Journal of Cellular Biochemistry 102:1542–1552 (2007)

Molecular Cloning and Characterization of the *Xenopus* Hypoxia-Inducible Factor 1α (xHIF1α)

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Abstract We report the molecular cloning and the characterization of the *Xenopus* homolog of mammalian hypoxia-inducible factor 1α (HIF1α), a member of the bHLH/PAS transcription factor family. Searches in *Xenopus* genome sequences and phylogenetic analysis reveal the existence of HIF1α and HIF2α paralogs in the *Xenopus laevis* species. Sequence data analyses indicate that the organization of protein domains in *Xenopus* HIF1α (xHIF1α) is strongly conserved. We also show that xHIF1α heterodimerizes with the *Xenopus* Arnt1 protein (xArnt1) with the proteic complex being mediated by the HLH and PAS domains. Subcellular analysis in a *Xenopus* XTC cell line using chimeric GFP constructs show that over-expression of xHIF1α and xArnt1 allows us to detect the xHIF1α/xArnt1 complex in the nucleus, but only in the presence of both partners. Further analyses in XTC cell line show that over-producing xHIF1α and xArnt1 mediates trans-activation of the hypoxia response element (HRE) reporter. The trans-activation level can be increased in hypoxia conditions. Interestingly such trans-activation properties can be also observed when human Arnt1 is used together with the xHIF1α. J. Cell. Biochem. 102: 1542–1552, 2007.

Key words: bHLH/PAS; PAS proteins; HIF1α; Arnt; *Xenopus*; normoxia; hypoxia; embryogenesis

Transcription factors belonging to the basic helix-loop-helix Per-Arnt-Sim (bHLH/PAS) family control a wide variety of biological processes in vertebrates and invertebrates. The processes include diverse roles in early development and adaptation to environmental stress [reviewed in Crews and Fan, 1999; Gu et al., 2000; Coumailleau, 2002; Kewley et al., 2004]. Members of this family were first identified in mammals and *Drosophila*. Recently, we and others have identified and characterized the developmental expression of the bHLH/PAS xSim and xArnt (xArnt1 and xArnt2) factors, *Xenopus* homologs of the *Drosophila Single-minded* and *Tango/Arnt* genes [Coumailleau et al., 2000; Bollérot et al., 2001; Rowatt et al., 2003]. *Xenopus* homologs of the Clock and the Aryl hydrocarbon receptor (AhR1α and AhR1β), two additional members of the bHLH/PAS family, have also been identified [Zhu et al., 2000; Ohi et al., 2003; Lavine et al., 2005].

Hypoxia-inducible factor (HIF1α) is another member of the bHLH/PAS family identified in mammals and invertebrates [Gorr et al., 2006]. HIF1α plays an important role in sensing environmental change such as hypoxia. Mammalian cell culture studies have been key in the considerable progress made towards elucidating the molecular mechanisms of oxygen sensing pathways (for review, see Ruas and Poellinger [2005]). Under normoxic conditions (21% O2), the HIF1α protein is ubiquitinylated and rapidly degraded. Degradation of HIF1α is mediated by an oxygen-dependent degradation (ODD) domain, in which conserved proline residues are covalently modified by prolyl hydroxylases. When hydroxylated, HIF1α is recognized by the

Abbreviations used: bHLH/PAS, basic helix-loop-helix/Per-Arnt-Sim; HIF1α, hypoxia-inducible factor 1α; ODD, oxygen degradation domain; TAD, trans-activation domain; NLS, nuclear localization signal; Arnt, aryl receptor nuclear translocator; Sim, single-minded; AhR, aryl hydrocarbon receptor; FIH-1α, factor inhibiting HIF1α.

Grant sponsor: French Research Ministry; Grant sponsor: French CNRS.

