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Hypoxia-inducible factor 1 is a basic–helix–loop–helix–PAS heterodimer regulated by cellular O_2 tension

(dioxin receptor/erythropoietin/hypoxia/transcription)

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ABSTRACT **Hypoxia-inducible factor 1 (HIF-1) is found in mammalian cells cultured under reduced O_2 tension and is necessary for transcriptional activation mediated by the erythropoietin gene enhancer in hypoxic cells. We show that both HIF-1 subunits are basic–helix–loop–helix proteins containing a PAS domain, defined by its presence in the *Drosophila* Per and Sim proteins and in the mammalian ARNT and AHR proteins. HIF-1α is most closely related to Sim. HIF-1β is a series of *ARNT* gene products, which can thus heterodimerize with either HIF-1α or AHR. HIF-1α and HIF-1β (ARNT) RNA and protein levels were induced in cells exposed to 1% O_2 and decayed rapidly upon return of the cells to 20% O_2, consistent with the role of HIF-1 as a mediator of transcriptional responses to hypoxia.**

Mammals require molecular oxygen (O_2) for essential metabolic processes including oxidative phosphorylation, in which O_2 serves as electron acceptor during ATP formation. Systemic, local, and intracellular homeostatic responses elicited by hypoxia (the state in which O_2 demand exceeds supply) include erythropoiesis by individuals who are anemic or at high altitude (1), neovascularization in ischemic myocardium (2), and glycolysis in cells cultured at reduced O_2 tension (3). These adaptive responses either increase O_2 delivery or activate alternative metabolic pathways that do not require O_2. Hypoxia-inducible gene products that participate in these responses include erythropoietin (EPO) (reviewed in ref. 4), vascular endothelial growth factor (5–7), and glycolytic enzymes (8, 9).

The molecular mechanisms that mediate genetic responses to hypoxia have been extensively investigated for the *EPO* gene, which encodes a growth factor that regulates erythropoiesis and, thus, blood O_2-carrying capacity (1, 4). Cis-acting DNA sequences required for transcriptional activation in response to hypoxia were identified in the *EPO* 3′ flanking region, and a trans-acting factor that binds to the enhancer, hypoxia-inducible factor 1 (HIF-1), fulfilled criteria for a physiological regulator of *EPO* transcription: inducers of *EPO* expression (1% O_2, $CoCl_2$, and desferrioxamine) also induced HIF-1 DNA-binding activity with similar kinetics, inhibitors of *EPO* expression (actinomycin D, cycloheximide, and 2-aminopurine) blocked induction of HIF-1 activity, and mutations in the *EPO* 3′ flanking region that eliminated HIF-1 binding also eliminated enhancer function (4). These results also support the hypothesis that O_2 tension is sensed by a hemoprotein (10) and suggest that a signal transduction pathway requiring ongoing transcription, translation, and protein phosphorylation participates in the induction of HIF-1 DNA-binding activity and *EPO* transcription in hypoxic cells (4).

EPO expression is cell type specific, but induction of HIF-1 activity by 1% O_2, $CoCl_2$, or desferrioxamine was detected in many mammalian cell lines (11) and the *EPO* enhancer directed hypoxia-inducible transcription of reporter genes transfected into non-EPO-producing cells (11, 12). RNAs encoding several glycolytic enzymes were induced by 1% O_2, $CoCl_2$, or desferrioxamine in EPO-producing Hep 3B cells or nonproducing HeLa cells whereas cycloheximide blocked their induction, and glycolytic gene sequences containing HIF-1 binding sites mediated hypoxia-inducible transcription in transfection assays (8, 9). HIF-1 may therefore play a general role in activating homeostatic responses to hypoxia. DNA affinity purification of HIF-1 from Hep 3B and HeLa cells revealed polypeptides with apparent molecular masses of 94, 93, and 91 kDa that had similar tryptic peptide maps and a 120-kDa polypeptide that had a distinct tryptic profile (13). Glycerol gradient sedimentation, UV crosslinking, and methylation interference studies suggested that HIF-1 was a heterodimer consisting of the 120-kDa HIF-1α subunit complexed with a 91- to 94-kDa HIF-1β subunit and that both subunits contacted DNA in the major groove (11, 13, 14). Here we show that HIF-1α and HIF-1β are basic–helix–loop–helix (bHLH)–PAS proteins whose expression is regulated by cellular O_2 tension.†

