pGal/ $\alpha$ 549–634/ARNT-ta, pGal/ $\alpha$ 549–611/ARNT-ta, pGal/ $\alpha$ 549–582/ARNT-ta, pGal/ $\alpha$ 572–652/ARNT-ta, and pGal/ $\alpha$ 572–634/ARNT-ta.

To generate plasmids bearing C-terminal subsequences from HIF-1 $\alpha$  similar PCR amplifications were used to create products with *Eco*RI and *Spe*I linkers suitable for in frame insertion into pCOTG to create pGal/ $\alpha$ 668–826, pGal/ $\alpha$ 708–826, pGal/ $\alpha$ 741–826, pGal/ $\alpha$ 767–826, and pGal/ $\alpha$ 775–826. The C-terminal deletions pGal/ $\alpha$ 652–794 and pGal/ $\alpha$ 652–813 were made by insertion of *Spe*I-*Pvu*II and *Spe*I-*Pst*I (after repair using Klenow) restriction fragments from pBluescript/HIF-1 $\alpha$ 3.2-3T7 into pCOTG.

Constructs bearing mutations altering individual amino acids in the HIF-1 $\alpha$  component of pGal/ $\alpha$ 530–652/ARNT-ta were generated using a commercially available site-directed mutagenesis kit (QuikChange; Stratagene, La Jolla, CA) and the following mutagenic oligonucleotides

with their complementary sequences; Y565 to F, 5'-GATGTTAGCTCCCTTTATCCCAATGGATG3'; S551, T552, T555 all to A, 5'-GAACCCATTTGCTGCTCAGGACGACAGATTAGAC3'; S577 to A, 5'-CTTCCAGTTACGTGCCTTCGATCAG3'; S581 to A, 5'-CTTCGATCAGTTGGCACCATTAGAAAG3'. S551, T552, T555, S577 all to A was created by the sequential use of the corresponding oligonucleotides. All PCR products and mutations were sequenced by the dideoxy method to confirm veracity.

**Luciferase and  $\beta$ -Galactosidase Assays**—Luciferase activities in cell lysates were determined at room temperature using a commercially available luciferase assay system (Promega, Madison, WI), according to the manufacturer's instructions and a TD-20e luminometer (Turner Designs, Sunnyvale, CA). Relative  $\beta$ -galactosidase activity in lysates was measured using *o*-nitrophenyl- $\beta$ -D-galactopyranoside (0.67 mg/ml) as substrate in a 0.1 M phosphate buffer (pH 7.0) containing 10 mM KCl, 1 mM MgSO<sub>4</sub>, and 30 mM  $\beta$ -mercaptoethanol incubated at 30 °C for 45–90 min. The A<sub>420</sub> was determined after stopping the reaction by the addition of 1 M sodium carbonate.

**Whole Cell Extracts and Western Blotting**—Cells were cooled rapidly by rinsing with ice-cold phosphate-buffered saline and harvested by scraping with a rubber policeman. The cell pellet was subjected to a single freeze-thaw cycle, and subsequent steps were performed at 4 °C. Cells were disrupted by passage 10 times through a 25-gauge needle in 2.5 volumes of extraction buffer (20 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM EGTA, 0.4 M NaCl, 20% glycerol, 0.5% Nonidet P-40, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, supplemented with leupeptin, pepstatin, and aprotinin all at 10  $\mu$ g/ml). Proteins were eluted by mixing on a shaking platform for 15 min. Supernatant was prepared by centrifugation at 13,000  $\times$  g for 10 min, mixed with an equal volume of 2  $\times$  Laemmli sample buffer, and denatured at 90 °C for 5 min prior to SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto Immobilon-P membrane (Millipore, Bedford, MA) by electrophoresis overnight at 20 V in Towbin buffer containing 10% methanol and 0.005% SDS. Membranes were blocked using phosphate-buffered saline supplemented with 5% dry milk powder and 0.1% Tween 20 prior to indirect immunostaining. Proteins were labeled with mouse monoclonal antibodies directed against either the C-terminal 19 amino acids of human ARNT (HIF-1 $\beta$ ) (2B.10) (35) or the Gal4 DNA binding domain (RK5C1; Santa Cruz Biotechnology) followed by peroxidase-conjugated swine anti-mouse immunoglobulin (DAKO). Peroxidase activity was detected by enhanced chemiluminescence (Super Signal Ultra; Pierce).

**Nuclear Extract and Electrophoretic Mobility Shift Assay**—Nuclear extract was prepared using a modification of the protocol described by Schreiber *et al.* (36). For EMSA, a Gal4 binding oligonucleotide, 5'-CCGGAGTACTGTCTCCG-3', was labeled using [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol) (Amersham, UK) and T4 polynucleotide kinase and then annealed with 4  $\times$  molar excess of the complementary strand. Binding reactions were performed in a 20- $\mu$ l volume containing (final concentration) 20 mM HEPES-KOH (pH 7.9), 2.4 mM EDTA, 0.3 mM dithiothreitol, 2 mM spermidine, 0.1  $\mu$ g/ $\mu$ l poly(dI)-poly(dC), 10 mg/ml bovine serum albumin, and 100 mM NaCl. Nuclear extract (5  $\mu$ g) was incubated with this mixture for 5 min at room temperature before probe (approximately 0.1 ng) was added. Incubation was continued for a further 30 min prior to electrophoresis (12.5 V/cm) at 4 °C using 5% polyacrylamide in 0.3  $\times$  TBE (30 mM Tris, 30 mM boric acid, 0.06 mM EDTA (pH 7.3), at 20 °C). Gels were vacuum dried prior to autoradiography.

## RESULTS

**HIF-1 $\alpha$  Sequences Confer Hypoxia-inducible Activity to a Truncated Glucocorticoid Receptor**—As a first step in studying the mechanism of oxygen-regulated transcriptional activation by HIF-1, plasmids expressing fusion proteins consisting of the N-terminal DNA binding domain (amino acids 1–500) of the human glucocorticoid receptor (pGR) linked to the complete coding sequence or near complete C-terminal sequence of HIF-1 $\alpha$  (pGR/ $\alpha$ 28-826) or HIF-1 $\beta$ /ARNT (pGR/ $\beta$ 1-789) were constructed. For comparison, a similar plasmid expressing the truncated glucocorticoid receptor fused to the related transcription factor, the mouse aryl hydrocarbon receptor (pGR/AHR5-805), was made. The plasmids were transfected with the reporter plasmid pMMTV-luc into HeLa and Hep3B cells. Results are given in Table I. The activator plasmid pGR was constitutively active in both cell types, increasing reporter gene expression 10.2- and 6.5-fold in HeLa and Hep3B cells, respectively.