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Received 7 November 2006; Accepted 19 March 2007

DOI 10.1002/jcb.21376

von-Hippel-Lindau protein (pVHL), ubiquitinylated, and degraded via the proteasomal pathway. By contrast, prolyl hydroxylation does not occur under conditions of hypoxia. The onset of hypoxia allows stabilization, translocation to the nucleus, dimerization with the Arnt protein, and finally activates transcription of several hypoxia-inducible genes. Trans-activation of gene expression also depends on oxygen tension because the interaction between HIF1α and CBP/300 is blocked by the oxygen-dependent hydroxylation of an asparagine residue in the COOH terminus of HIF1α. This hydroxylation is mediated by an asparaginyl hydroxylase: Factor inhibiting HIF1α (FIH-1; [reviewed in Wenger, 2002; Poellinger and Johnson, 2004; Schofield and Ratcliffe, 2004; Berra et al., 2006]).

In addition to being a crucial protein in oxygen sensing pathways, HIF1α is also a transcription factor required in the development of mice. Null mutations of HIF1α result in embryonic lethality (day 9), due to strong cardiovascular defects and mesenchymal cell death [Carmeliet et al., 1998; Iyer et al., 1998; Ryan et al., 1998; Kotch et al., 1999]. Hypoxia may regulate various morphological processes, for example angiogenesis [Pugh and Ratcliffe, 2003]. Target mutations of HIF2α, an isoform of HIF1α (known variously as Endothelial PAS domain protein 1, EPAS1; HIF1 related factor, HRF; HIF-like factor, HLF; Member Of PAS superfamily 2, MOP2 [Ema et al., 1997; Flamme et al., 1997; Hogenesh et al., 1997; Tian et al., 1997]), are lethal to embryos at mid-gestation, partly due to vascular defects [Tian et al., 1998; Peng et al., 2000].

Most of our understanding about the role of HIF proteins in oxygen sensing and in development pathways comes from studies on mammals and, more recently, on invertebrates (for review, see Gorr et al. [2006]). Recent data are now available for various fish species [reviewed in Nikinmaa and Rees, 2005], but there has been little work on this factor in amphibians and especially in *Xenopus laevis*. Here, we report the cloning and molecular characterization of an HIF1α cDNA fragment from *X. laevis*.

MATERIALS AND METHODS

Screening Procedure and Plasmid Vector Construction

A *X. laevis* stage 17 cDNA library (kindly provided by Prof. Kintner) was screened at low stringency; human HIF1α cDNA, encoding characteristic bHLH/PAS structural motifs, was used as a probe. Positive cDNA fragments were inserted into the *Eco*RI site of pSP72 and sequenced (pSP72/xHIF1α). pCMV4/xHIF1α was obtained by subcloning the *Kpn*I/*Cla*I-digested *Xenopus* HIF1α (xHIF1α) fragment into *Kpn*I/*Cla*I-digested CMV4 vector. Plasmids pUGP/xArnt1 and pUGP/xArnt1ΔbΔC, which encode Myc-GFP fusion proteins were constructed as previously described [Coumailleau et al., 2003]. Plasmids containing full-length human HIF1α (pCMV4/hHIF1α) and full-length human Arnt1 (pCMV4/hArnt1) have been previously described [Carrero et al., 2000].

Whole-Mount In Situ Hybridization

Whole-mount in situ hybridization was performed on embryos as described by Harland [1991]. The anti-sense probe was synthesized using pSP72/xHIF1α as the template linearized with *Xba*I and T7 RNA polymerase. The sense probe was synthesized using pSPP72/xHIF1α linearized with *Cla*I as the template and SP6 RNA polymerase. Anti-digoxigenin and the color reaction with NBT/BCIP were used for detection.

In Vitro Protein Interaction Assay

In vitro translation followed by co-immunoprecipitation experiments were performed as previously described [Coumailleau et al., 2003]. Wild-type proteins or truncated versions were translated in rabbit reticulocyte lysate, either in the presence or absence of [^{35}S] methionine, according to the manufacturer's (Promega) recommendations. Aliquots of the [^{35}S] methionine-labeled in vitro translation products were analyzed by SDS–PAGE and fluorography. For xHIF1α and xArnt1 co-immunoprecipitation experiments, [^{35}S] methionine-labeled in vitro translated xHIF1α or xArnt1 (5 μl) were mixed with equal concentrations of unlabeled full-length xArnt1 (Myc-xArnt1-GFP) or xArnt1 deletion mutants (Myc-xArnt1ΔbΔC-GFP). The samples were supplemented with 5 μg/ml of the protease inhibitors, aprotinine, leupeptin, and pepstatin, and 1 mM phenylmethylsulfonylfluoride and incubated overnight at 4°C. For negative controls, the incubations were also performed with the Myc-GFP protein. Protein mixtures were then incubated for 2 h at 4°C with the mouse monoclonal antibody anti-myc (9E10; 5 μl ascites solution). The mixtures were then