MATERIALS AND METHODS

Protein Microsequence Analysis. Purified HIF-1 subunits were fractionated by SDS/PAGE, and the 120- and 94-kDa polypeptides were transferred to poly(vinylidene difluoride) membranes and individually digested with trypsin *in situ*, and peptides were fractionated by reverse-phase HPLC (13). Protein microsequence analysis was performed at the Wistar Protein Microchemistry Laboratory (Philadelphia) (15).

cDNA Library Construction and Screening. Poly(A)$^+$ RNA was isolated from Hep 3B human hepatoma cells cultured for 16 hr at 37°C in a chamber flushed with 1% O_2/5% CO_2/94% N_2. cDNA was synthesized by using oligo(dT) and random hexamer primers, and bacteriophage libraries were constructed in λgt11 and Uni-ZAP XR (Stratagene). cDNA libraries were screened with ^{32}P-labeled cDNA fragments by plaque hybridization (16).

PCR. Degenerate oligonucleotide primers were designed by using codon preference rules (17). αF1 (5′-ATCGGATCCATCACIGARCTSATGGGITATA-3′) was based upon the amino terminus of HIF-1α peptide 87-1 and used as a forward primer. Two nested reverse primers, αR1 (5′-ATTAAGCTTTTGGTSAGGTGGTCISWGTC-3′) and αR2 (5′-ATTAAGCTTGCATGGTAGTAYTCATAGAT-3′), were based upon the carboxyl terminus of peptide 91-1. PCR was performed by denaturation of 10^8 phage or 10 ng of phage DNA at 95°C for 10

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Abbreviations: bHLH, basic–helix–loop–helix; EPO, erythropoietin; HIF-1, hypoxia-inducible factor 1; UTR, untranslated region.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. U22431).

min, addition of AmpliTaq DNA polymerase (Perkin–Elmer) at 80°C, and amplification for 3 cycles at 95°C, 37°C, and 72°C (30 sec each) followed by 35 cycles at 95°C, 50°C, and 72°C (30 sec each). Nested PCR with αF1/αR1 and then αF1/αR2 generated an 86-bp fragment which was cloned into pGEM-4 (Promega). For HIF-1β (ARNT), PCR was performed as described above, using primers 5′-ATAAAGCTTGTSTAYGTSTCIGAYT-CIG-3′ and 5′-ATCGAATTCYTCIGACTGIGGCTGGTT-3′ which resulted in the predicted 69-bp product. For analysis of the 5′ end of HIF-1β (ARNT), Hep 3B poly(A)$^+$ RNA was reverse transcribed with reagents from a 5′-RACE kit (Clontech). The cDNA was used as template to amplify nt 54–425 of ARNT cDNA (18), with the forward primer 5′-TACGGA-TCCGCCATGGCGGCGACTACTGA-3′ and nested reverse primers 5′-AGCCAGGGCACTACAGGTGGGTACC-3′ and 5′-GTTCCCCGCAAGGACTTCATGTGAG-3′ for 35 cycles at 95°C, 60°C, and 72°C (30 sec each). PCR products were cloned into pGEM-4 for nucleotide sequence analysis.

Nucleotide Sequence and Database Analysis. Complete unambiguous double-stranded nucleotide sequences were obtained by incorporation of fluorescence-labeled dideoxynucleotides into thermal-cycle sequencing reactions using T3, T7, and custom-synthesized primers. Reactions were performed with Applied Biosystems 394 DNA synthesizers and 373a Automated DNA sequencers in the Genetics Core Resources Facility of The Johns Hopkins University. Protein and nucleic acid database searches were performed at the National Center for Biotechnology Information with the programs BLASTP and TBLASTN (19).

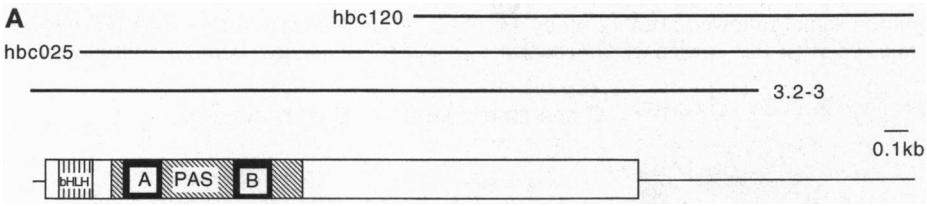
RNA Blot Hybridization. Total RNA (15 μg) was fractionated by 2.2 M formaldehyde/1.4% agarose gel electrophoresis, transferred to nitrocellulose membranes, and hybridized at 68°C in Quik-Hyb (Stratagene) to ^{32}P-labeled HIF-1α or ARNT cDNA. Gels were stained with ethidium bromide and RNA was visualized by UV illumination before and after transfer to ensure equal loading and transfer, respectively, in each lane. Based upon the migration of RNA size markers (GIBCO/BRL) on the same gels, the size of HIF-1α RNA was estimated to be 3.7 ± 0.1 kb. Two ARNT RNA species were identified as previously reported (18).