TABLE I

*Determination of the hypoxia inducible activity of fusion proteins in which the basic-helix-loop-helix transcription factors, HIF-1 $\alpha$ , HIF-1 $\beta$ /ARNT, or AHR were linked to the N-terminal 500 amino acids of the glucocorticoid receptor (pGR 500)*

Activator plasmids were co-transfected with a reporter plasmid containing an MMTV-promoted luciferase reporter gene and pSV $\beta$ GAL as transfection control. Corrected luciferase activity was normalized to the activity generated by pGR 500 in the normoxic cells. Values are given as mean  $\pm$  S.D., HeLa cells, *n* = 4; Hep3B cells, *n* = 3.

| Activator plasmid        | Normoxia, 21%   | Hypoxia, 1%     | Induction, hypoxia/normoxia |
|--------------------------|-----------------|-----------------|-----------------------------|
| <b>HeLa</b>              |                 |                 |                             |
| pGR 500                  | 1.0             | 1.3 $\pm$ 0.4   | 1.3 $\pm$ 0.4               |
| pGR 500/AHR5–805         | 0.19 $\pm$ 0.03 | 0.28 $\pm$ 0.05 | 1.5 $\pm$ 0.2               |
| pGR 500/ $\alpha$ 28–826 | 0.15 $\pm$ 0.02 | 1.23 $\pm$ 0.34 | 8.2 $\pm$ 1.6               |
| pGR 500/ $\beta$ 1–789   | 17 $\pm$ 6      | 26 $\pm$ 9      | 1.5 $\pm$ 0.2               |
| <b>Hep3B</b>             |                 |                 |                             |
| pGR 500                  | 1.0             | 1.7 $\pm$ 0.2   | 1.7 $\pm$ 0.2               |
| pGR 500/AHR5–805         | 0.02 $\pm$ 0.01 | 0.03 $\pm$ 0.02 | 1.5 $\pm$ 0.2               |
| pGR 500/ $\alpha$ 28–826 | 0.15 $\pm$ 0.03 | 0.91 $\pm$ 0.20 | 6.2 $\pm$ 1.9               |
| pGR 500/ $\beta$ 1–789   | 43 $\pm$ 8      | 55 $\pm$ 14     | 1.2 $\pm$ 0.2               |

In normoxic cells the fusions with HIF-1 $\alpha$  and the AHR both reduced activity; in contrast, the fusion with HIF-1 $\beta$ /ARNT showed increased transactivation in comparison with pGR. In hypoxic cells, a 5–10-fold increase in activity was observed for pGR/ $\alpha$ 28-826, whereas only a small increase in activity was observed in hypoxic cells with pGR, pGR/ $\beta$ 1-789, or pGR/AHR5-805, which was similar to that observed after transfection of the luciferase reporter gene alone. Thus HIF-1 $\alpha$  but not HIF-1 $\beta$ /ARNT could convey oxygen-regulated expression in this assay. When tested in Hepa-1 cells and the HIF-1 $\beta$ /ARNT-deficient derivative, c4, essentially similar results were obtained (data not shown), indicating that this property of HIF-1 $\alpha$  sequences was independent of any interaction between HIF-1 $\alpha$  and HIF-1 $\beta$ /ARNT.

To determine the extent to which regulation of the truncated glucocorticoid receptor/HIF-1 $\alpha$  fusion resembled that of endogenous HIF-1, we tested the response to a number of chemical agents. HeLa cells were co-transfected with pMMTV-luc and pGR/ $\alpha$ 28–826 or pGR, split into aliquots, and exposed in parallel to hypoxia, cobaltous chloride (100  $\mu$ M), DFO (100  $\mu$ M), azide (2 mM), or cyanide (1 mM). Results are given in Table II. The response characteristics were identical to those previously reported for HIF-1. Similar responses were induced by hypoxia, cobaltous ions, and DFO but not azide or cyanide.

**Definition of Regulatory Domains in HIF-1 $\alpha$** —To define the regions of the HIF-1 $\alpha$  gene that conveyed induction by hypoxia, cobaltous ions, and DFO on the activity of the truncated glucocorticoid receptor, deletions were made which removed either the 3'-untranslated region or portions of coding sequence from HIF-1 $\alpha$ . These experiments were first performed in HeLa cells that were co-transfected with plasmids expressing the chimeric genes and pMMTV-luc. To enable comparison of the regulatory properties of the chimera with those of HIF-1, transfected cells were again exposed to cobalt and desferrioxamine as well as hypoxia. Deletion of the HIF-1 $\alpha$  3'-untranslated region together with the C-terminal amino acid (pGR500/ $\alpha$ 28-825) did not affect the properties of the chimera indicating that the regulation was dependent on HIF-1 $\alpha$  coding sequences (data not shown). A series of 5 N terminus deletions of HIF-1 $\alpha$  was tested (Fig. 2). Chimeric genes containing the first 2 deletions (pGR500/ $\alpha$ 166-826 and pGR500/ $\alpha$ 244-826) showed very little activity in normoxic cells; some increase in activity in response to stimulation was observed but was difficult to quantify because of the low overall level of activity. pGR500/ $\alpha$ 329-826 had slightly higher activity which was clearly induced by cobalt,



TABLE II

Effect of different stimuli on the inducible activity of a truncated glucocorticoid receptor/HIF-1 $\alpha$  fusion protein in HeLa cells

For comparison the inducible activity of the truncated glucocorticoid receptor (pGR 500) alone is also given. Results are expressed as the ratio of corrected luciferase activity in stimulated cells to that observed in normoxic cells and represent the mean  $\pm$  S.D. ( $n = 4$ ).

| Stimulus        | Plasmid       |                          |
|-----------------|---------------|--------------------------|
|                 | pGR 500       | pGR 500/ $\alpha$ 28–826 |
| Normoxia        | 1.0           | 1.0                      |
| Hypoxia         | 1.4 $\pm$ 0.1 | 9.9 $\pm$ 2.9            |
| Cobalt          | 0.9 $\pm$ 0.1 | 14.1 $\pm$ 4.9           |
| Desferrioxamine | 0.8 $\pm$ 0.3 | 15.0 $\pm$ 8.4           |
| Cyanide         | 1.5 $\pm$ 0.2 | 0.9 $\pm$ 0.1            |
| Azide           | 1.2 $\pm$ 0.4 | 1.2 $\pm$ 0.4            |

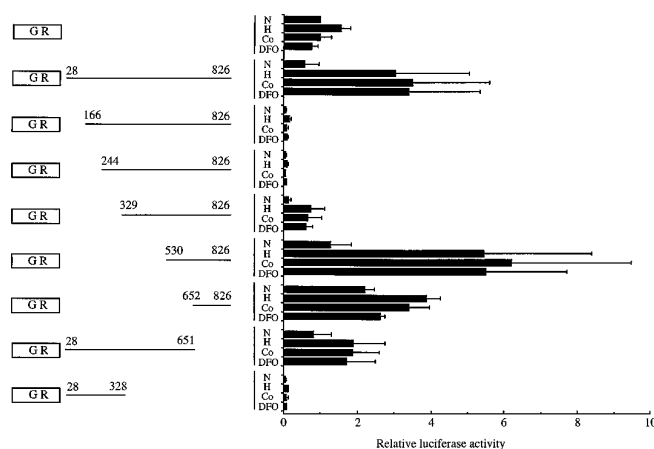


FIG. 2. **Transcriptional activity of glucocorticoid receptor/HIF-1 $\alpha$  fusion proteins in HeLa cells.** Activator plasmids encoded the indicated amino acids of HIF-1 $\alpha$  3' to a truncated glucocorticoid receptor gene. Cells were co-transfected with an activator plasmid, the MMTV-luciferase reporter plasmid, and pSV $\beta$ Gal (to provide a control for transfection efficiency) and harvested after 16 h incubation in the presence of normoxia (N), hypoxia (H), 100  $\mu$ M cobaltous ions (Co), or 100  $\mu$ M desferrioxamine (DFO). In each set of transfections corrected luciferase activity was normalized to that observed in normoxic cells transfected with the truncated glucocorticoid receptor (pGR). Values are the mean  $\pm$  1 S.D. of three experiments.

desferrioxamine, and hypoxia. pGR500/ $\alpha$ 530-826 had activity that was comparable to pGR in normoxic cells and that was induced by all three stimuli. The chimeric gene bearing the final deletion, pGR/ $\alpha$ 652-826 showed a further increase in activity in normoxic cells, but its activity was less inducible by any of the stimuli.