incubated with 40 μl of a 50% slurry of protein A-Sepharose in 1× PBS (pH 7.4) for 1.5 h. After rapid centrifugation, the Sepharose pellets were washed three times with 500 μl 1× of PBS buffer, supplemented with 0.1% Triton X-100. Co-immunoprecipitated proteins were analyzed by SDS–PAGE and fluorography.

Cell Culture and Transient Transfection

Xenopus XTC cells were propagated and maintained in L15 Leibovitz medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS), 250 IU penicillin-streptomycin, 1 mM NaPyruvate and 2 mM glutamine at room temperature (20°C). Transient transfections were performed using Fugene 6, as described by the manufacturer (Roche Molecular Biochemicals). Cells were grown and transfected on slides covered with fibronectine for confocal analysis. The GFP fluorescence in live cells after an overnight culture was observed under a confocal laser-scanning microscope (Leica DM IRBE, TCS SP).

Cell Transient Expression and Luciferase Assay

Xenopus and human HIF1α and Arnt1 trans-regulation properties were analyzed in XTC cell transfection experiments using the reporter pHRE/T81Luc. A sequence containing three copies of the hypoxia response element (HRE: 5′-GCC CTA CGT GCT GTC TCG-3′) was inserted into the *Sma*I/*Sal*I-digested luciferase reporter plasmid pT81Luc [Nordeen, 1988] and the resulting construct named pHRE/T81Luc. At 48 h after transfection, cells were harvested in TEN buffer (40 mM Tris-HCl, pH 7.9/10 mM EDTA, pH 8/150 mM NaCl), and washed with ice-cold 1× PBS. The cell pellet was resuspended in 50 μl lysis buffer (25 mM Tris-Acetate pH 7.8/1.5 mM EDTA pH 8/10% glycerol/1% Triton X-100/2 mM DTT/1 mM PMSF). Luciferase activity was determined using 20 μl of cell extract and 100 μl of luciferase assay reagents (Biothema). To normalize for transfection efficiency, cells were co-tranfected with pRSV/β-gal (1 μg) and assayed for β-galactosidase activity. Transfections were performed in duplicate and the results are presented as means ± standard error.

Hypoxia conditions were performed in XTC cells using an hypoxia-mimicking agent, 2,2′ dipyridil [Carrero et al., 2000]. Six hours after transfection, the medium was replaced and cells were induced 24 h later with 2,2′ dipyridil (100 μm), or treated with vehicle alone. Two hours after treatment, the medium was withdrawn and the cells were re-fed with fresh medium containing 10% FBS. Cells were harvested 24 h later.

RESULTS AND DISCUSSION

Cloning of *Xenopus laevis* HIF1α cDNA and Expression Analysis

A 2.5 kb cDNA fragment was isolated from a *X. laevis* stage 17 cDNA library using the human HIF1α clone as a probe and low stringency conditions. Amino acid sequence analysis of the full-length predicted protein revealed that this cDNA encodes a protein highly similar to the human HIF1α protein (66.5% amino acid identity) and the chick HIF1α sequence (70.7%) (Fig. 1A). By contrast, it was less similar (only 46% identity) to human HIF2α. These results strongly suggest that the 2.5 kb cDNA fragment isolated encodes the *Xenopus* ortholog of mammalian HIF1α proteins. A phylogenetic analysis confirmed the HIF1α identity (see Fig. 1D and the paragraph below). Our cDNA clone was therefore names xHIF1α and the cDNA sequence was submitted to Genbank (Genbank accession no. ***DQ529235***). The deduced amino acid sequence is a polypeptide of 800 amino acids with a predicted molecular mass of 89.8 kDa. Translation in vitro gave a single protein band migrating around 90 kDa on SDS–PAGE, which is consistent with the predicted molecular mass (Fig. 2A). Substantial sequence identity was obtained with the human HIF1α in the bHLH domain (55 amino acids, 87.2%) and the PAS domain (208 amino acids, 84.6%). The sequence identity for the C-terminal half of the protein was only 58.52%; the C-terminal half is thought to be the trans-regulation region encompassing the ODD/TAD-N and TAD-C domains (see Fig. 1A,B). Importantly, proline and asparagine residues, known in mammals to be hydroxylated under normoxia (Pro402, Pro564, Asn803 in human HIF1α), were conserved in the C-terminal half of the *Xenopus* sequence (Pro403, Pro559, Asn778, respectively). The two nuclear localization signal motifs (NLS-N and NLS-C) originally identified in the mammalian HIF1α [Guillemin and Krasnow, 1997; Kallio et al., 1998] were also conserved (Fig. 1A,B). No other HIF-α sequence was isolated from the screening of the neurula library, which suggests that

