Gene Expression, Vol. 6, pp. 287–299, 1997
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1052-2166/97 $10.00 + .00

Molecular Characterization of the Murine *Hif-1α* Locus

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Hypoxia inducible factor 1α (HIF-1α) is a basic helix-loop-helix-PAS (bHLH-PAS) transcription factor that mediates certain cellular responses to low oxygen tension, iron chelators, Co^{2+}, Ni^{2+}, Mg^{2+}, and low intracellular glucose concentration. Upon exposure to the above conditions, HIF-1α is upregulated and heterodimerizes with the Ah receptor nuclear translocator (ARNT, also known as HIF-1β), the heterodimeric complex binds TACGTG-containing genomic enhancer elements, and activates transcription of target genes. As a first step in developing genetic models to study the biology related to cellular hypoxia, we have cloned the murine HIF-1α cDNA, determined the tissue-specific expression of its mRNA, functionally analyzed its protein product, and characterized its promoter and its genomic structure. A comparison between the murine and human HIF-1α protein sequence reveals 95%, 99%, and 83% identity in the bHLH, PAS, and variable domains, respectively. RNAse protection assays demonstrate that in adult mice, the mHIF-1α mRNA is expressed at high levels in kidney, heart, brain, thymus, and placenta, with moderate expression in liver, spleen, testis, and lung and much lower expression in skeletal muscle testis. Northern blot analysis indicates that the mRNA of the murine HIF-1α is transcribed in two forms, a major 4-kb species and a minor 5-kb species; both are present in all tissues examined. The *Hif-1α* promoter is GC rich, does not have a TATA element near its transcriptional start site, and does not respond to hypoxia or Co^{2+}. The mHIF-1α structural gene is composed of 15 exons. The splice junction sites within the bHLH and the PAS domains of HIF-1α gene are highly conserved with respect to a number of previously characterized members of the bHLH-PAS superfamily. However, unlike other bHLH-PAS genes, where the variable domain is encoded by 2 exons, the variable region of the *mHIF-1α* gene is encoded by 7 exons. Furthermore, most of these splice junction sites in the variable region are conserved with that of *HIF-2α*, a recently cloned hypoxia-responsive bHLH-PAS protein (also known as MOP2, EPAS1, and HLF). These data suggest that HIF-1α, along with HIF-2α, represents a new subclass of the bHLH-PAS superfamily.

Murine *Hif-1α* Hypoxia inducible factor 1α bHLH-PAS superfamily
Molecular characterization

THE basic helix-loop-helix (bHLH) domain is common to a variety of transcription factors that regulate xenobiotic metabolism (8,27), neurogenesis (6), myogenesis (26,38), cellular proliferation (1), and hematopoiesis (37). The bHLH domain directs homotypic interactions between protein pairs, thus influencing the DNA binding specificity generated by the dimeric complex. An emerging superfamily of bHLH proteins also shares homology in a second region termed the PAS

Received May 20, 1997; revision accepted July 2, 1997.
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domain. The PAS domain is named for the first three proteins that were shown to contain it: PER, the *Drosophila* protein that controls circadian rhythm (5), ARNT, the dimeric partner of the AHR (15), and SIM, the *Drosophila* protein that controls specification of cell fate during midline cell differentiation (6,24). The PAS domain is a region of 200–300 amino acids containing two 60-amino acid degenerate repeats (24) that mediates protein dimerization (17). In the AHR, the PAS domain is required for dimerization with ARNT and is also necessary for interactions with the 90-kDa heat shock protein (Hsp90), as well as ligand binding (7,13).

HIF-1α is a newly identified member of the bHLH-PAS superfamily (37). This transcription factor responds to conditions of low oxygen tension and activates hypoxia-responsive genes such as erythropoietin (EPO), vascular endothelial growth factor (VEGF) (12), and certain glycolytic enzymes (22,34). Under normal physiological conditions, HIF-1α protein levels are very low or undetectable in cells. Upon exposure to low oxygen tension, or treatment with Co^{2+} or desferroxamine, the HIF-1α protein is substantially increased in nuclear fractions of cells (37). The observed responsiveness of HIF-1α to low O_2 tension, Co^{2+}, Ni^{2+}, Mg^{2+}, and desferroxamine treatment suggests that a heme sensor regulates the intracellular level of HIF-1α (14). A model supported by evidence from a number of laboratories suggests that HIF-1α concentration drives the formation of HIF-1α/ARNT complexes in vivo, which in turn activates the expression of genes that increase erythrocyte production, and energy production via glycolysis. In an effort to develop genetic models to study the biology related to HIF-1α gene, we have characterized the murine HIF-1α locus and its encoded mRNA and protein.

MATERIALS AND METHODS

cDNA Cloning

Polymerase chain reaction (PCR) was employed to obtain the murine HIF-1α cDNA using an Uni-ZAP C57BL/6 mouse kidney phage cDNA library as a template (Stratagene, La Jolla, CA). Typical amplification reactions employed 10^9 plaque forming units of the phage library and 2.5 units of Taq polymerase in 50 μl reactions buffered with 50 mM KCl, 10 mM Tris-HCl, and 2.0 mM $MgCl_2$, at pH 8.3. Initially, a fragment of the murine HIF-1α cDNA was obtained using the human HIF-1α-specific oligonucleotides OL358 and OL365. Once murine-specific sequences were obtained in this initial PCR, succeeding fragments of the murine cDNA were amplified using one murine-specific oligonucleotide and one human-specific oligonucleotide (bold) in the following combinations: **OL404** vs. OL412, OL394 vs. **OL363**, OL413 vs. **OL430**, and OL433 vs. **OL431**. Sequences corresponding to the 5′ and 3′ UTRs were obtained by PCR amplification with primer pairs corresponding to the T3 promoter site of the Uni-ZAP vector plus OL395 and primer pairs corresponding to the T7 promoter site of the Uni-ZAP vector plus OL458, respectively. PCR products were purified by gel electrophoresis, subcloned into pGEM-T vector (Promega, Madison, WI), and sequenced using the dideoxy chain termination method (31).

PCR was employed to construct an HIF-1α cDNA that contained the entire ORF. Oligonucleotide primers OL404 and OL363 were used to amplify a 1475-bp fragment containing the initial methionine and the 5′ half of the ORF and primers OL411 and OL431 were used to amplify a 1464-bp fragment containing the 3′ half of the ORF up to the stop codon. These two cDNA fragments were subcloned into separate pGEM-T vectors in the T7 orientation and then digested with *Csp45*I and *Not*I. The resulting cDNA fragments were ligated to generate an ORF-containing plasmid, termed pGmHIF-1α. To obtain a vector with greater expression levels for in vitro experiments, the *Sac*II and *Not*I of pGmHIF-1α was subcloned into the corresponding sites of pBSK. The *Nco*I/*Eco*RI fragment of this plasmid was subcloned into the corresponding sites of pSPUTK (Stratagene, La Jolla, CA) and designated pSmHIF-1α. In vitro transcription and translation of the murine HIF-1α protein were performed using the TNT coupled reticulocyte lysate as described previously (7).

Antibody Generation and Western Blot

The human HIF-1α antibody was generated as described previously (16). The ARNT-specific antisera was a generous gift of Dr. Alan Poland of the McArdle Laboratory for Cancer Research, University of Wisconsin, Madison (28). Western blots were performed as described previously (3).

Gel Shift Assays

Synthetic oligonucleotides containing core TACGTG (OL414) or CACGTG (OL445) sequences were end-labeled with [γ-^{32}P]ATP by T4 nucleotide kinase. The radiolabeled oligonucleotide probes were annealed with a 10-fold molar excess of the complementary oligonucleotides OL415 and OL446, respectively. The clone pm-ARNT, containing the complete ORF of the C57BL/6J mouse ARNT cDNA, was a generous gift from Alan Poland (University of Wisconsin-Madison, WI). Approximately 0.2 fmol of in vitro expressed mHIF-1α and mARNT proteins were incubated for 10 min at 30°C, followed by the addition of the nonspecific competitor, poly(dI-dC) (100 ng, 10 min at RT) and ^{32}P-labeled complementary oligonucleotides (1 ng, 70,000 cpm, 10 min at RT). For the antibody blocking experiments, antisera was added to this reaction and incubated for 10 min at room temperature. In oligonucleotide competition experiments, equal amounts of mHIF-1α and mARNT were incubated with an unlabeled complementary pair of synthetic oligonucleotides and poly(dI-dC) for 10 min at room temperature. Following this incubation, ^{32}P-labeled oligonucleotides were added for 10 min at room temperature prior to analysis by PAGE (3).