Two 3' deletions of HIF-1 $\alpha$  coding sequence were tested in a similar manner. pGR500/ $\alpha$ 28-329 had little activity in normoxic cells, and induction by cobalt, DFO, and hypoxia was unimpressive. pGR500/ $\alpha$ 28-652 showed a higher level of activity, and induction by all three stimuli was observed but was of lower magnitude than that observed for the near full-length fusion protein pGR500/ $\alpha$ 28-826 or for pGR500/ $\alpha$ 530-826.

When this series of fusion genes was tested in Hep3B cells, similar behavior was observed except that, in these cells, the fusion containing the C terminus of HIF-1 $\alpha$ , (pGR500/ $\alpha$ 652-826) also showed clear inducible activity.

These results demonstrate that amino acids 530–826 of HIF-1 $\alpha$  mediate transactivating function and are sufficient to convey an inducible response similar in amplitude to that observed with the near full-length sequence, 28–826. Although levels of protein expression were not defined in these experiments, sequences 5' to amino acid 530 appeared to be capable of a repressive action. The difference in inducible behavior between pGR/ $\alpha$ 530-826 and pGR/ $\alpha$ 652-826 suggested that

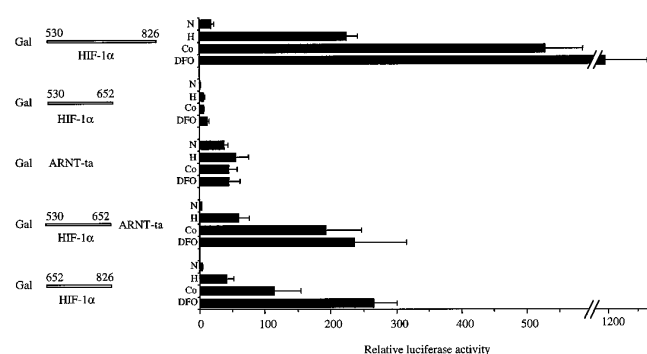


FIG. 3. **Transcriptional activity of Gal4/HIF-1 fusion proteins in Hep3B cells.** Schematics show the structure of the expressed fusion proteins. In these activator plasmids the DNA for the indicated HIF-1 $\alpha$  sequences was either encoded 3' to the DNA binding domain (amino acids 1–147) of the yeast transcription factor Gal4 (Gal) or between this domain and the sequence for the constitutively active C-terminal transactivator (amino acids 697–789) from HIF-1 $\beta$ /ARNT (ARNT-ta). Cells were harvested after 16 h incubation in the presence of normoxia (N), hypoxia (H), 100  $\mu$ M cobaltous ions (Co), or 100  $\mu$ M desferrioxamine (DFO). Cells were co-transfected with an activator plasmid and the pUAS-tk-luc reporter. In each set of transfections corrected luciferase activity was normalized to that observed in normoxic cells transfected with the truncated Gal4 gene (pCOTG). Values are the mean  $\pm$  1 S.D. of three experiments.

amino acids 530–652 contained one or more sequences that were responsive to hypoxic stimulation. In Hep3B cells, the inducible activity of pGR/ $\alpha$ 652-826 suggested that other sequences within the C-terminal domain might also be responsive to regulation. Furthermore, since pGR/ $\alpha$ 530-826 showed both a reduction in normoxic activity and an increase in hypoxic activity when compared with pGR/ $\alpha$ 652-826, the interaction between sequences 530–652 and 652–826 appeared to be complex.

Since the N-terminal 500 amino acids of the glucocorticoid receptor themselves contain sequences with transactivating potential, we considered the possibility that the inducible transcriptional activity of the GR/HIF-1 $\alpha$  fusion genes might be dependent on interactions with this region of the glucocorticoid receptor or might be specific for this type of chimeric gene. We therefore fused sequences derived from HIF-1 $\alpha$  to the N terminus (amino acids 1–147) of the transcription factor Gal4. Following the results presented above this analysis was focused on the amino acids 530–826 of HIF-1 $\alpha$ .

In contrast with the truncated glucocorticoid receptor, the truncated Gal4 gene (Gal) had negligible intrinsic transcriptional activity, increasing reporter gene expression only 1.4-fold. Results for the first series of chimeric activator genes are shown in Fig. 3. When the C-terminal sequences of HIF-1 were fused to Gal 1–147 (pGal/ $\alpha$ 530-826) substantial activity was observed which was induced a further 12–60-fold by hypoxia, cobalt, and desferrioxamine, confirming that amino acids 530–826 of HIF-1 $\alpha$  are sufficient to convey an inducible transcriptional response to these stimuli in a heterologous system. When the N-terminal portion of this sequence was tested (pGal/ $\alpha$ 530-652), little activity was observed in normoxic cells but some inducible activity (albeit at a much lower level) was retained, confirming that at least one of the sites able to confer induction was contained within amino acids 530–652. We next determined if this domain of HIF-1 $\alpha$  could confer regulation on an otherwise constitutive activation domain. A chimeric gene was constructed in which the Gal4 N terminus was linked to the C-terminal activation domain of HIF-1 $\beta$ /ARNT (amino acids 697–789). As expected from the first series of experiments, this fusion protein showed considerable activity which was not inducible (Fig. 3). A fusion gene was then constructed in which

amino acids 530–652 from HIF-1 $\alpha$  were encoded between the Gal4 N terminus and the HIF-1 $\beta$ /ARNT C terminus (pGal/ $\alpha$ 530-652/ARNT-ta). This gene showed a low level of activity in normoxic cells but was very strongly inducible by all three stimuli, demonstrating that this 123-amino acid sequence from HIF-1 $\alpha$  was able to confer a high amplitude modulation on a constitutive activation domain. The interactions with the HIF-1 $\beta$ /ARNT activation domain were both negative and positive involving a 10–15-fold repression in normoxic cells and a 4–5-fold activation in cells treated with DFO.

Finally we tested the C-terminal portion of the HIF-1 $\alpha$  gene, amino acids 652–826, for the ability to confer inducible responses in this system. pGal/ $\alpha$ 652-826 showed a low level of activity in normoxic cells, which was more strikingly inducible than the equivalent glucocorticoid receptor fusion. Thus, two portions of the HIF-1 $\alpha$  gene were defined, each of which could act independently to confer inducible responses to these three stimuli. Further experiments were performed to analyze these sequences in more detail.