Antibody Production. Rabbits were immunized with recombinant proteins in which glutathione-*S*-transferase (GST) was fused to aa 329–531 of HIF-1α or aa 496–789 of ARNT. To generate antibodies against HIF-1α, a 0.6-kb *Eco*RI fragment from hbc025 was cloned into pGEX-3X (Pharmacia) and transformed into *Escherichia coli* DH5α cells (GIBCO/BRL). GST/HIF-1α fusion protein was isolated by exposure of bacteria (OD_{600} 0.8) to 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) at room temperature for 1 hr, sonication in 50 mM Tris·HCl, pH 7.4/1 mM EDTA/1 mM EGTA/1 mM phenylmethylsulfonyl fluoride, centrifugation at 10,000 × *g* for 10 min, incubation of supernatant with glutathione-agarose (Pharmacia) in the presence of 1% (vol/vol) Nonidet P-40 for 1 hr at 4°C, and elution with 5 mM reduced glutathione/50 mM Tris·HCl, pH 8.0/150 mM NaCl. To generate antibodies against HIF-1β, ARNT nt 1542–2428 were amplified from Hep 3B cDNA by PCR with *Taq* polymerase using forward primer 5′-ATAGGATCCTCAGGTCAGCTGGCACCCAG-3′ and reverse primer 5′-CCAAAGCTTCTATTCTGAAAAGGGG-GG-3′. The product was digested with *Bam*HI and *Eco*RI, to generate a fragment corresponding to ARNT nt 1542–2387, and cloned into pGEX-2T (Pharmacia). Fusion protein was isolated as described above, except that induction was with 1 mM IPTG for 2 hr and binding to glutathione-agarose was in the presence of 1% (vol/vol) Triton X-100 rather than Nonidet P-40. Fusion proteins were excised from SDS/10% polyacrylamide gels and used to immunize New Zealand White rabbits (HRP, Denver, PA) according to an institutionally approved protocol. Antibodies raised against HIF-1α were affinity purified by binding to GST/HIF-1α coupled to CNBr-activated Sepharose 4B (Pharmacia).

Immunoblot Analysis. Aliquots (15 μg) of nuclear protein extracts were resolved in SDS/6% polyacrylamide gels and transferred to nitrocellulose membranes in 20 mM Tris·HCl, pH 8.0/150 mM glycine/20% (vol/vol) methanol. Membranes were blocked with 5% (vol/vol) nonfat dry milk/TBS-T (20 mM Tris·HCl, pH 7.6/137 mM NaCl/0.1% Tween 20), incubated with affinity-purified HIF-1α antibodies or HIF-1β antiserum (diluted 1:400 or 1:5000, respectively), washed, incubated with horseradish peroxidase anti-immunoglobulin conjugate (diluted 1:5000), washed, and developed with ECL reagents (Amersham) and autoradiography. Incubations were for 1 hr in 5% nonfat dry milk/TBS-T and washes were for a total of 30 min in TBS-T at room temperature.

RESULTS AND DISCUSSION

Isolation and Characterization of HIF-1α cDNA Sequences. The purified 120-kDa HIF-1α polypeptide was digested with trypsin, peptides were fractionated by reverse-phase HPLC (13), and fractions 87 and 92 were subjected to microsequencing. Each fraction contained two tryptic peptides, for which virtually complete amino acid sequences were obtained: ITELMGYEPEELLGR (peptide 87-1), XIILIPSDLAXR (87-2), SIYEYYHALDSDHLTK (91-1), and SFFLR (91-2). When 87-1 and 91-1 were entered as contiguous sequences, database searches identified similarities to the *Drosophila*