HIF1α is the major isoform expressed during early developmental stages.

Recent studies have described homologs of HIFα in fish [Nikinmaa and Rees, 2005]. We compared amino acid sequences of our xHIF1α protein and various fish homologs: there was 52% amino acid identity with the published rainbow trout HIF1α (rtHIF1α; see Fig. 1A,D; [Soitamo et al., 2001]) and 53% identity with a zebrafish HIF1α sequence found in the database (Genbank accession no. ***AAQ91619***; Fig. 1D). Recently, another zebrafish HIF1α-like protein was identified (Genbank accession no. ***AY835381***; [Galloway et al., 2005]) and weak amino acid identity could be detected with xHIF1α (36%) and hHIF1α (31%) (Fig. 1D). A xHIF1α-like sequence can be found in Genbank library (Genbank accession no. ***CAB96628***). This unpublished sequence, obtained from blastulae stage, displays only 87.5% amino acid identity with our xHIF1α protein. We compared both our sequence and the unpublished sequence with the human HIF1α amino acid sequence; our sequence was more similar (66.5% identity vs. 59%). By aligning the two *Xenopus* sequences with human HIF1α, we detected some sequence errors in the ***CAB96628*** clone. This results in the appearance of the coding sequence alternatively in the three phases (data not shown). An additional xHIF1α-like sequence (Genbank accession no. ***AAH43769***) obtained from embryo stage 31/32 and provided by the *Xenopus* Initiative was also recently made available in the library. A very strong amino acid identity was observed between our sequence and this recently deposited sequence (92% identity). The slight differences between the two sequences could be due to errors associated with the large-scale of the sequencing in the *Xenopus* Initiative [Klein et al., 2002]. To exclude any sequence anomalies in our xHIF1α clone, we additionally sequenced two separate clones isolated in the process of the library screening and we confirmed the sequence shown in Figure 1A. Another explanation could be the existence of multiple HIF1α paralogs in *X. laevis*, a phenomenon common to pseudotetraploid species.