RNA Analysis

Northern blots were performed using 2 μg of poly(A)$^+$ RNA per lane from eight different mouse tissues (Clontech, Palo Alto, CA). A probe was constructed from the *Apa*I-*Sac*I fragment of PL428 and random primed in the presence of [α-^{32}P]CTP (10). Hybridization was performed overnight at 65°C in a solution containing 5× SSPE, 2× Denhardt's, 0.5% SDS, and 100 mg/ml heat-denatured salmon sperm DNA (Life Technologies, Gaithersburg, MD). Following overnight hybridization, the blot was washed four times with 2× SSC (0.3 M NaCl, 0.03 M sodium citrate), 0.5% SDS at room temperature for 5 min, and then once with 0.1× SSC, 0.1% SDS at 65°C for 15 min. The blot was exposed to film for 24 h at −80°C.

Ribonuclease protection assays were performed with 10 μg of total RNA, prepared from 10-month-old C57BL/6 mouse tissues using the TRIzol reagent (Life Technologies, Gaithersburg, MD). The riboprobe for ribonuclease protection was generated from the plasmid PL428. This plasmid was linearized with *Hind*III and used as template for synthesis of a 370-nucleotide antisense riboprobe with SP6 polymerase in the presence of [α-^{32}P]UTP (10 mCi/ml, 3000 Ci/mmol). This riboprobe was designed to protect a 291-bp fragment of mHIF-1α mRNA. The riboprobe was added to total RNA in molar excess such that the signals of all protected fragments were within the linear range of the assay. Hybridization was performed using Hybspeed Buffer (Ambion Inc., Austin,TX) at 68°C for 1 h. After hybridization, the RNA was subjected to RNAse A_1/T_1 for 30 min at 37°C. The protected bands were quantified on BAS2000 phosphoimaging system. For presentation, the dried gel was exposed to film for 24 h at −70°C. A 109-nucleotide riboprobe from the conserved region of human 18S subunit of ribosomal RNA was used as an internal control (Ambion Inc., Austin, TX). The 18S riboprobe often results in multiple bands ranging from 66 to 80 nucleotides (data not shown) (2).

Identification of the Transcriptional Start Site

The RNAse protection assay was performed essentially as described above. PL529 containing 1.7 kb *Pst*I fragment upstream of the translation starting codon ATG was cut with *Xba*I to make a riboprobe of approximately 1.4 kb using T7 polymerase in the presence of [α-^{32}P]UTP (3000 Ci/mmol). UTP (1 mM) was added to ensure generation of full-length riboprobe. Riboprobe (450 pg) (45,000 cpm; S.A. = 1 × 10^8 cpm/μl) and 15 μg of total RNA from E17.5d mouse placenta were used for the assay.

Cell Culture and Transient Transfection

Human Hep3B cells were maintained in MEM medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, 1 mM nonessential amino acids, 1 mM sodium pyruvate, and 2 mM L-glutamine at 37°C in 5% CO_2. For transient transfection experiments, a 1.9-kb *Sac*I fragment containing the 5′ UTR of the murine HIF-1α cDNA was subcloned into the *Sac*I site of the pGL2-basic plasmid (Promega, Madison, WI) to produce a construct that contained the HIF-1α promoter fused to the luciferase gene. This fragment was also subcloned in the reverse orientation using the *Xba*I/*Bgl*II sites of pGL-2 basic plasmid. Transient transfections were performed by the lipofectamine method (Life Technologies, Gaithersburg, MD) in six-well polystyrene tissue culture. Cells were incubated with the DNA liposome complex for 5 h in OPTI-MEM I reduced serum medium. Following incubation, fresh media were added with or without 75 μM $CoCl_2$ and incubated at 37°C overnight. To control for transfection efficiency, 0.1 μg of β-galactosidase control plasmid pCH110 was cotransfected in each plate. To quantify activity, cells were harvested and the luciferase and the β-galactosidase activities were determined using the luciferase and Galacto-Light™ protocols