B
1 MEGAGGANDKKKISSERRKEKSRDAARSRRSKESEVFYELAHQLPLPHNVSSHLDKASVMR
62 LITISYLRVRKLLDAGDLDIEDDMKAQMNCFYLKALDGFVMVLTDDGDMIYISDNVNKYMGL
123 TQFELTGHSVFDFTHPCDHEEMREMLTHRNGLVKKGKEQNTQRSFFLRMKCTLTSRGRTMN
184 IKSATWKVLHCTGHIHVYDTNSNQPQCGYKKPPMTCLVLICEPIPHPSNIEIPLDSKTFLS
245 RHSLDMKFSYCDERITELMGYEPEELLGRSIYEYYHALDSDHLTKTHHDMFTKGQVTTGQY
306 RMLAKRGGYVWVETQATVIYNTKNSQPQCIVCVNYVVSGIIQHDLIFSLQQTECVLKPVES
367 SDMKMTQLFTKVESEDTSSLFDKLKKEPDALTLLAPAAGDTIISLDFGSNDTETDDQQLEE
428 VPLYNDVMLPSPNEKLQNINLAMSPLPTAETPKPLRSSADPALNQEVALKLEPNPESLELS
489 FTMPQIQDQTPSPSDGSTRQSSPEHNSPSEYCFYVDSDMVNEFKLELVEKLFAEDTEAKNP
550 FSTQDTDLDLEMLAPYIPMDDDFQLRSFDQLSPLESSSASPESASPQSTVTVFQQTQIQEP
611 TANATTTTATTDELKTVTKDRMEDIKILIASPSPTHIHKETTSATSSPYRDTQSRTASPNR
672 AGKGVIEQTEKSHPRSPNVLSVALSQRTTVPEEELNPKILALQNAQRKRKMEHDGSLFQAV
733 GIGTLLQQPDDHAATTSLSWKRVKGCKSSEQNGMEQKTILIPSDLACRLLGQSMDESGLP
794 QLTSYDCEVNAPIQGSRNLLQGEELLRALDQVN

FIG. 1. HIF-1α is a bHLH–PAS protein. (*A*) cDNAs encoding HIF-1α. Bold lines indicate extent of clones hbc120, hbc025, and 3.2-3 relative to the full-length RNA-coding sequence shown below. Box, amino acid coding sequences; thin line, untranslated sequences; A and B, internal homology units within the PAS domain. A composite sequence was derived from the complete nucleotide sequences determined for clones 3.2-3 (nt 1–3389), hbc025 (nt 135–3691), and hbc120 (nt 1739–3720). (*B*) Derived amino acid sequence of HIF-1α. Sequences of four tryptic peptides obtained from the purified 120-kDa HIF-1α polypeptide are underscored (two peptides are contiguous).