To test this last hypothesis we have performed intensive search in the database for HIF1α/HIF2α/HIF3α full-length proteins from various species, including the *Xenopus tropicalis* diploid species [Gerhard et al., 2004; Morin et al., 2007]. We also performed a multiple sequence alignment and constructed a phylogenetic tree to identify possible evolutionary relationships (Fig. 1D). The phylogenetic analysis clearly demonstrated, as already suggested in Figure 1A, that our *X. laevis* cDNA clone (***DQ529235***) was an HIF1α ortholog. Rigorous database searching allowed us to identify to date three HIF1α full-length sequences for *X. laevis* (Genbank accession no. ***CAB96628, AAH43769, AAI26006***), in addition to our HIF1α sequence (Genbank accession no. ***DQ529235***). The ***AAI26006*** and ***DQ529235*** sequences were highly related (99% amino acid sequence identity) and may correspond to the same sequence (Fig. 1D). Interestingly, the phylogenetic tree strongly suggested that the xHIF1α sequences, ***AAH43769*** and ***CAB96628***, are paralogs of ***DQ529235***, which is expected for tetraploid species (the ***AAH43769*** amino acid sequence is 92% identical to that of ***DQ529235***). In our database search, we found only one cDNA clone without annotation that encoded an HIF1α ortholog of *X. tropicalis* (Genbank accession no. ***CT030482***; Fig. 1D). This cDNA sequence was consistent with the diploid character of *X. tropicalis*. The predicted *X. tropicalis* HIF1α sequence was closely related to our *X. laevis* HIF1α protein, and the full-length proteins had 92% amino acid identity. Two full-length sequences of *X. laevis* HIF2α could be identified in the database (Genbank accession no. ***MGC80589*** and ***MGC80468***), and both share 92% amino acid sequence identity. The phylogenetic analysis strongly suggested that these two sequences were also paralogs (Fig. 1D). Once again, a single HIF2α ortholog was found for *X. tropicalis* (Genbank accession no. ***NP_001005647***). The *X. laevis* HIF2α sequence (***AAH73244***) displayed 64% amino acid identity with the human HIF2α sequence and only 45% amino acid identity with our *Xenopus* xHIF1α. In summary, the multiple sequence alignment and the deduced phylogenetic tree clearly demonstrated that at least two HIF1α paralogs, identified as HIF1α-a and HIF1α-b, and two HIF2α paralogs, identified as HIF2α-a and HIF2α-b, could be found for *X. laevis*. Only one ortholog for each HIF1α and HIF2α, was identified in *X. tropicalis* (Fig. 1D). This is consistent with the *X. laevis* genome having undergone allotetraploidization, whereas that of *X. tropicalis* did not and remains diploid. No additional forms of HIF-α proteins, for example HIF3α, were identified in our database search.