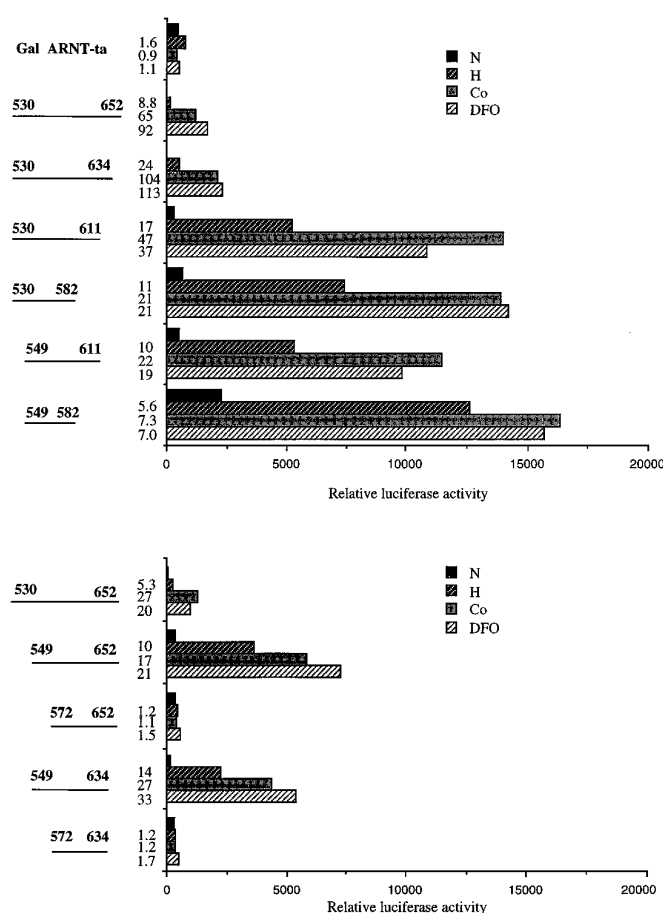
**Analysis of a Regulatory Domain within Amino Acids 530–652 of HIF-1 $\alpha$** —A series of deletions was made from both the 5' and 3' ends of this sequence to define the minimal region which could confer regulation by hypoxia on the constitutively active Gal/ARNT-ta fusion gene. As before, sequences from HIF-1 $\alpha$  were linked in frame between the DNA binding domain of Gal4 and the C-terminal activation domain of ARNT.

Results of a series of experiments in which Hep3B cells were co-transfected with this series of activator plasmids and pUAS-tk-luc are shown in Fig. 4. Consideration of the 3' deletions shows that whereas deletion to amino acid 634 had little effect on activity, further deletions of HIF-1 $\alpha$  sequence to 611 and 582 were associated with marked increases in constitutive activity in normoxic cells. In comparison with pGal/ $\alpha$ 530-632/ARNT-ta, similar plasmids bearing HIF-1 $\alpha$  sequences 530–611 and sequences 530–582 showed approximately 10- and 20-fold increases in normoxic expression (Fig. 4). Although the ratio of stimulated to unstimulated activity was reduced, inducibility was clearly retained. Similar increases in constitutive expression were observed using independently constructed plasmids bearing these 3' deletions in the context of a 5' deletion of the HIF-1 $\alpha$  sequence to amino acid 549.

When the series of 5' deletions of HIF-1 $\alpha$  sequence was considered two different effects were observed. The deletion of amino acids 530–549 also increased normoxic activity, with the remaining sequence retaining inducible activity. In contrast deletion of the HIF-1 $\alpha$  sequence to amino acid 572 caused complete loss of inducible activity. Again this effect was observed in independently constructed plasmids bearing this deletion in the context of different 3' termini; for instance, compare plasmids expressing amino acids 549–652 with 572–652 and 549–634 with 572–634 (Fig. 4).

Taken together these findings indicate that subsequences within amino acids 530 and 652 have the potential to generate high levels of transcriptional activation in this context, with amino acids 549–572 being essential for this effect. Sequences 582–652 and 530–549 contain elements that effectively reduce the activity of the chimeric transcription factor. Although the amplitude of induction was less than with sequences 530–652, inducible activity could clearly be conferred on the Gal/ARNT-ta fusion protein by the 33-amino acid HIF-1 $\alpha$  sequence 549–582.

To define further the function of this domain of HIF-1 $\alpha$ , we wished to determine if the sequence could convey changes in the level of activator plasmid product. Whole cell extracts were prepared from transfected Hep3B cells and were subject to Western analysis. Of several antibodies and antisera tested, a

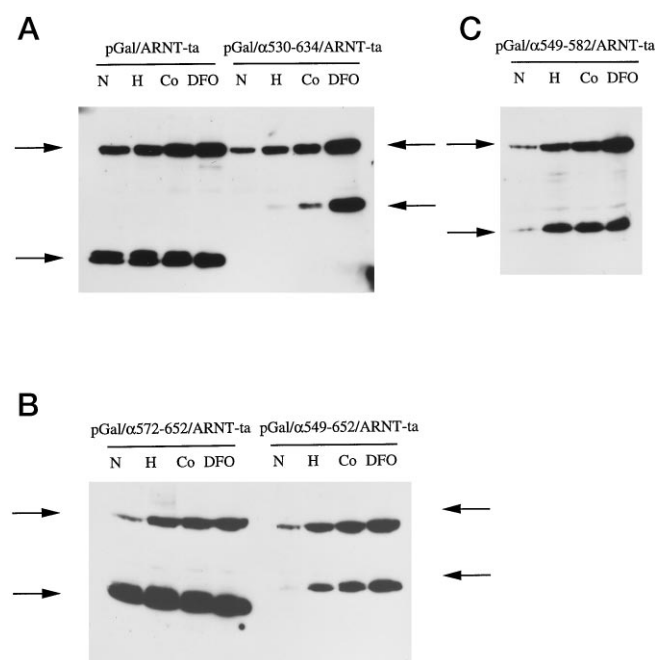


**FIG. 4. Definition of activation and regulatory domains within amino acids 530–652 of HIF-1 $\alpha$ .** Hep3B cells were co-transfected with the chimeric Gal4 activator plasmid and the Gal4-responsive reporter (pUAS-tk-luc). The top data set shows the activity of a Gal4/ARNT-ta fusion consisting of the DNA binding domain of Gal4 (amino acids 1–147) linked to the C-terminal activation domain of HIF-1 $\beta$ /ARNT (amino acids 697–789). Below are results for similar activator plasmids encoding the indicated amino acids of HIF-1 $\alpha$  between these domains. The figure shows the results of two series of transfection experiments. In each experiment, the complete HIF-1 $\alpha$  sequence under analysis (amino acids 530–652) was included to provide direct comparison with deleted sequences. Columns show the corrected luciferase activity after 16 h incubation in the presence of normoxia (N), hypoxia (H), 100  $\mu$ M cobaltous ions (Co), or 100  $\mu$ M desferrioxamine (DFO). The amplitude of induction (stimulated/normoxic activity) is indicated to the left of each column.

monoclonal antibody (2B.10) that recognizes an epitope in the C-terminal 19 amino acids of ARNT (HIF-1 $\beta$ ) (35) was found to give the best sensitivity. This antibody also permitted the use of the endogenous HIF-1 $\beta$ /ARNT signal as an internal control for comparison between Western blots and was therefore used in preference to the Gal antibody (RK5C1) for experiments using the Gal/ARNT-ta fusions, although similar results were obtained with both reagents. Although a clear signal was obtained from the Gal4-ARNT fusion protein in cells transfected with pGal/ARNT-ta, products of cells transfected with pGal/ $\alpha$ 530-652/ARNT-ta were below the limit of detection. This indicated that amino acids 530–652 of HIF-1 $\alpha$  could greatly reduce the level of the fusion protein but precluded an assessment of whether levels were regulated in response to exposure to hypoxia, cobalt, or DFO. To increase these levels into a range that could be detected by Western analysis, Hep3B cells were co-transfected with activator plasmid and amplifying plasmid pCMV-TAg, after which the cells were split for parallel 48-h cultures in normoxia and stimulating conditions. Whereas levels of the Gal4-ARNT fusion protein were high and not regu-