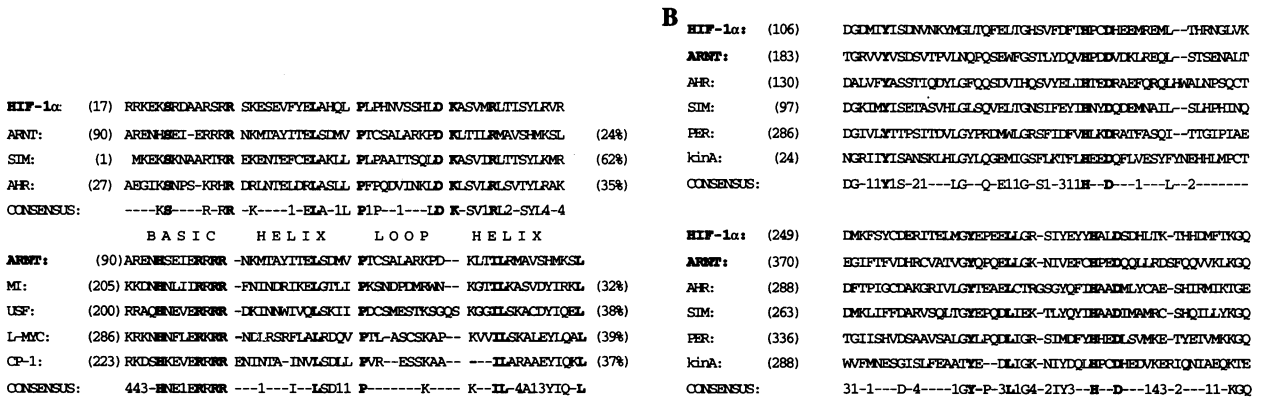


FIG. 2. Alignments of HIF-1α and HIF-1β (ARNT) with other bHLH and PAS proteins. (*A*) Analysis of bHLH domains. Coordinate of first residue of each sequence and amino acid identity with HIF-1α or HIF-1β (ARNT) are given in parentheses at left and right, respectively. Hyphen indicates gap introduced into sequence to maximize alignment, except in consensus, where it indicates a lack of agreement. Consensus indicates at least three proteins with identical or similar residue at a given position. Similar residues: 1, F/I/L/M/V; 2, S/T; 3, D/E; 4, K/R. Invariant residues are shown in bold type. (*B*) Analysis of PAS domains. Alignments of PAS A (*Upper*) and B (*Lower*) subdomains are shown. Consensus indicates at least four proteins with identical or similar residue at a given position. GenBank accession nos: ARNT, M69238; AHR, L19872; Sim, M19020; MI, Z23066; USF, X55666; L-Myc, X13945; CP-1, M34070; Per, M30114; KinA, M31067.

proteins period (Per) and single-minded (Sim) and the mammalian aryl hydrocarbon receptor (AHR) and aryl hydrocarbon receptor nuclear translocator (ARNT) proteins, which all contain sequences of 200–350 aa that constitute the PAS (Per-ARNT-AHR-Sim) domain (18, 20–23). Degenerate oligonucleotides were synthesized based upon the 87-1 and 91-1 sequences and used for PCR with cDNA prepared from hypoxic Hep 3B cells. Nucleotide sequence analysis revealed that the cloned PCR product encoded the predicted amino acids, demonstrating that 87-1 and 91-1 were contiguous peptides.

Database analysis also identified an expressed-sequence tag (EST) whose derived amino acid sequence showed similarity to bHLH–PAS proteins. We obtained the 3.6-kb cDNA from which the EST was derived, hbc025 (24). Complete nucleotide sequence analysis revealed that it encoded all four tryptic peptides. Another EST was identified which shared identity with hbc025 and was encoded by a 2.0-kb cDNA, hbc120 (24). Sequence analysis of hbc120 revealed that it was colinear with the 3′ end of hbc025 (Fig. 1*A*), differing only in the length of the poly(A) tail. The 5′ end of hbc025 was used to screen a Hep 3B cDNA library, resulting in the isolation of an overlapping 3.4-kb cDNA, 3.2-3, which extended to an initiator codon. The composite cDNA of 3720 bp encoded a 2478-bp open reading frame that included a translation initiation codon, a 28-bp 5′ untranslated region (UTR) that contained an in-frame termination codon, and a 1211-bp 3′ UTR that ended with a canonical polyadenylylation signal followed after 12 bp by 43 adenine residues. Compared with the consensus translation initiation sequence GCCRCCATGG (25), the HIF-1α cDNA sequence is TTCACCATGG. The HIF-1α cDNA open reading frame predicted an 826-aa polypeptide (Fig. 1*B*) of 93 kDa that contained a bHLH–PAS domain at its amino terminus.

Identification of HIF-1β. Analysis of two tryptic peptides isolated from the 94-kDa HIF-1β polypeptide (13) yielded partial amino acid sequences, VVYVSDSVTPVLNQPQSE and TSQFGVGSFQTPSSFSSMXLPGAPTASPGAAAY. With degenerate oligonucleotide primers based upon the second peptide sequence, a PCR product of the predicted size was amplified from Hep 3B cDNA. Database searches identified both peptides within the sequence of ARNT, a bHLH–PAS protein previously shown to heterodimerize with AHR to form the functional dioxin receptor (26). Two isoforms of ARNT have been identified which differ by the presence or absence of a 15-aa sequence encoded by a 45-bp alternative exon (18). Analysis of Hep 3B RNA by reverse transcription–PCR revealed the presence of both sequences, as well as additional isoforms that have not been analyzed yet (data not shown). These primary sequence differences may account for the purification of three (91-, 93-, and 94-kDa) HIF-1β polypeptides (13). The apparent molecular mass of both HIF-1α and HIF-1β in denaturing polyacrylamide gels was greater than the mass predicted from the cDNA sequence. For HIF-1α the apparent mass was 120 kDa, compared with a calculated mass of 93 kDa; for the HIF-1β subunits, the apparent masses were 92–94 kDa, compared with calculated masses of 85 and 87 kDa for the 774- and 789-aa isoforms of ARNT, respectively. The HIF-1α and ARNT sequences contain multiple consensus sites for protein