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    CGCGAGGACTGTCCTCGCCGCCGTCGCGGGCAGTGTCTAGCCAGGCCTTGACAAGCTAGCCGGAGGAGCGCCTAGGAACCCGAGCCGGAGCTCAGCGA  -160
    GCGCAGCCTGCAGCTCCCGCCTCGCCGTCCCGGGGGGCGTCCCGCTCCCACCCCGCCTCTGGACTTGTCTCTTTCTCCGCGCGCGGACAGAGCCGGCGT  -61
    TTAGGCCCGAGCGAGCCCGGGGGCCGCCGGCCGGGAAGACAACGCGGGCACCGATTCGCCATGGAGGGCGCCGGCGGCGAGAACGAGAAGAAAAAGATG  39
1                                                               M  E  G  A  G  G  E  N  E  K  K  K  M
    AGTTCTGAACGTCGAAAAGAAAAGTCTAGAGATGCAGCAAGATCTCGGCGAAGCAAAGAGTCTGAAGTTTTTTATGAGCTTGCTCATCAGTTGCCACTT  138
14  S  S  E  R  R  K  E  K  S  R  D  A  A  R  S  R  R  S  K  E  S  E  V  F  Y  E  L  A  H  Q  L  P  L
    CCCCACAATGTGAGCTCACATCTTGATAAAGCTTCTGTTATGAGGCTCACCATCAGTTATTTACGTGTGAGAAAACTTCTGGATGCCGGTGGTCTAGAC  237
47  P  H  N  V  S  S  H  L  D  K  A  S  V  M  R  L  T  I  S  Y  L  R  V  R  K  L  L  D  A  G  G  L  D
    AGTGAAGATGAGATGAAGGCACAGATGGACTGTTTTTATCTGAAAGCCCTAGATGGCTTTGTGATGGTGCTAACAGATGACGGCGACATGGTTTACATT  336
80  S  E  D  E  M  K  A  Q  M  D  C  F  Y  L  K  A  L  D  G  F  V  M  V  L  T  D  D  G  D  M  V  Y  I
    TCTGATAACGTGAACAAATACATGGGGTTAACTCAGTTTGAACTAACTGGACACAGTGTGTTTGATTTTACTCATCCATGTGACCATGAGGAAATGAGA  435
113 S  D  N  V  N  K  Y  M  G  L  T  Q  F  E  L  T  G  H  S  V  F  D  F  T  H  P  C  D  H  E  E  M  R
    GAAATGCTTACACACAGAAATGGCCCAGTGAGAAAAGGGAAAGAACTAAACACACAGCGGAGCTTTTTTCTCAGAATGAAGTGCACCCTAACAAGCCGG  534
146 E  M  L  T  H  R  N  G  P  V  R  K  G  K  E  L  N  T  Q  R  S  F  F  L  R  M  K  C  T  L  T  S  R
    GGGAGGACGATGAACATCAAGTCAGCAACGTGGAAGGTGCTTCACTGCACGGGCCATATTCATGTCTATGATACCAACAGTAACCAACCTCAGTGTGGG  633
179 G  R  T  M  N  I  K  S  A  T  W  K  V  L  H  C  T  G  H  I  H  V  Y  D  T  N  S  N  Q  P  Q  C  G
    TACAAGAAACCACCCATGACGTGCTTGGTGCTGATTTGTGAACCCATTCCTCATCCGTCAAATATTGAAATTCCTTTAGATAGCAAGACATTTCTCAGT  732
212 Y  K  K  P  P  M  T  C  L  V  L  I  C  E  P  I  P  H  P  S  N  I  E  I  P  L  D  S  K  T  F  L  S
    CGACACAGCCTCGATATGAAATTTTCTTACTGTGATGAAAGAATTACTGAGTTGATGGGTTATGAGCCGGAAGAACTTTTGGGCCGCTCAATTTATGAA  831
245 R  H  S  L  D  M  K  F  S  Y  C  D  E  R  I  T  E  L  M  G  Y  E  P  E  E  L  L  G  R  S  I  Y  E
    TATTATCATGCTTTGGATTCTGATCATCTGACCAAAACTCACCATGATATGTTTACTAAAGGACAAGTCACCACAGGACAGTACAGGATGCTTGCCAAA  930
278 Y  Y  H  A  L  D  S  D  H  L  T  K  T  H  H  D  M  F  T  K  G  Q  V  T  T  G  Q  Y  R  M  L  A  K
    AGAGGTGGATATGTCTGGGTTGAAACTCAAGCAACTGTCATATATAATACGAAGAACTCCCAGCCACAGTGCATTGTGTGTGTGAATTATGTTGTAAGT  1029
311 R  G  G  Y  V  W  V  E  T  Q  A  T  V  I  Y  N  T  K  N  S  Q  P  Q  C  I  V  C  V  N  Y  V  V  S
    GGTATTATTCAGCACGACTTGATTTTCTCCCTTCAACAAACAGAATCTGTGCTCAAACCAGTTGAATCTTCAGATATGAAGATGACTCAGCTGTTCACC  1128
344 G  I  I  Q  H  D  L  I  F  S  L  Q  Q  T  E  S  V  L  K  P  V  E  S  S  D  M  K  M  T  Q  L  F  T
    AAAGTTGAATCAGAGGATACAAGCTGCCTTTTTGATAAGCTTAAGAAGGAGCCTGATGCTCTCACTCTGCTGGCTCCAGCTGCCGGCGACACCATCATC  1227
377 K  V  E  S  E  D  T  S  C  L  F  D  K  L  K  K  E  P  D  A  L  T  L  L  A  P  A  A  G  D  T  I  I
    TCTCTGGATTTTGGCAGCGATGACACAGAAACTGAAGATCAACAACTTGAAGATGTTCCATTATATAATGATGTAATGTTTCCCTCTTCTAATGAAAAA  1326
410 S  L  D  F  G  S  D  D  T  E  T  E  D  Q  Q  L  E  D  V  P  L  Y  N  D  V  M  F  P  S  S  N  E  K
    TTAAATATAAACCTGGCAATGTCTCCTTTACCTTCATCGGAAACTCCAAAGCCACTTCGAAGTAGTGCTGATCCTGCACTGAATCAAGAGGTTGCATTA  1425
443 L  N  I  N  L  A  M  S  P  L  P  S  S  E  T  P  K  P  L  R  S  S  A  D  P  A  L  N  Q  E  V  A  L
    AAATTAGAATCAAGTCCAGAGTCACTGGGACTTTCTTTTACCATGCCCCAGATTCAAGATCAGCCAGCAAGTCCTTCTGATGGAAGCACTAGACAAAGT  1524
476 K  L  E  S  S  P  E  S  L  G  L  S  F  T  M  P  Q  I  Q  D  Q  P  A  S  P  S  D  G  S  T  R  Q  S
    TCACCTGAGAGACTTCTTCAGGAAAACGTAAACACTCCTAACTTTTCCCAGCCTAACAGTCCCAGTGAATATTGCTTTGATGTGGATAGCGATATGGTC  1623
509 S  P  E  R  L  L  Q  E  N  V  N  T  P  N  F  S  Q  P  N  S  P  S  E  Y  C  F  D  V  D  S  D  M  V
    AATGTATTCAAGTTGGAACTGGTGGAAAAACTGTTTGCTGAAGACACAGAGGCAAAGAATCCATTTTCAACTCAGGACACTGATTTAGATTTGGAGATG  1722
542 N  V  F  K  L  E  L  V  E  K  L  F  A  E  D  T  E  A  K  N  P  F  S  T  Q  D  T  D  L  D  L  E  M
    CTGGCTCCCTATATCCCAATGGATGATGATTTCCAGTTACGTTCCTTTGATCAGTTGTCACCATTAGAGAGCAATTCTCCAAGCCCTCCAAGTATGAGC  1821
575 L  A  P  Y  I  P  M  D  D  D  F  Q  L  R  S  F  D  Q  L  S  P  L  E  S  N  S  P  S  P  P  S  M  S
    ACAGTTACTGGGTTCCAGCAGACCCAGTTACAGAAACCTACCATCACTGCCACTGCCACCACAACTGCCACCACTGATGAATCAAAAACAGAGACGAAG  1920
608 T  V  T  G  F  Q  Q  T  Q  L  Q  K  P  T  I  T  A  T  A  T  T  T  A  T  T  D  E  S  K  T  E  T  K
    GACAATAAAGAAGATATTAAAATACTGATTGCATCTCCATCTTCTACCCAAGTACCTCAAGAAACGACCACTGCTAAGGCATCAGCATACAGTGGCACT  2019
641 D  N  K  E  D  I  K  I  L  I  A  S  P  S  S  T  Q  V  P  Q  E  T  T  T  A  K  A  S  A  Y  S  G  T
    CACAGTCGGACAGCCTCACCAGACAGAGCAGGAAAGAGAGTCATAGAACAGACAGACAAAGCTCATCCAAGGAGCCTTAAGCTGTCTGCCACTTTGAAT  2118
674 H  S  R  T  A  S  P  D  R  A  G  K  R  V  I  E  Q  T  D  K  A  H  P  R  S  L  K  L  S  A  T  L  N
    CAAAGAAATACTGTTCCTGAGGAAGAATTAAACCCAAAGACAATAGCTTCGCAGAATGCTCAGAGGAAGCGAAAAATGGAACATGATGGCTCCCTTTTT  2217
707 Q  R  N  T  V  P  E  E  E  L  N  P  K  T  I  A  S  Q  N  A  Q  R  K  R  K  M  E  H  D  G  S  L  F
    CAAGCAGCAGGAATTGGAACATTATTGCAGCAACCAGGTGACTGTGCACCTACTATGTCACTTTCCTGGAAACGAGTGAAAGGATTCATATCTAGTGAA  2316
740 Q  A  A  G  I  G  T  L  L  Q  Q  P  G  D  C  A  P  T  M  S  L  S  W  K  R  V  K  G  F  I  S  S  E
    CAGAATGGAACGGAGCAAAAGACTATTATTTTAATACCCTCCGATTTAGCATGCAGACTGCTGGGGCAGTCAATGGATGAGAGTGGATTACCACAGCTG  2415
773 Q  N  G  T  E  Q  K  T  I  I  L  I  P  S  D  L  A  C  R  L  L  G  Q  S  M  D  E  S  G  L  P  Q  L
    ACCAGTTACGATTGTGAAGTTAATGCTCCCATACAAGGCAGCAGAAACCTACTGCAGGGTGAAGAATTACTCAGAGCTTTGGATCAAGTTAACTGAGCG  2514
806 T  S  Y  D  C  E  V  N  A  P  I  Q  G  S  R  N  L  L  Q  G  E  E  L  L  R  A  L  D  Q  V  N  *  836

    TTTCCTAATCTCATTCCTTTTGATTGTTAATGTTTTTGTTCAGTTGTTGTTGTTTGTTGGGTTTTTGTTTCTGTTGGTTATTTTTGGACACTGGTGGCT  2613
    CAGCAGTCTATTTATATTTTCTATATCTAATTTTAGAAGCCTGGCTACAATACTGCACAAACTCAGATAGTTTAGTTTTCATCCCCTTTCTACTTAATT  2712
    TTCATTAATGCTCTTTTTAATATGTTCTTTTAATGCCAGATCACAGCACATTCACAGCTCCTCAGCATTTCACCATTGCATTGCTGTAGTGTCATTTAA  2811
    AATGCACCTTTTTATTTATTTATTTTTGGTGAGGGAGTTTGTCCCTTATTGAATTATTTTTAATGAAATGCCAATATAATTTTTTAAGAAAGCAGTAAA  2910
    TTCTCATCATGATCATAGGCAGTTGAAAACTTTTTACTCATTTTTTTCATGTTTTACATGAAAAATAATGCTTTGTCAGCAGTACATGGTAGCCACAATT  3009
    GCACAATATATTTTCTTTAAAAAACCAGCAGTTACTCATGCAATATATTCTGCATTTATAAAACTAGTTTTTAAGAAATTTTTTTTGGCCTATGGAATT  3108
    GTTAAGCCTGGATCATGAAGCGTTGATCTTATAATGATTCTTAAACTGTATGGTTTCTTTATATGGGTAAAGCCATTTACATGATATAAAGAAATATGC  3207
    TTATATCTGGAAGGTATGTGGCATTTATTTGGATAAAATTCTCAATTCAGAGAAGTTATCTGGTGTTTCTTGACTTTACCAACTCAAAACAGTCCCTCT  3306
    GTAGTTGTGGAAGCTTATGCTAATATTGTGTAATTGATTATGAAACATAAATGTTCTGCCCACCCTGTTGGTATAAAGACATTTTGAGCATACTGTAAA  3405
    CAAACAAACAAAAAAATCATGCTTTGTTAGTAAAATTGCCTAGTATGTTGATTTGTTGAAAATATGATGTTTGGTTTATGCACTTTGTCGCTATTAACA  3504
    TCCTTTTTTCATATAGATTTCAATAAGTGAGTAATTTTAGAAGCATTATTTTAGGAATATAGAGTTGTCATAGTAAACATCTTGTTTTTTCTATGTACA  3603
    CTGTATAAATTTTTCGTTCCCTTGCTCTTTGTGGTTGGGTCTAACACTAACTGTACTGTTTTGTTATATCAAATAAACATCTTCTGTGGACCAGGAAAA  3702
    AAAAAAAAAAAAAA  3716
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FIG. 1. cDNA sequence and the deduced protein sequence of the murine HIF-1α gene. Numbers on the right indicate the nucleotide numbering with the A in the initiation codon ATG defined as +1. Numbers on the left side indicate the amino acid position of the deduced mHIF-1α protein. The star at the end of the protein sequence indicates the stop codon. Positions of the in-frame termination codon (TAG) upstream of the initiation codon ATG and the polyadenylation signal are underlined in the 5′ and 3′ ends, respectively. To obtain the entire cDNA, fragments of the murine cDNA were amplified and subcloned into pGEM-T vectors: OL358 and OL365 generated plasmid PL426, OL404 vs. OL412 generated PL430, OL394 vs. OL363 generated PL428, OL413 vs. OL430 generated PL431, OL433 vs. OL431 generated PL432, a T3 promoter oligo vs. OL395 generated PL505, and a T7 promoter oligo vs. OL458 generated PL506.