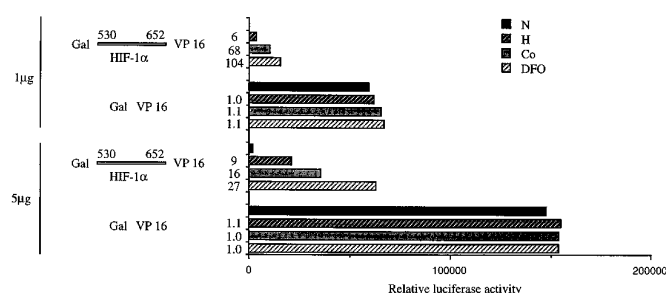
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**FIG. 5. Expression of Gal/HIF-1 $\alpha$ /ARNT fusion proteins in transfected Hep3B cells.** Whole cell extracts (50  $\mu$ g of protein) were analyzed by SDS-polyacrylamide gel electrophoresis, and expressed fusion proteins were detected by enhanced chemiluminescence after indirect immunostaining with a mouse monoclonal antibody 2B.10 directed against the C-terminal 19 amino acids of HIF-1 $\beta$ /ARNT followed by peroxidase-conjugated goat anti-mouse immunoglobulin. Cells were transfected with activator plasmid and pCMV-Tag and extracts prepared after 48 h incubation. N, normoxia; H, hypoxia; Co, cobaltous ions 100  $\mu$ M; DFO, desferrioxamine 100  $\mu$ M. Signals from the endogenous HIF-1 $\beta$ /ARNT protein, allowing comparison between panels (upper arrows), and the transfection product (lower arrows) are observed. A, cells transfected with pGal/ARNT-ta (1st to 4th lanes) and pGal/ $\alpha$ 530-634/ARNT-ta (5th to 8th lanes). The product of pGal/ARNT-ta is expressed at a relatively high level and is not inducible. Inclusion of the HIF-1 $\alpha$  sequences in the chimeric gene greatly reduced the level of fusion protein product in normoxic cells (compare 1st and 5th lanes) but also conferred marked regulation by all three stimuli (6th to 8th lanes). B, cells transfected with pGal/ $\alpha$ 572-652/ARNT-ta (1st to 4th lanes) and pGal/ $\alpha$ 549-652/ARNT-ta (5th to 8th lanes). Deletion of amino acids 549–572 ablates the regulation of the level of the fusion protein which is then expressed at a high constitutive level (compare 1st and 5th lanes). C, cells transfected with pGal/ $\alpha$ 549–582/ARNT-ta. Regulation of the fusion protein level is observed, although the level of the product is somewhat higher in normoxic cells than for pGal/ $\alpha$ 530-634/ARNT-ta, and pGal/ $\alpha$ 549-652/ARNT-ta (compare 1st lane with 5th lane in A and B).

lated by the applied stimuli, the products of pGal/ $\alpha$ 530-652/ARNT-ta and pGal/ $\alpha$ 530-634/ARNT-ta were very much lower in normoxic cells and regulated by each of the stimuli, levels being raised strikingly by DFO and cobalt and increased to a lesser extent by hypoxia (Fig. 5A). This indicated that amino acids 530–634 of HIF-1 $\alpha$  also contained sequences that affected the level of expressed activator protein and that might contribute to the functional response through that mechanism. To explore that possibility further we studied the products of other activator plasmids in the same way. Fig. 5B shows results for similar activator plasmids bearing sequences 549–652 and 572–652. Whereas the product from the former was highly inducible with a low level in normoxic cells, the latter manifested a constitutively high level of expression. This indicated that sequences between 549 and 572 were critical for the regulated effects on protein level as well as for transactivation, the striking finding being that deletion of this sequence led to a high level of product with much reduced transcriptional activity. Since we had observed that removal of surrounding sequences 532–549 and 582–652 markedly increased the activity



**FIG. 6. Action of the HIF-1 $\alpha$  modulatory domain on the transcriptional activity of a Gal4/VP16 fusion gene.** Transfection of Hep3B cells was as outlined in Fig. 4. The HIF-1 $\alpha$  sequences were expressed between the Gal4 DNA binding domain and amino acids 410–490 of the herpes simplex virus protein VP16. The figure shows the results of experiments performed with two different quantities of activator plasmid. Columns show the corrected luciferase activity after incubation in the presence of normoxia (N), hypoxia (H), 100  $\mu$ M cobaltous ions (Co), or 100  $\mu$ M desferrioxamine (DFO). The amplitude of induction (stimulated/normoxic activity) is indicated to the left of each column.

of this series of plasmids, we also compared the level of product from the activator plasmid pGal/ $\alpha$ 549-582/ARNT-ta. This fusion protein was expressed at a slightly higher level in normoxic cells, although the difference was much less than the difference in activity, particularly under stimulated conditions (compare Figs. 4 and 5).

Two further experiments were performed on this modulatory sequence. First we tested whether modulation could be conveyed on an activator function that was not derived from the HIF-1 complex. In this experiment we replaced the HIF-1 $\beta$ /ARNT transactivation domain with amino acids 410–490 from the herpes simplex virus protein VP16. In keeping with the known properties of the VP16 activation domain, this plasmid (pGal/VP16) showed powerful constitutive transactivation. Inclusion of the HIF-1 $\alpha$  domain (pGal/ $\alpha$ 530-652/VP16) conveyed modulation on this fusion protein, activity being increased approximately 30-fold by exposure to DFO, from an activity in normoxic cells which was almost 80-fold less than that of pGal/VP16 (Fig. 6). The effect of the HIF-1 $\alpha$  sequence was therefore negative under all conditions. This action was different from that of HIF-1 $\alpha$  sequence on the Gal/ARNT-ta fusions where both positive and negative effects were observed. Since we were concerned that the high activities had saturated the reporter system, we tested a lower concentration of the Gal/VP16 plasmids. Similar results were obtained, inducible activity being observed from a much reduced normoxic base line.

Finally we tested the effect of a number of point mutations. Results are shown in Table III. Mutations of phosphoacceptor amino acids within and close by the critical amino acids 549–572 had no discernible effect on the function of pGal/ $\alpha$ 530-652/ARNT-ta.

**Analysis of a Regulatory Domain within Amino Acids 652–826 of HIF-1 $\alpha$** —To determine the sequences that were critical for the inducible activity demonstrated in Gal4 chimeras containing the C-terminal portion of HIF-1 $\alpha$  (amino acids, 652–826), a further set of fusions was made containing progressive deletions of this sequence. Results of co-transfection experiments are shown in Fig. 7. The first three deletions from the 5' end of this sequence had little effect on activity so that pGal/ $\alpha$ 652-826, pGal/ $\alpha$ 668-826, pGal/ $\alpha$ 708-826, and pGal/ $\alpha$ 741-826 all showed similar basal and inducible activity. In contrast, pGal/ $\alpha$ 767-826 and pGal/ $\alpha$ 775-826 showed an increase in basal expression but retained inducibility by hypoxia, cobalt, and desferrioxamine from this higher normoxic activity.