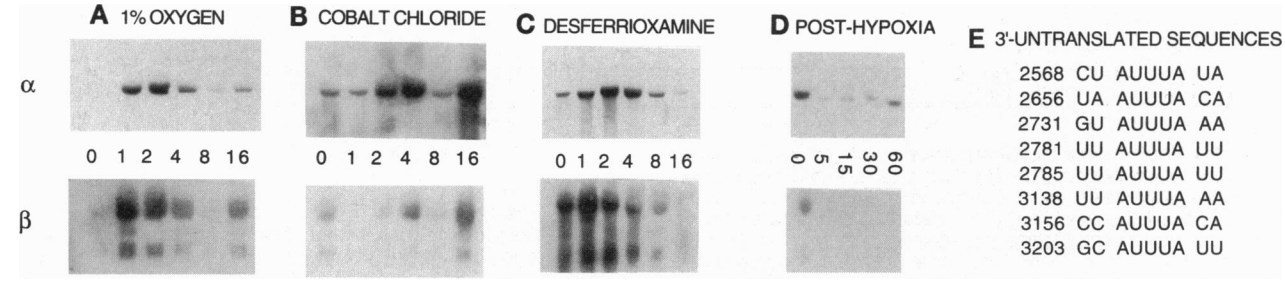


FIG. 3. Analysis of HIF-1α and HIF-1β RNA. Hep 3B cells were exposed to 1% O_2 (*A*), 75 μM $CoCl_2$ (*B*), or 130 μM desferrioxamine (*C*) for 0 (no treatment) 1, 2, 4, 8, or 16 hr, or cells were exposed to 1% O_2 for 4 hr and then returned to 20% O_2 for 0, 5, 15, 30, or 60 min (*D*) prior to RNA isolation. Samples (15 μg) of total cellular RNA were analyzed by blot hybridization using HIF-1α or HIF-1β (ARNT) cDNA probes. (*E*) AUUUA-containing elements from the HIF-1α 3′ UTR are shown with the first nucleotide numbered according to the composite cDNA sequence. Agreement with the consensus for RNA instability elements (5′-UUAUUUAWW-3′) requires identity with the core sequence and no more than two substitutions in flanking residues (34).

phosphorylation, and HIF-1 has been shown to require phosphorylation for DNA binding (14).

Alignments of bHLH and PAS domains. HIF-1α and HIF-1β (ARNT) belong to different classes of bHLH domains, which consist of contiguous DNA binding (b) and dimerization (HLH) motifs. The bHLH domain of HIF-1α is most similar to the other bHLH–PAS proteins Sim and AHR (Fig. 2*A*). HIF-1β (ARNT) has greatest similarity to the bHLH domains found in a series of mammalian (MI, USF, L-Myc) and yeast (CP-1) proteins that bind to 5′-CACGTG-3′ (27), a sequence which resembles the HIF-1 [5′-BACGTGCK-3′ (9)] and dioxin receptor [5′-KNGCGTGMSA-3′ (28)] binding sites. These transcription factors share bHLH domains of related sequence which occur in different dimerization contexts: MI, L-Myc, and USF are bHLH–leucine zipper proteins, ARNT is a bHLH–PAS protein, and CP-1 contains only a bHLH domain.

Analysis of PAS domains, which have been implicated in both ligand binding and protein dimerization (29–31), revealed that HIF-1α is most similar to Sim. Our alignment established consensus sequences that include a previously unreported motif, HXXD, present in the A and B repeats of all PAS proteins (Fig. 2*B*). We also found that KinA of *Bacillus subtilis* (32) contains a PAS domain at its amino terminus and is thus, to our knowledge, the first prokaryotic member of this protein family, indicating a remarkable degree of evolutionary conservation. KinA, like Per, possesses a PAS but not a bHLH domain and is thus unlikely to bind DNA. *B. subtilis* undergoes sporulation in response to adverse environmental conditions and KinA functions as a sensor that transmits signals via a carboxyl-terminal kinase domain (33).