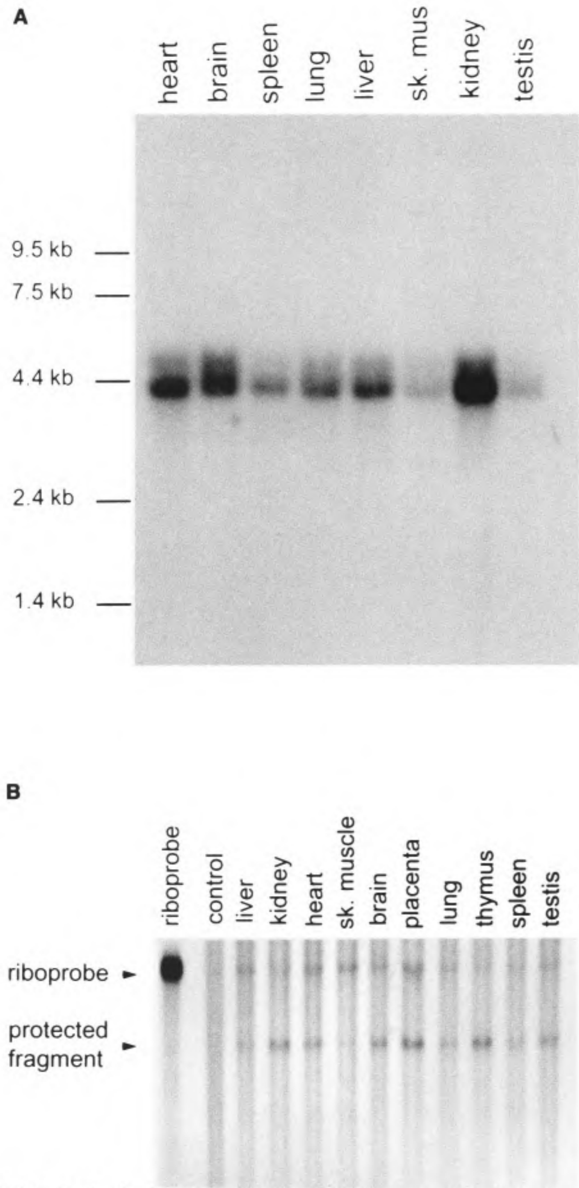


FIG. 2. Analysis of the murine HIF-1α expression in mouse tissues. (A) Northern blot. Approximately 2 μg of poly(A) RNA per lane from eight different mouse tissues was hybridized with a random primed fragment of murine HIF-1α cDNA. (B) RNAse protection assay. Approximately 10 μg of total RNA per lane from 10 different C57BL/6 mouse tissues was hybridized at 68°C for 1 h with a 370-bp [α-^{32}P]UTP-labeled riboprobe synthesized from a *Hind*III linearized murine HIF-1α cDNA (PL428). The protected mRNA fragment is 290 nucleotides. The amount of total RNA in each lane was normalized using a 109-nucleotide riboprobe from the conserved region of human 18S subunit of ribosomal RNA. The film was exposed for 24 h at −70°C.

according to manufacturer's instructions (Promega, TROPIX, Inc.). All luciferase values were normalized to β-galactosidase activity (32).

Determination of the HIF-1α Gene Structure

Two overlapping P1 phage clones (P1mHIF-1α 5635 and P1mHIF-1α 5636) containing the murine HIF-1α gene were identified from pools of phage by PCR using the primer pair OL406 and OL407 (Genome Systems, St. Louis, MO). Subclones were obtained by either restriction digest or PCR. Exon–intron splice junctions and their corresponding locations were identified by nucleotide sequencing. The PCR was typically performed using 10^3 colony forming units of P1 phage and various combinations of mHIF-1α-specific oligonucleotide primers. The PCR products were analyzed by Southern blot and subcloned into the pGEM-T vector as described above. PCR fragments typically contained at least one exon–intron boundary. Intron size was estimated from the size difference of the PCR products obtained using the genomic and cDNA as template. The plasmids were sequenced by the dideoxy chain termination method (31).

Oligonucleotide primers used (from 5′ to 3′) are given below:

OL145	AGCTCGAAATTAACCCTCACTAAAGG,
OL146	GAATTGTAATACGACTCACTATAGGG,
OL358	AACTCAGTTTGAACTAACTGG,
OL363	CCAGTGACTCTGGATTTGGTTC,
OL365	GACAGTTGCTTGAGTTTCAACC,
OL394	CAGCAACGTGGAAGGTGCTT,
OL404	GCGGTACCGGGACCGATTCACCATG GAG,
OL411	CGGCGACACCATCATCTCTCTG,
OL412	AATATGGCCCGTGCAGTGAAGC,
OL413	GCAATGTCTCCTTTACCTTCAT,
OL430	TAGGGCTTCTTGGATGAGATTT,
OL431	AAGTTTGTGCAGTATTGTAG,
OL433	CCAGTTACAGAAACCTACCATC,
OL406	CATGACGTGCTTGGTGCTGAT,
OL407	GAGGCTGTGTCGACTGAGAAATGT.

Complementary pairs of oligonucleotides used in gel shift assays are given below. Only the 5′ oligonucleotide is shown. Note constant flanking sequences, with variable core sequences underlined:

OL316/317	TCGAGCTGGGCAGGTCATGTGGC AAGGC,
OL323/324	TCGAGCTGGGCAGGTCAGCTGGC AAGGC,
OL396/397	TCGAGCTGGGCAGGTAACGTGGC AAGGC,
OL398/399	TCGAGCTGGGCAGGTGACGTGGC AAGGC
OL414/415	TCGAGCTGGGCAGGGTACGTGGC AAGGC,
OL445/446	TCGAGCTGGGCAGGTCACGTGGC AAGGC.