When deletions of the 3' end of this sequence were made, it was found that both the basal activity and inducible property



TABLE III

Effect of phosphoacceptor amino acid mutations on the function of pGal/α530–652/ARNT-ta

Hep3B cells were transfected with pGal/α53C-652/ARNT-ta as described previously or with similar plasmids encoding the indicated amino acid mutations in the HIF-1α sequence. Amplitude of induction (stimulated/normoxic activity) in response to hypoxia (H), cobaltous ions (Co), and DFO is given together with constitutive activity normalized to the activity of pCOTG in normoxic cells. Data for pGal/ARNT-ta is given for comparison.

|   | Fold induction |     |     | Constitutive activity |
|---|----------------|-----|-----|-----------------------|
|   | H              | Co  | DFO |                       |
| pGal ARNT-ta                                      | 1.5            | 1.1 | 0.9 | 13                    |
| pGal <sup>530</sup> HIF-1α <sup>652</sup> ARNT-ta |                |     |     |                       |
| 549 PFSTQDTLDLLEMLAPYIPMDDDFQLRSFDQLSP 582        | 6              | 26  | 35  | 0.9                   |
| —AA—A—  | 9              | 25  | 50  | 0.8                   |
| —AA—A—  | 11             | 33  | 39  | 1.1                   |
| —AA—A—  | 14             | 39  | 52  | 1.4                   |
| —AA—A—  | 16             | 37  | 49  | 1.2                   |
| —AA—A—  | 13             | 25  | 26  | 2.9                   |

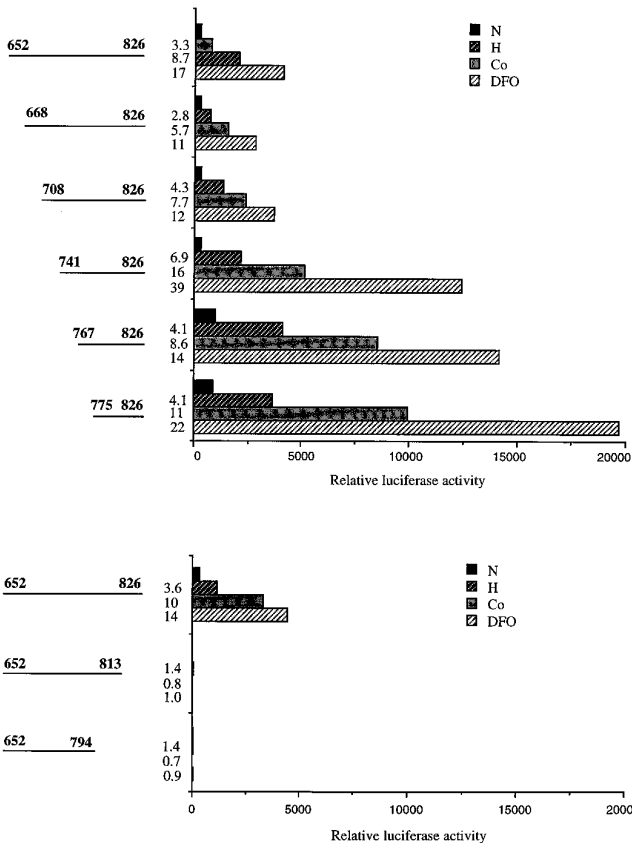


FIG. 7. Definition of activation and regulatory domains within amino acids 652–826 of HIF-1α. Transfection of Hep3B cells was as outlined in Fig. 4. Activator plasmids encoded DNA for the indicated sequences of HIF-1α 3' to that for the DNA binding domain of Gal4 (amino acids 1–147). The figure shows the results of two series of transfection experiments. In each experiment the complete HIF-1α sequence under analysis (amino acids 652–826) was included to provide direct comparison with deleted sequences. Columns show the corrected luciferase activity after incubation in the presence of normoxia (N), hypoxia (H), 100 μM cobaltous ions (Co), or 100 μM desferrioxamine (DFO). The amplitude of induction (stimulated/normoxic activity) is indicated to the left of each column.

were almost entirely ablated by removal of the C-terminal 13 amino acids. These experiments therefore defined a second domain of HIF-1α that conveyed responses to hypoxia, cobalt, and DFO.

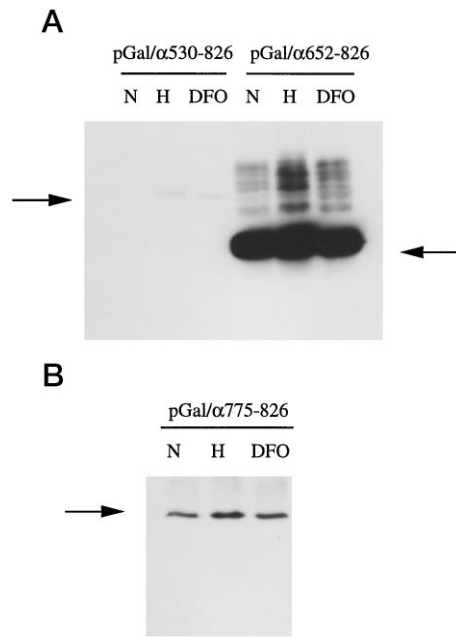
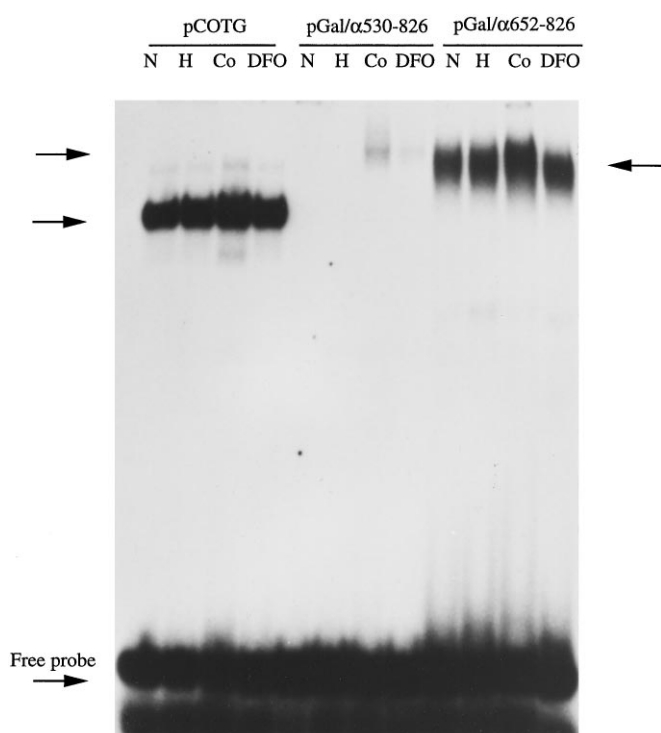


FIG. 8. Expression of Gal/HIF-1α fusion proteins in transfected Hep3B cells. Whole cell extracts (50 μg of protein) were analyzed by Western blotting. Fusion proteins were detected by enhanced chemiluminescence using anti-Gal4 mouse monoclonal RK5C1 and peroxidase-conjugated goat anti-mouse immunoglobulin. N, normoxia; H, hypoxia; DFO, desferrioxamine 100 μM. A, comparison of the expressed products of pGal/α530-826 (1st to 3rd lanes), and pGal/α652-826 (4th to 6th lanes). Cells were transfected with either activator plasmid. pCMV-TAG was included in each transfection. Despite plasmid amplification pGal/α530-826 product is undetectable in normoxic cells (1st lane) but is increased into the detectable range by hypoxia or DFO (2nd and 3rd lanes). In contrast, pGal/α652-826 gives a very strong signal in both normoxic (4th lane) and stimulated cells (5th and 6th lanes). B, expressed products of pGal/α775-826. pCMV-TAG was not included in the transfection. The product is readily detectable without plasmid amplification, and in contrast with the functional activity of this plasmid (see Fig. 7), its protein product is present at similar levels in normoxic cells (1st lane) and stimulated cells (2nd and 3rd lanes).