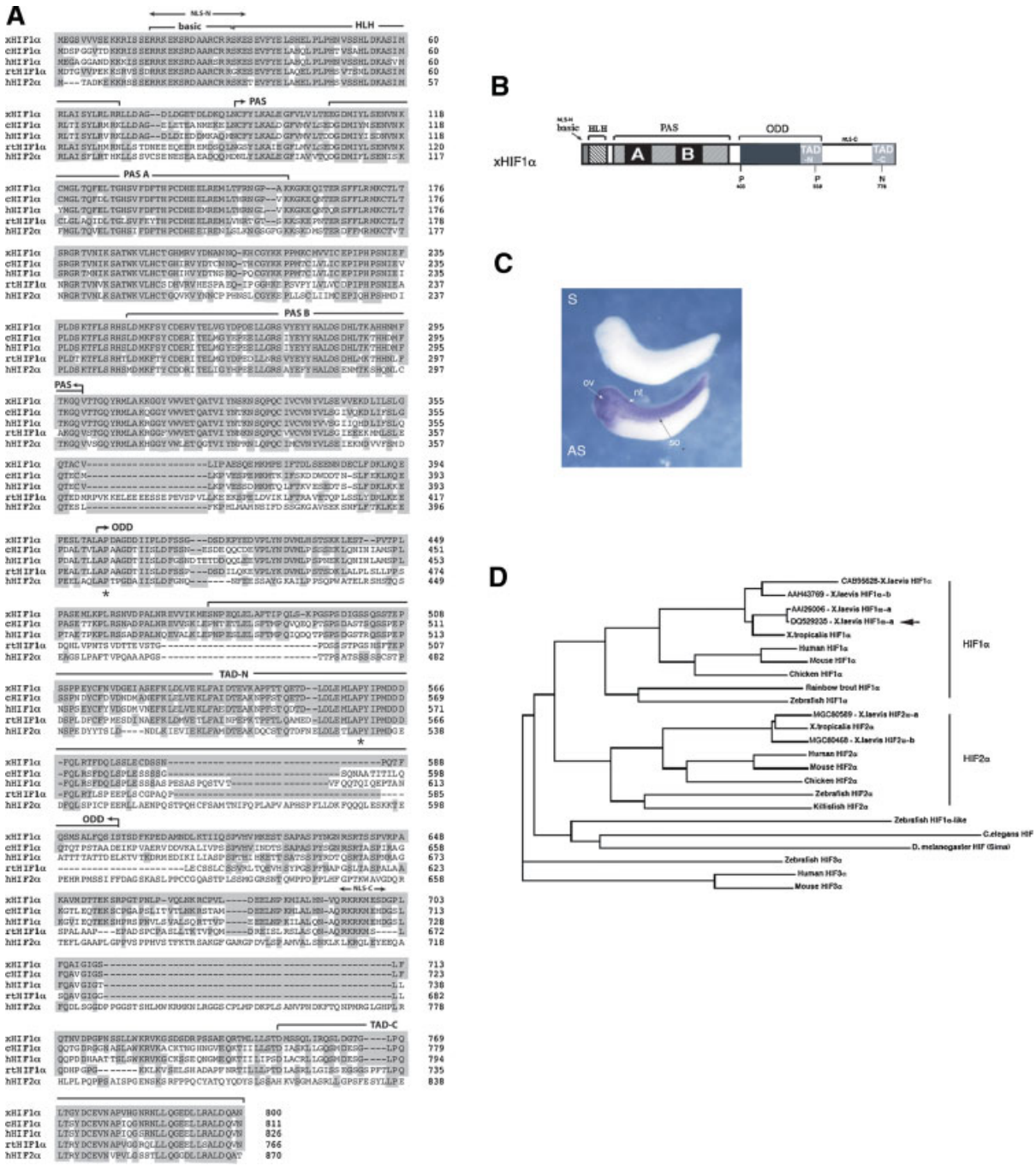

Fig. 1. **A**: Predicted amino acid sequence of the *Xenopus* HIF1α (xHIF1α, Genbank accession no. ***DQ529235***) and sequence alignment with human HIF1α (hHIF1α, Genbank accession no. ***U22431***, [Wang et al., 1995]), human HIF2α (hHIF2α, Genbank accession no. ***NP_001421***, [Tian et al., 1997]), chicken HIF1α (cHIF1α, Genbank accession no. ***ABO013746***, [Takahashi et al., 2001]), rainbow trout HIF1α (rtHIF1α, Genbank accession no. ***AF304864***; [Soitamo et al., 2001]). Conserved amino acid residues are highlighted. Dashes, deletions to maximize the sequence identity. Comparisons were performed using the ClustalW method [Thompson et al., 1994]. Basic, helix-loop-helix (HLH), Per-Arnt-Sim (PAS), PAS-A, and PAS-B motifs, oxygen degradation domain (ODD), trans-activation domain N- and C-terminal (TAD-N and TAD-C), nuclear localization signal N- and C-terminal (NLS-N and NLS-C), hydroxylation sites P and N (P, Proline; N, Asparagine; asterisk) are indicated. **B**: The sequence organization of the *Xenopus* HIF1α derived from cDNA clone sequences is shown along with important sequence motifs. **C**: Whole-mount in situ hybridization with xHIF1α antisense (AS) or sense probes (S). Lateral views of stage 32 embryos, anterior to the left. OV, optical vesical; nt, neural tube; SO, somites. **D**: A phylogram of predicted HIF-α amino acid sequences from amphibian (*Xenopus laevis* and *Xenopus tropicalis*) and other representative vertebrates. The Genbank accession numbers used in addition to those in part A of the figure are *X. tropicalis* HIF1α, ***CT030482***; mouse HIF1α, ***Q61221***; zebrafish HIF1α, ***AAQ91619***; *X. tropicalis* HIF2α, ***NP_001005647***; mouse HIF2α, ***NM010137***; chicken HIF2α, ***NP_990138***; zebrafish HIF2α, ***XP_698713***; killifish HIF2α, ***Q8QGM4***; zebrafish HIF1α-like, ***AY835381***; zebrafish HIF3α, ***Q6EGR9***; human HIF3α, ***Q9Y2N7***; mouse HIF3α, ***Q9Z2I5***; *C. elegans* HIF, ***NP_508008***; *D. melanogaster* HIF (Sima), ***AAC47303***. Deduced amino acid sequences were aligned with ClustalW and the tree shown was the result of a parsimony analysis of phylogenetic relatedness of the aligned HIF-α sequences using PAUP4.0. [Color figure can be viewed in the online issue, which is available at www. interscience.wiley. com.]