RESULTS AND DISCUSSION

Cloning of the Murine HIF-1α cDNA

To develop genetic models of HIF-1α function, we cloned the murine HIF-1α cDNA using se-

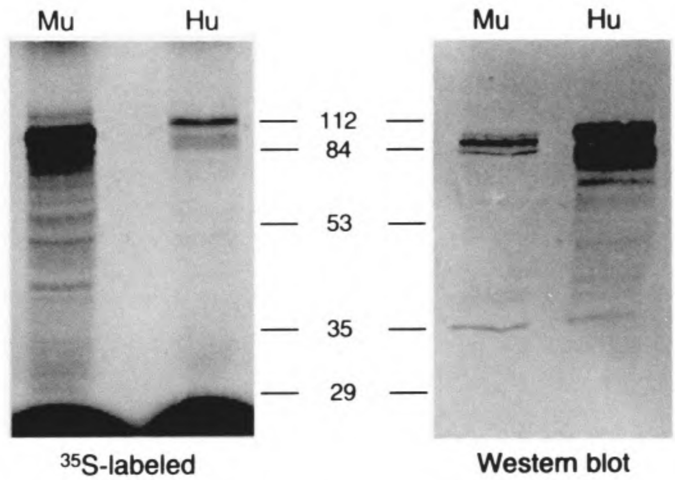


FIG. 3. In vitro expression of the murine HIF-1α protein and the Western Blot analysis. Left: The murine HIF-1α was expressed in TNT reticulocyte lysate using ^{35}S-labeled methionine and resolved in 7.5% SDS-PAGE. Right: The same gel (left) was analyzed by Western Blot using the human HIF-1α specific antibody. Hu: human; Mu: murine.

quence information from the human cDNA (16, 37). Figure 1 provides the murine HIF-1 cDNA sequence obtained by PCR. The ORF contains 2511 nucleotides that encode a protein of 836 amino acids with a predicated molecular weight of 95 kDa. Nucleotide number one is defined as the first A of the initiation codon (ATG). The initiation codon is defined by the presence of an upstream in-frame termination codon (found at −201). The cloned 5′ and 3′ UTRs of mHIF-1α contains 257 and 1205 nucleotides, respectively. A consensus polyadenylation signal is located at nucleotide 3674, 24 nucleotides upstream of a poly(A) sequence. A comparison of the murine and human HIF-1α amino acid sequences revealed that the murine HIF-1α protein shared with its human homologue 95% identity in the bHLH domain, 99% identity in the PAS domain, and 83% identity in the C-terminal variable region (data not shown). While this work was in progress, two independent groups also reported cDNA sequences for murine HIF-1α (22,39). The open reading frame for the sequence we report here is essentially identical to that reported by Li et al. (22). Although these two sequences are in agreement, their prediction of initiation codon is in contrast to that reported by Wenger et al. (39), where the initiation codon is placed at a position corresponding to our nucleotide at position 37. At the present time, we cannot rule out either a true polymorphism, the existence of an additional exon, or a sequencing error as the underlying cause of this difference.

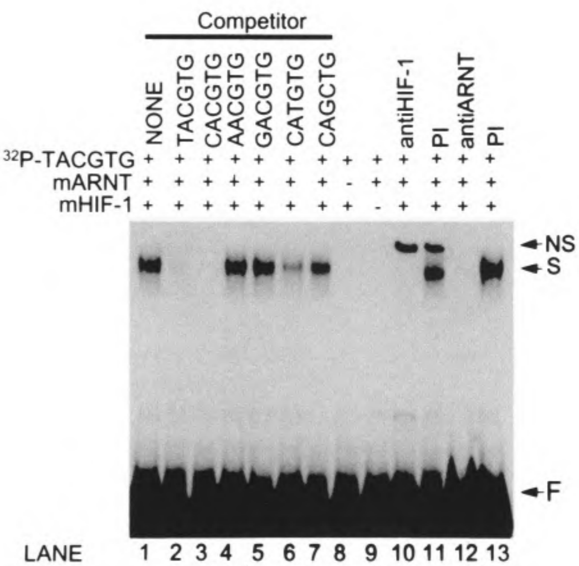


FIG. 4. DNA binding specificity of the murine HIF-1α/ARNT complex. Approximately 0.2 fmol of reticulocyte lysate-expressed murine HIF-1α and ARNT were analyzed by gel shift assays using ^{32}P-end labeled double-strand oligonucleotide corresponding to the consensus HRE (TACGTG, OL415/OL416). The murine HIF-1α and ARNT complex specifically binds to TACGTG (lane 1). Competition assays with divergent core sequences were performed as described in the text (lanes 2–7). No specific binding was seen with the murine HIF-1α or ARNT alone (lane 8 and 9). The anti-HIF-1α or anti-ARNT immuglobin abolished the specific binding, but the nonspecific IgG does not (lanes 10–13). NS: nonspecific binding; S: specific binding; F: free ^{32}P-end labeled oligonucleotides.

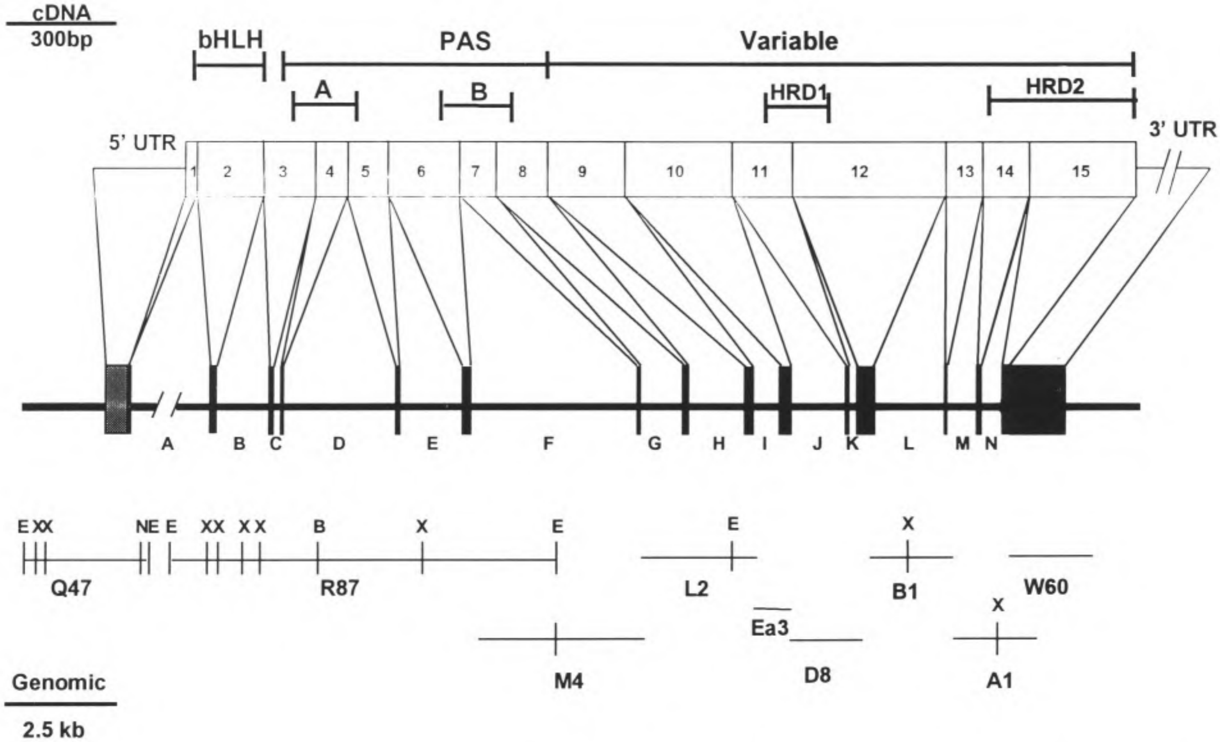


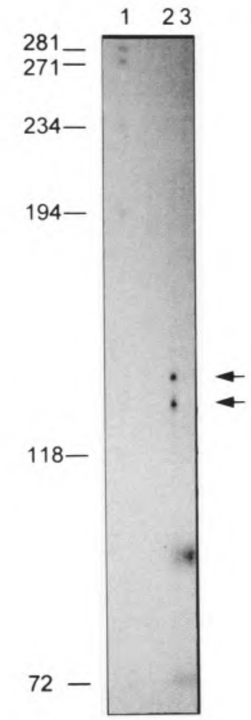
FIG. 5. The murine HIF-1α gene structure. The exons of the murine HIF-1α cDNA are represented by numbers. Boxes represent the coding regions (exons) of the genomic DNA. Shaded boxes respresent the 5′ and 3′ untranslated regions (UTRs). Letters represent the introns. Scale bars are shown for the cDNA (top left) and for the genomic DNA (bottom left), respectively. Restriction sites of the murine HIF-1α gene are indicated as follows: B: *Bam*HI; E: *Eco*RI; N: *Not*I; X: *Xba*I. This restriction map does not include a 9-kb fragment of the first intron. The clone comprising the region between exon 1 and intron A was obtained by digesting the P1 phage clone 5635 with *Eco*RI and identifying the fragment by Southern blot. The corresponding band was subcloned into the *Eco*RI site of pBSK and referred to as PLQ47. Similarly, an 8.5-kb fragment was obtained by Southern blot with a random primed exon 2 cDNA fragment (PLR87).