We next determined the extent to which these functional results reflected differences in the levels of fusion protein generated by the activator plasmid. When pGal/α652-826 was co-transfected with pCMV-TAG in a manner analogous to that used to assess the previous set of plasmids, a very high level of protein product was observed which was not inducible. This suggested that the behavior of sequences 530–652 and 652–826 was different. To explore this pGal/α530-826 and pGal/α652-826 were compared directly (Fig. 8A). In contrast with the high level of expression of pGal/α652-826, pGal/α530-826 showed a much lower level of expression that was inducible, indicating that the addition of sequences 530–652 markedly reduced and modulated the protein level in this context as well as the previously assayed fusion proteins.

It also appeared that the 15–30-fold induction observed for the functional activity of the HIF-1α C-terminal fusions was not reflected in changes in protein level. To consider this further we performed additional experiments in which no amplifying plasmid was used. Protein levels were much lower but clearly within the detectable range. Results for the most active Gal/HIF-1α fusion pGal/α775-826 are shown in Fig. 8B; again no regulation of expressed protein level was observed.

To determine whether inducible functional activity was reflected in changes in DNA binding activity, nuclear extracts were analyzed by electrophoretic mobility shift assays (EMSA) using Gal4 binding oligonucleotides. Cells were transfected and exposed to stimuli using conditions identical to the functional assays. Fig. 9 shows an EMSA using nuclear extracts prepared



**FIG. 9. Electrophoretic mobility shift assays of Gal4 DNA binding activity in nuclear extracts prepared from Hep3B cells expressing Gal4/HIF-1 $\alpha$  fusion proteins.** 1st to 4th lanes show binding activity from cells transfected with a plasmid (pCOTG) expressing the truncated DNA binding domain of Gal4 (amino acids 1–147) alone. 5th to 12th lanes show binding activity for similar experiments using pGal/ $\alpha$ 530–826 (5th to 8th lanes) and pGal/ $\alpha$ 652–826 (9th to 12th lanes). Transfected cells were incubated for 16 h in the presence of normoxia (N), hypoxia (H), 100  $\mu$ M cobaltous ions (Co), or 100  $\mu$ M desferrioxamine (DFO). A high level of DNA binding activity was observed in cells transfected with either pCOTG or pGal/ $\alpha$ 652–826, which was similar under all the conditions tested. In contrast cells transfected with pGal/ $\alpha$ 530–826 had greatly reduced DNA binding activity, which was below the limit of detection on this autoradiograph in normoxic and hypoxic cells but induced to a detectable level in cells treated with cobalt and DFO.

from cells transfected with the plasmid containing the GAL4 DNA binding domain alone (pCOTG), pGal/ $\alpha$ 530–826, and pGal/ $\alpha$ 652–826. In keeping with the results of the Western analysis of protein levels, the Gal4 DNA binding activity was low and showed increases upon stimulation in cells transfected with pGal/ $\alpha$ 530–826. In contrast, cells transfected with pGal/ $\alpha$ 652–826 showed much higher levels of DNA binding activity, which were not regulated by the applied stimuli, and resembled those from cells transfected with the plasmid encoding the DNA binding domain of Gal4 alone.

#### DISCUSSION

We have demonstrated that sequences from the  $\alpha$  subunit of HIF-1 convey hypoxia-inducible activity when fused to the DNA binding domain of heterologous transcription factors. As has been established for the activation of HIF-1, the chimeric transcription factors also responded to cobaltous ions and desferrioxamine but not to mitochondrial inhibitors. Such responses were not observed for the HIF-1 $\beta$ /ARNT subunit, defining a regulatory function for HIF-1 $\alpha$ .

In our initial analysis of HIF-1 $\alpha$ , we fused sequences from this gene to the N-terminal 500 amino acids of the glucocorticoid receptor, a sequence which itself possesses transactivation activity localized to the N terminus. This strategy was designed to enable sequences from HIF-1 $\alpha$  to be assayed for regulatory properties independently of their own transactivation capability.

When the activity of such fusion proteins was tested, an inducible response was observed for fusions containing C-terminal sequences lying distal to the portions of the molecule known to be involved in DNA binding and dimerization (37). When fused to the truncated glucocorticoid receptor, HIF-1 $\alpha$  sequences 530–826 and 28–826 conferred similar inducible behavior. Since, despite the intrinsic transcriptional activity of the truncated glucocorticoid receptor, several fusions containing N-terminal sequences had very low activities which made induction difficult to assess (Fig. 2, and data not shown), this result does not necessarily exclude inducible properties within HIF-1 $\alpha$  sequences lying N-terminal to amino acid 530. However, the findings did indicate that sequences lying distal to amino acid 530 were sufficient to convey highly inducible activity and focused our detailed analysis on this portion of the molecule.

This analysis defined two regions within these sequences which could independently confer inducible characteristics on heterologous transcription factors. One region was defined within HIF-1 $\alpha$  amino acids 530–652. That this region was responsive to the inducing stimuli was first suggested by comparison of the activity of different glucocorticoid receptor/HIF-1 $\alpha$  fusion proteins in HeLa cells (Fig. 2) and confirmed by its action on constitutively active chimeric transcription factors constructed from the DNA binding domain of Gal4 and activation domains from HIF-1 $\beta$ /ARNT or the herpes simplex virus protein VP16 (Figs. 3 and 6). The second region was defined within amino acids 652–826. Although inducible activity was clearly observed when this sequence was tested as a Gal4 fusion in Hep3B cells, the sequence had only constitutive activity when tested as a glucocorticoid receptor fusion in HeLa cells. This difference could not be assigned to reporter system or cell type specificity, since the relevant glucocorticoid receptor/HIF-1 $\alpha$  fusion showed inducible activity in Hep3B cells, and the relevant Gal4/HIF-1 $\alpha$  fusion showed activity in HeLa cells albeit of lower amplitude. Whatever the reason for the differences, these experiments did define two regulatory domains of HIF-1 $\alpha$  that were capable of independent action. Deletional analysis demonstrated that in each case the property was located within a relatively short amino acid sequence (amino acids 549–582 for the first domain and amino acids 775–826 for the second), and functional analysis demonstrated that in each case the inducible characteristic included stimulation by cobaltous ions and DFO as well as hypoxia. Somewhat surprisingly, in Hep3B cells, stimulation by cobaltous ions and DFO was more effective than hypoxia, a difference that is not generally observed in the regulation of endogenous HIF-1-dependent genes (20, 22, 23) and that was not observed in the transactivation assays performed in HeLa cells.