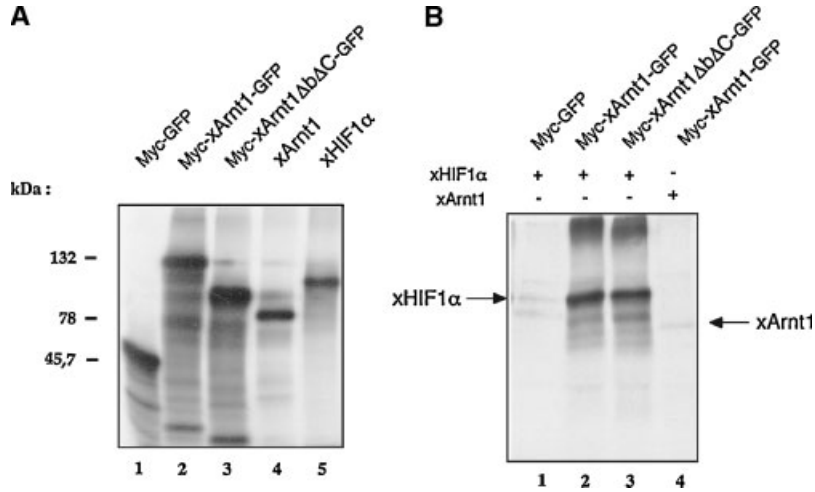

Fig. 2. In vitro interaction assay between xHIF1α and xArnt1. **A**: In vitro translation of the *Xenopus* HIF1α and wild-type/chimeric xArnt1. Proteins were synthesized by in vitro translation in rabbit reticulocyte lysate and assessed by 7.5% SDS–PAGE and autoradiography after the incorporation of [^{35}S] methionine. **B**: Co-immunoprecipitation assay of xHIF1α by chimeric proteins containing the Myc-GFP protein fused to either full-length (Myc-xArnt1-GFP, **lane 2**) or truncated (Myc-xArnt1 ΔbΔC-GFP, **lane 3**) xArnt1 factor using Myc antibody. Myc-GFP is used as a negative control (**lane 1**). Co-immunoprecipition assay of xArnt1 by the chimera protein Myc-xArnt1-GFP is shown in **lane 4**.

Sipe et al. [2004] detected maternal and zygotic expression of a xHIF1α gene during embryonic development by using xHIF1α upstream regulatory regions. By whole-mount in situ hybridization and using xHIF1α coding sequences from our cDNA, we confirmed this expression pattern (Fig. 1C and data not shown). xHIF1α was ubiquitously expressed at low levels with significantly elevated expression from the neurula stage in the head, developing nervous system, and somites (see Fig. 1D, AS; [Sipe et al., 2004]); this is consistent with previously published results. No signal was detected with a sense probe at any stage (Fig. 1D, S). A similar expression pattern has also been described for the mammalian HIF1α gene [Ema et al., 1997; Tian et al., 1997; Jain et al., 1998] and invertebrate orthologs [Nambu et al., 1996; Sonnefeld et al., 1997; Jiang et al., 2001]. Initially, no sequence homology could be identified between our HIF1α cDNA and the sequences published by Sipe et al. (Accession numbers ***AY18982, AY262064, AY262065, AY262066, AY262067***, and ***AY262068***). This may be because these sequences are mostly HIF1α upstream regulatory regions. We found an unpublished and partial coding sequence, with the accession number ***AAO72733*** and related to Sipe's studies, in the database. This sequence encompassed approximately the first 450 amino acids of HIF1α. We performed a multiple sequence alignment and phylogenetic analysis with this partial HIF1α sequence and the various *X. laevis* HIF1α full-length sequences. Our HIF1α clone had the highest homology with this partial coding sequence, ***AAO72733*** (almost 100% amino acid identity; data not shown). Sequence data analysis and gene expression patterns thus strongly suggest that both sequences correspond to the same HIF1α paralog.