TABLE 1
INTRON-EXON SPLICE JUNCTIONS OF THE MURINE HIF-1α GENE

Position	Exon No.	Exon Seq.	Intron Size (bp)	Intron Seq.	Exon No.	Exon Seq.
35/36	1	G/AAA/AA	>10000	GTAAG . . . TGCAG	2	G/ATG/AG
226/227	2	AT/GCC/G	1100	GTGAG . . . TGCAG	3	GT/GGT/C
372/373	3	ACT/CAG/	82	GTAAA . . . TGCAG	4	TTT/GAA/
457/458	4	GA/AAT/G	2500	GTGAG . . . TTCAG	5	GC/CCA/G
570/571	5	TGG/AAG/	1300	GTAGC . . . TTCAG	6	GTG/CTT/
773/774	6	T/GAA/AG	3700	GTAAA . . . AACAG	7	A/ATT/AC
880/881	7	AT/GAT/A	900	GTAAG . . . CCCAG	8	TG/TCT/A
1028/1029	8	TT/GTA/A	1250	GTAAT . . . GACAG	9	GT/GGT/A
1249/1250	9	GC/GAT/G	500	GTCAG . . . TGCAG	10	AC/ACA/G
1533/1534	10	CCT/GAG/	1100	GTTGG . . . AACAG	11	AGA/CTT/
1698/1699	11	ACT/CAG	108	GTATG . . . GAAAG	12	GAC/ACT/
2123/2124	12	T/CAA/AG	1500	GTATT . . . TCCAG	13	A/AAT/AC
2232/2233	13	CCA/ATT/	600	GTAAG . . . TTTAG	14	GGA/ACA/
2359/2360	14	CC/TCC/G	400	GTTAG . . . TTTAG	15	AT/TTA/G
3740	15	AATAAA				

The positions of the murine HIF-1α intron–exon splice junctions and their adjacent sequences are shown. The consensus splice donor and acceptor dinucleotides of each intron are underlined. The genetic codons are separated by slashes.

A



B

Pst I GR/PR AP3
CTGCAGCCATTGTTCTTAACACTGTACTTTCAGGGTGTTACACTACATATGTGTAATATATATATATATATATATATATATATATATATATGTATAT -2042

ATATACACACAAATATATGTATATATATACACACATATACATATATATATGTATGTATGTATATGTATATATATTTCCCATGATTAAAAACAAAACAAA -1943

MBF-1 TFIID GR/PR
ATGAAAAACAAGTAATTAGTTTTGGTCTTTTACTCTTTTTATATATACCCTGTAGCTCTTTCAGAGGGTATGTTCTAGATTTCAAACAAAAATAGTGCT -1844

TFIID LBP-1 TEF
CAAATCTATTTATATAGTCCTAACTTCTTTTTTGAAGCCAGGGCCTTGTGCATGCTACACCACTCCTCTACTACTGAGCTAAATCTCCAGCTGTAATCA -1745

TEF Xba I MBF-1
TATCTTTATGAGTATTGAGTTGCTTCATGCATTGTTTATTGTCTTATTAGACCTGAACATTATGTGTATTTCTAGAGGGCATTGTTTTTGGATTTGAAT -1646

Core BF
GCAATTTAAATGTACCACAAATATCTACATTATTATACAACCCAAAGTTAGATTGTAACAATGTACTAGCAAGGCAAAAAAAAAATACTAAACAGTCTA -1547

GTGTCAGGTCAGTTTCAAAAACCCCAAATTCAGACTCAGTTGTGTCTATAACACGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTACACGGGCA -1448

TCF-1
CGAAGTGTTCCTTTGTTGGGCTTGGAATACTTTTAAAACAAACACACACCATACCCACAGACCTAACATTTATGCTTCCAAAGACCGACTAAAAATGAT -1349

GR/PR
GGCTTTAATCCATAGCTCCCAGCATAGCCGGTGAGCAGTCCTGTCAAGGTCTGTAGGTTGAAGGACTTGTTCTTTCAGTCTAAACAGTGAACTAAACTC -1250

NF-BA1
AGTCTGAAACAATTCTTGCACCTTTTCTGCATCCATTTAAGATGATCTTTGTCAGTTTTTAAAGCAACATGAAAGCAAAATAAGTCTATTCAATGCCTT -1151

Elf-1 LVc
TTCCTGTCTTGTCAAATTCAGGCTTGTGCTCTCACTTATTGAAGCTGTTGTTTATTTGTAAATGCTTAAGATAGTTTATTTTTAAATGGCCCTGCAACT -1052

TFIID GATA3
TCCCTCCCCTTGAGAGGTTTGGCAAGACGGATTTGTTTTATATTTTTATCTGCATTTGCTCAGTAGTCAAACTTTGGCCCAGAAAATCTTAATCACTTG -953

CIIIB1 LBP-1 NFμE4 H4TF2
GAGACTTCCCTTGTTGTATAGCAAAGAGCGGAGACTGCAGACTGAGTGGTGTCCGGTCACCTGGGAAGGAGGGACCCCACTCTAACATCAGGGGGGCTG -854

H4TF2
GCTGCCAGAACGCAAAGTTGGCTTTTTTTTTCAACTGGAAACTCGGGCGGGATGGGCGGCGACCGGGTCCAGCCACGCAGTTAAGGAGTAAGCGAGCCT -755

TFII-I LBP-1 H4TF2 LYF-1
CAGAGAACTCTATAGAGAGCCGGTGCGGTGCGAGCCAGGCTCGGACCCAAGTTTAGCAGCCCACTCGCTCCAGCAGCGCCTCCCAAAGCCAGACGCAGC -656

LBP-1 SIF R1 MCB
TTTCCACTGAAGGTGCAGAGACACCCCCCCCCCCCAGACCTGGGTCAGCCGCACCCGTCACTCGCCCCTTTTTCTTCCCTCCCTAGCTGCACACAGAC -557

IF2 CBP/CRF CP2 LBP-1 LVc
AAGCACGTGAGCGGGGCAGCTCGTCCCAGCTGGGGCTCAGCTGACCTCCTCCTGATTGGCTGAGAGCAACGTGGGCTGGGGTGGGGCCTGGCCGCCTGC -458

IF2
GTCCTTCACCATTGGCTCTCCGGGAACCCCTCCGCTCAGGTGAGGCGGGCCCGCGGGCGCGCGTTGGGGGCTGAGCGGGCGCGCGCACCCCTCGGCT -359

Sp1 Sp1
TTTCCCTCCCCTCGCCGCGCGCCCGAGCGCGCCTCCGCCCTTGCCCGCCCCCTCCCACTCCTTCAGCGCCTCAGTGCACAGAGCCTCCTCGGCTGAGGG -260

Sac I
GA*CGCGAGGACTGTCCTCGCCGCCGTCGCGGGCAGTGTCTAGCCAGGCCTTGACAAGCTAGCCGGAGGAGCGCCTAGGAACCCGAGCCGGAGCTCAGCG* -161

Pst I
AGCGCAGCCTGCAGCTCCCGCCTCGCCGTCCCGGGGGGCGTCCCGCTCCCACCCCGCCTCTGGACTTGTCTCTTTCTCCGCGCGCGGACAGAGCCGGCG -62

TTTAGGCCCGAGCGAGCCCGGGGGCCGCCGGCCGGGAAGACAACGCGGGCACCGATTCGCC ATG

Tissue-Specific Expression of Murine HIF-1α mRNA

Northern blot analysis was first performed to determine the size of HIF-1α mRNA transcript. RNAse protection assays were performed to determine the level of HIF-1α mRNA expression in different tissues (Fig. 2A, B). Northern blots revealed a major 4-kb transcript and a minor 5-kb transcript in all tissues examined (Fig. 2A). The size of the major transcript is consistent with the 3973-bp size of the full-length murine HIF-1α cDNA that we obtained by PCR. Given the rare nature of the 5-kb transcript, this species was not pursued further. RNAse protection assays were performed on 10 different tissues from the C57BL/6 mouse (Fig. 2B) and a 109-nucleotide riboprobe from the conserved region of human 18S subunit of ribosomal RNA was use as the internal control [(2), data not shown]. The results show that the mHIF-1α mRNA was abundantly expressed in placenta, kidney, heart, brain, and thymus. Moderate expression of mHIF-1α was found in liver, spleen, testis, and lung, and low expression was found in skeletal muscle (Fig. 2B). Given that HIF-1α protein expression has recently been reported to be regulated by a posttranscriptional mechanism [(18); our unpublished observations], these data identify tissues where HIF-1α has the potential to play an important physiological role.