Amino acids in both the critical regions are 100% conserved between human and mouse genes although, overall, amino acids 530–826 of the human sequence are only 83% conserved in the mouse (16, 24, 38). In keeping with the functional significance of this conservation, the C-terminal 52-amino acid domain that we have defined in the human HIF-1 $\alpha$  lies within the region homologous to an 83-amino acid-inducible activation domain defined in studies of mouse HIF-1 $\alpha$  published during the course of this work (16). Our finding of a second regulatory domain lying N-terminal to this region does not imply a species difference in the mode of regulation since in the analysis of the mouse gene that domain was not analyzed independently or in the context of a heterologous activation domain.

An important aspect of our analysis of these regulatory domains was the finding that sequences within amino acids 530–652 of HIF-1 $\alpha$  had a striking effect on the levels of fusion protein in the transfected cells. In normoxic cells, the level of



the Gal/ARNT fusion was dramatically reduced by this sequence, the reduction being relieved by exposure of cells to hypoxia, cobalt, and DFO in a manner that correlated with the functional results. Studies of the regulation of endogenous HIF-1 have demonstrated large increases in HIF-1 $\alpha$  protein level in hypoxic cells (24, 26) despite relative stability of HIF-1 $\alpha$  mRNA levels (15, 17, 26). Based on the apparent stability of HIF-1 $\alpha$  protein in hypoxic cells and its rapid degradation when cells are re-oxygenated, it has been proposed that the regulation of HIF-1 involves changes in stability of the  $\alpha$  subunit (26). Although similar measurements are difficult in transiently transfected cells, and we have not formally addressed the mechanism by which the fusion protein levels are regulated, our results are most consistent with this proposal and with the regulatory domain we have defined containing a regulated determinant of protein stability. First, the effect of the HIF-1 $\alpha$  sequences was always to reduce protein levels, reduction being profound in normoxic cells and relieved to a greater or lesser extent in stimulated cells. Second, these effects were observed on gene products expressed using the powerful constitutive cytomegalovirus promoter and optimized translational initiation sequences from different heterologous genes. Third, this region of HIF-1 $\alpha$  is rich in proline, glutamic acid/aspartic acid, serine, and threonine residues that have been implicated as signals directing protein degradation (39).

Further analysis indicated that sequences 549–572 were critically important for the effect on protein level. Deletion of sequences surrounding this region also had effects; for instance, deletion of amino acids 582–634 increased fusion protein levels in normoxic cells, suggesting that these sequences might also contribute to the mechanism of regulation. In the functional analysis, successive deletions of amino acids 582–634 led to a progressive increase in the activity of the chimeric transcription factor in normoxic cells and a progressive reduction in the amplitude of the inducible response. Although in the overall analysis of HIF-1 $\alpha$  sequences 530–652 correlation was clearly present between the functional effects and protein levels, we cannot be sure whether regulation of protein level could fully account for the effects on activity. Substantial quantitative discrepancies were apparent between the two measurements, but given the likelihood of a nonlinear relationship in the process of transcriptional activation, and the fact that we used a plasmid amplification system in these experiments, it is difficult to know whether such differences are evidence for additional mechanisms of transcriptional regulation at this site.

In the analysis of the C-terminal domain of HIF-1 $\alpha$  much more convincing support for this possibility was obtained. Gal4/HIF-1 $\alpha$  fusion proteins containing C-terminal sequences were expressed at a much higher level than fusion proteins containing amino acids 530–652, and irrespective of whether the plasmid amplification system was used, their levels were not regulated by the inducing stimuli. Moreover, when Gal4 DNA binding activity was assayed by EMSA, activity in cells transfected with the Gal/HIF-1 $\alpha$  C-terminal fusion was similar to that obtained in cells transfected with the plasmid encoding the Gal4 DNA binding domain alone. Thus the inducible activation associated with this domain did not appear to result from changes in protein level or DNA binding activity. Together with the analysis of amino acids 530–652 our results strongly suggest the operation of at least two different mechanisms of transcriptional regulation for HIF-1 $\alpha$ , one based on post-translational enhancement of activation and the other involving regulation of transcription factor levels, most probably through changes in protein stability.

One further point in relation to the analysis of amino acid

sequences 530–652 is the co-localization of a potential stability determinant and activation domain, which raises an issue as to the relation between the two processes. Both processes may be separately and actively regulated or one may occur as a default consequence of lack of activation of the other. These possibilities are difficult to distinguish, although the increased protein level and loss of functional activity observed when the critical amino acids 549–572 were deleted shows that increases in protein level can be independent of the process of transactivation. Interestingly, transcriptional activation was only modest when amino acids 530–652 were considered in isolation (Fig. 3), even when the highly active core sequence 549–582 was assessed as a simple Gal4 fusion (data not shown), nor was a positive interaction observed when the sequences were placed adjacent to the VP16 activation domain (Fig. 5). In contrast, in stimulated cells, a strongly positive interaction was observed with both the constitutive C-terminal activation domain of HIF-1 $\beta$ /ARNT and the inducible C-terminal activation domain of HIF-1 $\alpha$ , allowing the possibility that such interactions *in cis* or *in trans* could be important in the function of the native HIF-1 heterodimer. In considering the functional data alone, the power of this interaction is disguised. Thus, in stimulated cells the activity of chimeric genes expressing HIF-1 $\alpha$  sequences 530–826 was only a little greater than those expressing sequences 652–826, but when the differences in protein level and DNA binding activity are considered (Figs. 8 and 9), it can be seen that the specific activation potential of the product containing the additional amino acids 530–652 must be very much greater.

Overall, our results suggest a model in which the function of an inducible activation domain is amplified by modulation of protein level, most probably occurring through changes in protein stability. There are many precedents for post-translational modifications that enhance transactivation through phosphorylation, ligand-dependent conformational change, or co-factor recruitment (40). Less well recognized is the regulation of transcription through changes in the stability of transcription factors, although several examples have recently been described, dependent either on the action of a specific protease or on the inducible targeting of the protein to the ubiquitin-dependent proteosomal system of degradation (39, 41). Aside from the preponderance of proline, glutamic acid/aspartic acid, serine, and threonine residues, examination of the critical sequences defined in the deletional analysis did not reveal any known recognition motifs for such systems nor did mutation of phosphoacceptor sites at residues 551, 552, 555, 565, 576, and 581 affect the operation of this regulatory domain. Nevertheless detailed analysis of these sequences should now permit important new insights to be gained into this mechanism of transcriptional regulation and the underlying processes of oxygen sensing.

**Acknowledgments**—We thank Sylvia Bartlett for her expert technical assistance in this work and the following for donation of experimental materials: Krishna Chatterjee (pRShGR, pMMTV-CAT, pUAS-tk-luc), Stephen Goodbourn (pCOTG), Oliver Hankinson (pBM5/Neo/hARNT, pcDNA3/ $\beta$ AHR, Hepa 1 cells, and their ARNT-deficient mutant derivative, c4), Gary Perdew (mouse monoclonal antibody 2B.10), Gregg Semenza (pBluescript/HIF-1 $\alpha$  3.2-3T7), and Dave Simmons (pCMV-TAg).

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*J. Biol. Chem.* 1997, 272:11205-11214.

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