Expression of HIF-1α and HIF-1β RNA. We analyzed expression of HIF-1 RNAs in response to inducers of HIF-1 DNA-binding activity. When Hep 3B cells were exposed to 1% O_2, HIF-1α and HIF-1β (ARNT) RNA levels peaked at 1–2 hr, declined to near basal levels at 8 hr, and showed a secondary increase at 16 hr of continuous hypoxia (Fig. 3*A*). In response to 75 μM $CoCl_2$, HIF-1 RNAs peaked at 4 hr, declined at 8 hr, and increased again at 16 hr (Fig. 3*B*). In cells treated with 130 μM desferrioxamine, a single peak at 1–2 hr was seen (Fig. 3*C*). When cells were incubated at 1% O_2 for 4 hr and then returned to 20% O_2, both HIF-1α and HIF-1β RNA decreased to below basal levels within 5 min, the earliest time point assayed (Fig. 3*D*). Thus, as in the case of HIF-1 DNA-binding activity (14), HIF-1 RNA levels are tightly regulated by cellular O_2 tension. The marked instability of HIF-1α RNA in post-hypoxic cells may involve the 3′ UTR, which contains eight AUUUA sequences (Fig. 3*E*) that have been identified in RNAs with short half-lives and shown to have a destabilizing effect when introduced into heterologous RNAs (35). Seven of the HIF-1α AUUUA sequences conform to a more stringent consensus for RNA instability elements, 5′-UUAUUUAWW-3′ (34). cDNA sequences encoding the complete 3′ UTR of ARNT have not been reported.

Expression of HIF-1α and HIF-1β Protein. To analyze HIF-1 protein expression, polyclonal antisera were raised in rabbits against HIF-1α and HIF-1β (ARNT). We first used these antisera to demonstrate that the proteins encoded by the cloned HIF-1α cDNA and *ARNT* are components of HIF-1 DNA-binding activity (Fig. 4*A*). When crude nuclear extracts from hypoxic cells were incubated with probe DNA and either antiserum (lanes 2 and 4), the HIF-1/DNA complex seen in the absence of antiserum (lane 1) was replaced by a more slowly migrating HIF-1/DNA/antibody complex, whereas addition of preimmune sera (lanes 3 and 5) had no effect on the HIF-1/DNA complex.

Immunoblot analysis revealed that the antisera detected polypeptides in crude nuclear extracts from hypoxic Hep 3B or $CoCl_2$-treated HeLa cells which comigrated with polypeptides present in purified HIF-1 protein preparations (Fig. 4*B*). Analysis of nuclear and cytoplasmic extracts prepared from Hep 3B cells exposed to 1% O_2 (Fig. 4*C*) revealed that peak