Functional Expression of mHIF-1α

To investigate the biochemical properties of the mHIF-1α protein, mHIF-1α cDNA was expressed using a reticulocyte lysate system (Promega, Madison, WI). Quantitiation of protein expression was performed by monitoring incorporation of [^{35}S]methionine by autoradiography and by Western blot with antisera raised against the recombinant human protein (Fig. 3). The results of these analyses revealed that the expressed mHIF-1α protein has an apparent molecular weight of ~86 kDa, which is within 10% of the 95 kDa predicted from its open reading frame. The human HIF-1α antibody displays much greater sensitivity at detecting the human HIF-1α than the murine HIF-1α (Fig. 3). This is not surprising given that the antigen used is from the variable region of the hHIF-1α (residues 329 to 531), where the amino acid sequence identity between the HIF-1α orthologues is only 85%.

To determine the DNA binding specificity of the mHIF-1α protein, a gel shift assay was performed using double-stranded oligonucleotides containing the known hypoxia-responsive element found within the human EPO gene enhancer (TACGTG). In the presence of equimolar amounts of mHIF-1α and mARNT, specific interactions were obtained with the radiolabled TACGTG (OL414/415) sequence (Fig. 4). In the presence of mHIF-1α or mARNT alone, no reactivity was observed. The binding specificity to the TACGTG sequence was demonstrated by the observation that oligonucleotides containing AACGTG (OL396/397), GACGTG (OL398/399), or CAGCTG (OL323/324) did not compete for mHIF-1α/ARNT binding. Like its human orthologues, the mHIF-1α/ARNT complex also showed specific binding for the core sequences of CACGTG (OL445/446) and CATGTG (OL316/317) (16,20). The presence of both mouse HIF-1α and ARNT proteins in the complex was demonstrated by the observation that both the hHIF-1α antibody and the ARNT antibody completely abolished the DNA–protein interaction (Fig. 4).

Characterization of the mHIF-1α Gene

To determine the complete structure of the mHIF-1α gene, nine genomic DNA clones were obtained by either PCR or standard subcloning from P1 phage clones (Fig. 5). Sequence analysis

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FIG. 6, Identification and characterization of the murine HIF-1α promoter. (A) RNAse protection assay to identify transcriptional start sites (see text). Left: Size in bp of DNA molecular weight markers. Lane 1: radiolabeled, double-stranded DNA markers. Lane 2: 15 μg of total RNA from 17.5-day mouse placenta. Lane 3: 15 μg of yeast RNA as a control. The sizes of the two protected fragments were calculated from a linear regression analysis of the migration of the DNA standards. As a result, we estimate that the sizes of the protected fragments are approximately 130 and 137 nucleotides in length. (B) DNA sequence of the murine *Hif-1α* gene 5′ region. The *Pst*I and *Xba*I sites used to generate the plasmid for the riboprobe for RNAse protection assays are underlined. Putative transcriptional start sites are depicted as follows: Italics: 5′ UTR of cDNA as described in Fig. 1. Overline: approximate boundaries of region predicted to contain two independent start sites by RNAse protection. Double underline: sequences closely conforming to a reported consensus initiation site, KCABHYBY, where K = G/T, B = C/G/T, and Y = C/T (21). Arrows: Predicted transcriptional start sites based on all of the above information. To identify potential binding sites for known transcription factors, the "Matrix Search" program (http://bimas.dcrt.nih.gov/molbio/signal) was used and sites with a "match ratio" score of greater than 1 are depicted by a box (4). Two consensus Sp1 sites were also identified with scores of 0.77 and 0.75. Given the importance of these sites in initiation from TATA-less promoters, these sites are also identified by circles.

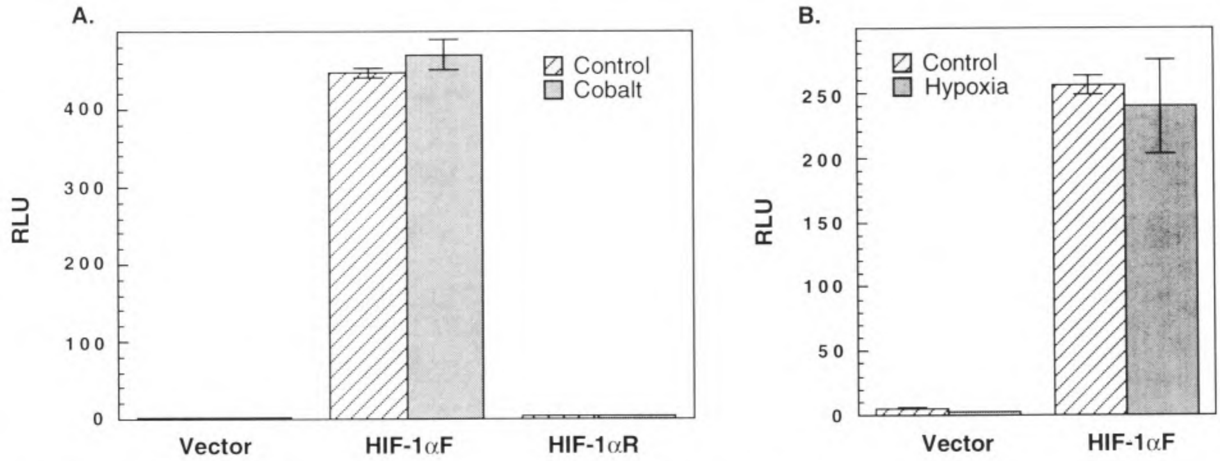

FIG. 7. Functional analysis of the of the murine HIF-1α promoter. A 1.9-kb fragment upstream of the *Sac*I site of the murine HIF-1α promoter (Fig. 6B) was subcloned in either forward (HIF-1αF) or reverse (HIF-1αR) orientation into the luciferase expression vector pGL2-basic (vector). (A) 2 μg or (B) 1 μg of each expression plasmid along with 0.1 μg of the β-galactosidase control plasmid was transiently transfected into Hep3B cells. The cells were incubated in the absence or presence of either 75 μM $CoCl_2$ (A) or 1% O_2 (B) for 48 h before being harvested. The value of luciferase activity was normalized to that of β-galactosidase control plasmid and the data represent mean ± SE of triplicate samples. RLU: relative luciferase units. Parallel experiments to demonstrate induction of HIF-1 protein under these conditions were performed (data not shown).

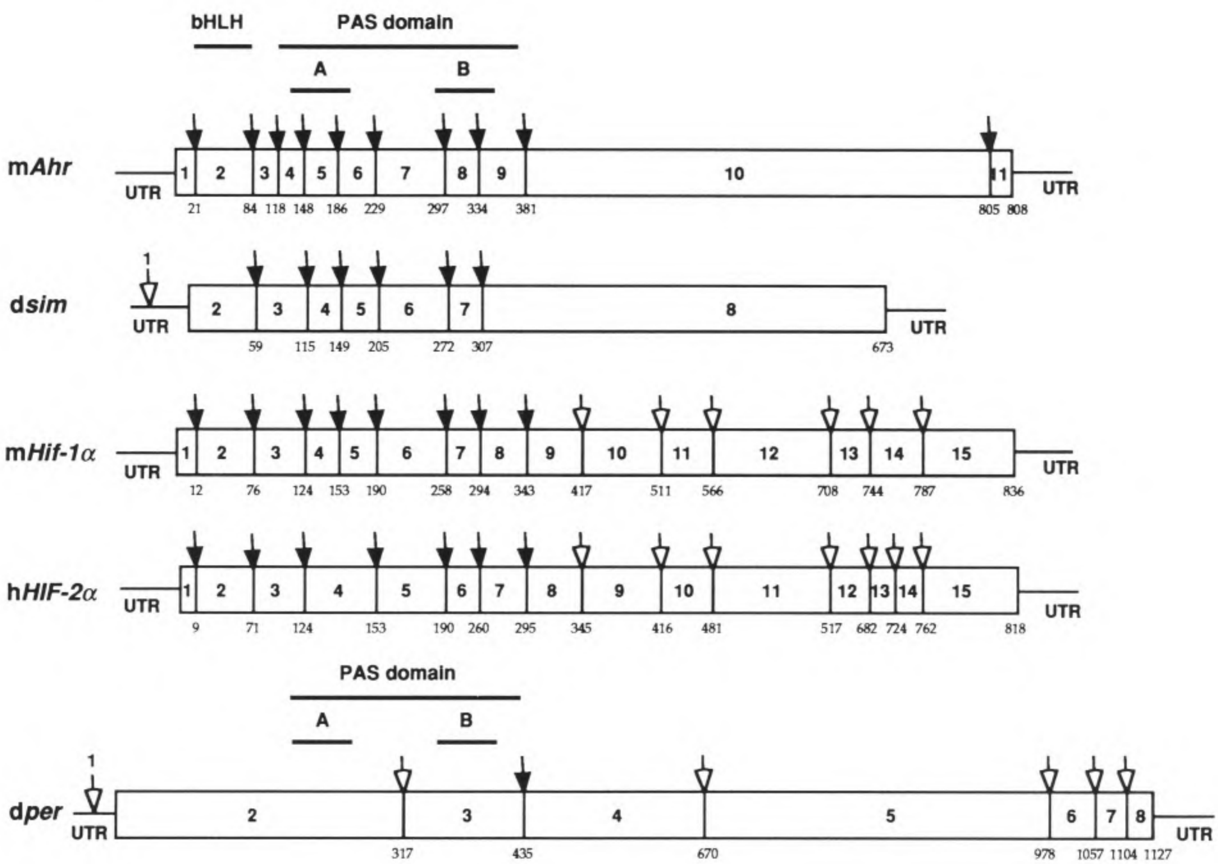

FIG. 8. Schematic comparison of splice junction sites of mouse AHR, *Drosophila* Sim, mouse HIF-1α, human HIF-2α, and *Drosophila* Per. The protein sequences of mAHR (33), dSIM (25), mHIF-1α, hHIF-2α (36), and dPER (19) were aligned by the clustal method using the DNASTAR program (DNA Star, Madison, WI). The numbers of amino acids at which the splicing occurs were marked underneath each protein. The filled arrows represent splice junction sites that are conserved compared with that of mAHR. The hollow arrows represent those splice junction sites that are not conserved. The numbers of exons were labeled either between splice junctions or on top of the arrows above 5′ UTR.

of these clones revealed that the mHIF-1α gene contains 14 introns and 15 exons. Table 1 shows the intron–exon splice junction of the murine HIF-1α gene. All the splice junctions have the consensus GT . . . AG donor and acceptor dinucleotides. Figure 5 illustrates the structural organization of the murine HIF-1α genomic DNA and relates the 15 exons to the functional domains of the encoded protein. Exon 2 contains the bHLH domain, exons 3 through 8 comprise the PAS domain, and exons 9 through 15 encode the variable region. Recently, two hypoxia-responsive domains (HRD) have been characterized (22,30). HRD1 is within exons 11 and 12 whereas HRD2 is within exons 14 and 15.

Using RNAse protection assay, we identified two transcriptional start sites that mapped to approximately 137 and 130 bp upstream of the *Pst*I site (Fig. 6A, B). In support of the conclusion that this region comprises bona fide transcriptional start sites, we offer two supporting observations. First, the boundaries of this region are within 18 nucleotides of the 5′ end of the HIF-1α cDNA obtained by RACE (Fig. 6B) and within 4 nucleotides of the 5′ end of a murine HIF-1α cDNA sequence independently obtained by another group (22). Second, the boundaries of the predicted transcriptional start sites are proximal to two 8-nucleotide sequences that have high homology to the consensus for transcriptional initiation (21). Based upon the relative intensity of the two protected species in the RNAse protection assay, we conclude that the HIF-1α promoter uses two nearby independent transcriptional start sites with equal efficiency (Fig. 6B). Analysis of the regions upstream of these start sites indicated that the promoter is extremely GC rich (68% GC in the 500 bp upstream of the start site region), similar to promoters found in many "housekeeping" genes. Although consensus recognition sequences for a number of well-characterized transcription factors were identified, no TATA or CCAAT boxes were found in the sequences proximal to the transcriptional start sites. In keeping with many TATA-less promoters, two SP1 sites were found in a region 20–40 nucleotides upstream of the proposed transcriptional starts (21). Interestingly, these characteristics of the promoter region of the murine HIF-1α gene are similar to that of the murine AHR gene, another member of the bHLH-PAS superfamily (33).

Once the genomic region harboring transcriptional start sites was mapped, we subcloned the corresponding 1.9-kb fragment upstream of a luciferase reporter gene (pGL2-basic by Promega, WI) in both the forward and the reverse orientations. These constructs were used in transient transfection of human Hep3B hepatoma cell lines and the activity of each reporter construct was measured in the absence or presence of either 75 μM $CoCl_2$ or hypoxia treatment (Fig. 7A, B). The results indicate that the mHIF-1α promoter greatly activates reporter gene activity in human hepatoma cells and the promotor is only active in the forward orientation. However, this promoter was not induced by either $CoCl_2$ or hypoxia treatment (Fig. 7A, B). This observation is consistent with the notion that HIF-1α protein levels are regulated by a posttranscriptional mechanism [(18); unpublished observations].

PAS Gene Family Splice Junction Comparison

Recent evidence has demonstrated that a superfamily of PAS-encoding genes exist within the mammalian genome (9,16,29,36,40). We are interested in a comparison of the structural organization of these genes as a first step in understanding evolutionary relationships and to help guide our efforts to generate informative chimeric molecules to explore related signaling pathways. We have previously provided a complete characterization of the murine *AHR* gene and partial characterization of the murine *ARNT* gene (23,32). Among other members of the bHLH-PAS proteins, the genomic DNA structures of *Drosophila* Per and Sim were also previously characterized (19,25). Recently, our laboratory and others have characterized a homologue of HIF-1α, that we now refer to as HIF-2α (9,16,36). [HIF-2α had also been referred to as MOP2, EPAS1, and HLF (9,16,36). The fact that this homologue has 50% sequence identity with HIF-1α, that it also dimerizes with ARNT, and that it is also hypoxia responsive leads us to suggest that this gene product is more appropriately referred to as HIF-2α.] In an effort to better understand the evolutionary relationship among bHLH-PAS proteins, we compared the structural organization of the mouse *Hif-1α* gene with the available information of other members of the bHLH-PAS superfamily, mouse *Ahr*, *Drosophila sim*, human *HIF-2α*, and *Drosophila per* (19,25,32,36) (Fig. 8). This comparison demonstrates that the bHLH domain is always encoded by a single exon and that the PAS domains are commonly encoded by 5–7 exons with highly conserved splice junction sites. However, the variable region is composed of 1 or 2 exons in *Sim* or *Ahr*, and 7 and 8 exons in the *Hif-1α* and *HIF-2α*, respectively. The splice junctions in the variable region are highly conserved between *Hif-1α* and *HIF-2α*. These results suggest that *Hif-1α* and *HIF-2α* are derived from a common primordial gene and belong to a new subclass of the bHLH-PAS gene superfamily. Similarly, *Ahr* and *Sim* belong to a separate subclass in the superfamily (16,32). The poor splice site conservation in *Drosophila per* protein, coupled with the observation that this gene does not encode a bHLH motif, suggests that per represents another subclass of the bHLH-PAS superfamily.

Conclusion

The murine *Hif-1α* locus will undoubtedly be the focus of continued scientific investigation in coming years. This expanding interest arises from its role in the regulation of EPO, VEGF, and glycolysis under conditions of low oxygen and low glucose (12,23,34,35). The observation that an endothelial-specific homologue, *Hif-2α*, also exists suggests that the corresponding promoters are likely to utilize distinct tissue-specific regulatory elements to control promoter activity. Our characterization of the Hif-1α promoter and its structural gene is a first step in understanding how these HIFs are regulated under normal condition and physiological stress. Description of the structural gene is also a requisite step in attempts to generate informative murine strains harboring null or mutant alleles at this locus. Such mutant strains have already provided great insights into the biology of other bHLH-PAS proteins (11, 23,33).

ACKNOWLEDGMENTS

We thank Drs. Peggy Farnham and Tom Gilmore for their help and Alan Poland for the gift of pmARNT.

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