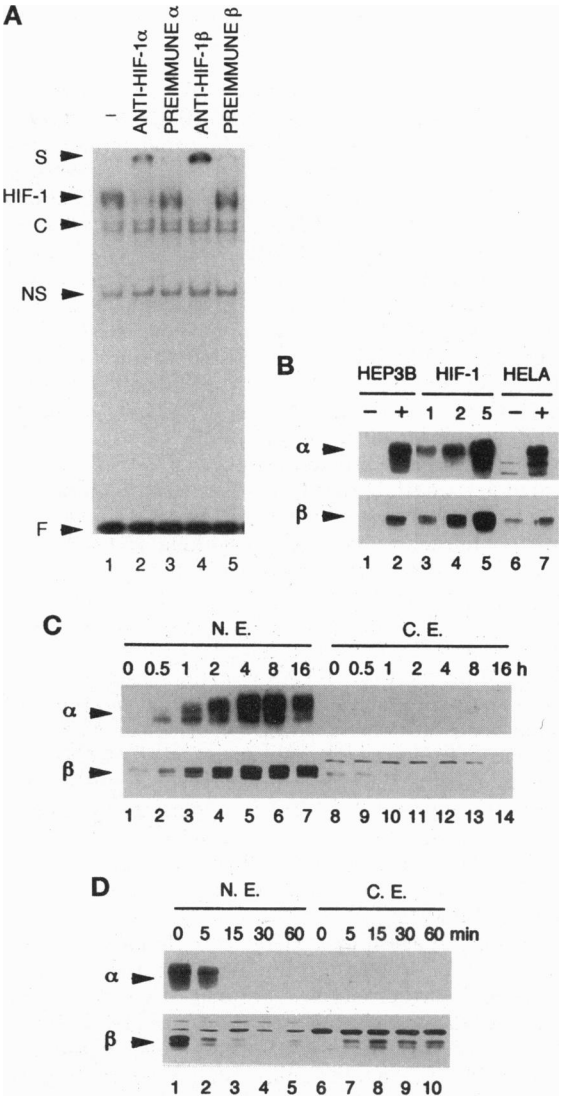


FIG. 4. Analysis of HIF-1α and HIF-1β protein. (*A*) Supershift analysis. Nuclear extract from hypoxic Hep 3B cells was incubated with oligonucleotide probe W18 (14) for 10 min on ice, sera were added (lanes 2–5 only) and incubated for 20 min on ice, and DNA/protein complexes were analyzed by polyacrylamide gel electrophoresis. Preimmune sera (lanes 3 and 5) and antisera (lanes 2 and 4) were obtained from two rabbits before and after immunization, respectively, with GST/HIF-1α (lanes 2 and 3) or GST/HIF-1β (lanes 4 and 5). HIF-1, constitutive (C), and nonspecific (NS) DNA-binding activities, free probe (F), and supershifted HIF-1/DNA/antibody complex (S) are indicated. (*B*) Antisera recognize HIF-1 subunits present in purified protein preparations and crude nuclear extracts. Nuclear extracts from Hep 3B cells which were untreated (lane 1) or exposed to 1% O_2 for 4 hr (lane 2) and from HeLa cells which were untreated (lane 6) or exposed 75 μM $CoCl_2$ for 4 hr (lane 7) were fractionated in an SDS/6% polyacrylamide gel in parallel with 1, 2, and 5 μl of affinity-purified HIF-1 (13) from $CoCl_2$-treated HeLa cells (lanes 3–5). Protein was transferred to a nitrocellulose membrane and incubated with antiserum to HIF-1α (*Upper*) or HIF-1β (*Lower*). (*C*) Induction kinetics of HIF-1α and HIF-1β protein in hypoxic cells. Hep 3B cells were exposed to 1% O_2 for 0–16 hr prior to preparation of nuclear (N.E.) and cytoplasmic (C.E.) extracts, and immunoblot analysis with antiserum to HIF-1α (*Upper*) or HIF-1β (*Lower*). (*D*) Decay kinetics of HIF-1α and HIF-1β in post-hypoxic cells. Hep 3B cells were exposed to 1% O_2 for 4 hr and returned to 20% O_2 for 0–60 min prior to preparation of extracts and immunoblot analysis. Arrowheads distinguish HIF-1 subunits from crossreacting proteins of unknown identity.

levels of HIF-1α and HIF-1β were present in nuclear extracts at 4–8 hr of continuous hypoxia, similar to the induction

kinetics of HIF-1 DNA-binding activity (14). For HIF-1α, the predominant protein species accumulating at later time points migrated to a higher position in the gel than protein present at earlier time points, suggesting that posttranslational modification of HIF-1α may occur. For HIF-1β, the 94- and 93-kDa species were resolved from the 91-kDa form but not from each other and no shifts in migration were seen. The post-hypoxic decay of HIF-1 proteins was also remarkably rapid (Fig. 4*D*), indicating that, as with the RNAs, these proteins are unstable in post-hypoxic cells. For both HIF-1α and ARNT, 31% of all amino acids are proline, glutamic acid, serine, or threonine (PEST) residues, which have been implicated in protein instability (36). In HIF-1α, two 20-aa sequences (499–518 and 581–600; Fig. 1*B*) each contain 15 PEST residues. For HIF-1β (ARNT), redistribution between nuclear and cytoplasmic compartments also appeared to play a role in both the induction and decay of nuclear protein levels.

Together with our previous studies of HIF-1, the results presented here indicate that HIF-1 is a heterodimeric bHLH–PAS transcription factor consisting of a 120-kDa HIF-1α subunit complexed with a 91- to 94-kDa HIF-1β (ARNT) isoform. Thus, *ARNT* encodes a series of common subunits utilized by both HIF-1 and the dioxin receptor, analogous to the heterodimerization of *E2A* gene products with various bHLH proteins (37). These results and the similarity of HIF-1α and Sim within the bHLH–PAS domain suggest that ARNT may also heterodimerize with Sim. In *Drosophila*, several Sim-regulated genes are characterized by enhancer elements that include one to five copies of the sequence 5′-RWACGTG-3′ (38). The observation that the HIF-1, dioxin receptor, and Sim binding sites share the sequence 5′-CGTG-3′ supports the hypothesis that ARNT is capable of combinatorial association with HIF-1α, AHR, and Sim, since this half-site is also recognized by the transcription factors with which ARNT shows greatest similarity in the bHLH domain (see Fig. 2*A*).

Our results suggest that HIF-1 DNA-binding activity is regulated by both transcriptional and posttranscriptional mechanisms. Further studies are necessary to determine whether the changes in steady-state levels of HIF-1 RNAs and proteins reflect changes in rates of synthesis, degradation, or both. In addition, the possibility that one or both HIF-1 subunits are capable of ligand binding has not been excluded. The cDNA and antisera described here will permit a detailed analysis of the molecular mechanisms by which HIF-1 activity is regulated and the role of HIF-1 in mammalian O_2 homeostasis.

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