xHIF1α Heterodimerizes With xArnt1

The substantial sequence identity between xHIF1α and HIF1α proteins from other species predicts that xHIF1α should participate in protein: protein interactions in much the same manner as mammalian HIF1α, that is involving the Arnt protein. This prediction is reinforced by the similar expression patterns of xArnt1 and xHIF1α genes in *Xenopus* embryos (Fig. 1C, [Sipe et al., 2004; Bollérot et al., 2001]). To determine whether xHIF1α interacts with xArnt1, we performed immunoprecipitation analyses in vitro to estimate protein dimerization. We performed in vitro translations of wild-type (xHIF1α, xArnt1), mutated and/or chimeric (Myc-GFP, Myc-xArnt1-GFP; Myc-xArnt1ΔbΔC-GFP) proteins in rabbit reticulocyte lysates. The translations were followed by immunoprecipitation as described in "Cell Transient Expression and Luciferase Assay" Section. As shown in Figure 2A, all proteins used in this assay could be translated in vitro

and we obtained proteins of the expected sizes. When the chimeric xArnt1 protein (Myc-xArnt1-GFP) was pre-incubated with radiolabeled xHIF1α, a co-immunoprecipitation of xHIF1α was observed with an anti-Myc antibody (Fig. 2B, lane 2). This suggests that the two proteins physically interacted in vitro. In the control experiment using the Myc-GFP protein, no signal was detected (Fig. 2B, lane 1); thus, xHIF1α only interacts with xArnt1. In addition, this result showed that Myc-GFP fusion did not impede the interaction between the two bHLH/PAS proteins. As a negative control, we performed a co-immunoprecipitation assay between Myc-xArnt1-GFP and xArnt1. No xArnt1 homodimerization should be detected in vitro [Coumailleau et al., 2003], and indeed, no signal was detected in our assay after immunoprecipitation (Fig. 2B, lane 4). In mammals, bHLH and PAS regions are involved in the dimerization process [Reisz-Porszask et al., 1994; Lindebro et al., 1995]. To test if the conserved HLH and PAS domains on their own were responsible for xHIF1α/xArnt1 heterodimerization, radiolabeled xHIF1α was preincubated with a truncated version of the xArnt1 protein (Myc-xArnt1ΔbΔC-GFP; xArnt1 deleted of the basic and the C-terminal half region). After immunoprecipitation, a signal with similar intensity to the wild-type protein, xArnt1, was still detected (Fig. 2B, lane 3 compared to lane 2). This demonstrates that HLH and PAS domains were sufficient for heterodimerization between xHIF1α and xArnt1. These domains were previously shown also to be involved in heterodimerization between xArnt1 and xSim (*Xenopus* single-minded protein; [Coumailleau et al., 2003]).

These results together demonstrate that xHIF1α heterodimerizes in vitro with xArnt1, and that this complex formation is mediated by the HLH and PAS domains. Interestingly, the *Xenopus* Arnt1 factor appears to be a dimerization partner for at least two *Xenopus* bHLH/PAS proteins, xHIF1α and xSim. In *Drosophila* and mammals, Arnt (or Tango in *Drosophila*) is a dimerization partner for several bHLH/PAS proteins (Ahr, HIF1/2, Sim1/2, NPAS in mammalian; Sim, trachealess, Spineless, Similar in *Drosophila*). It would be interesting to investigate if xArnt1, as in mammals and invertebrates, is an heterodimeric partner of other bHLH/PAS factors, for example the recently identified *Xenopus* aryl hydrocarbon receptors (AhR1, AhR2) and other yet unidentified bHLH/PAS factors.

xHIF1α Allows Nuclear Translocation of xArnt1

Previously, we demonstrated that most *Xenopus* Arnt1 protein is in the cytoplasm in various cell lines and co-expression of a heterodimeric partner, for example xSim, allowed its nuclear accumulation [Coumailleau et al., 2003]. A similar subcellular localization was also observed in *Drosophila* embryos (for review, see Crews and Fan [1999]). We tested the ability of another *Xenopus* bHLH/PAS factor in targeting xArnt1 to the nucleus; we performed transient over-expression studies in embryonic *Xenopus* cell lines (XTC) using the chimeric xArnt1 construct (Myc-xArnt1-GFP) alone or together with xHIF1α or xSim. GFP fluorescence was then detected in living cells by confocal imaging (Fig. 3). Myc-xArnt1-GFP expressed alone resulted in the protein being detected in the cytoplasm of XTC cells (Fig. 3a). Conversely, when Myc-xArnt1-GFP was co-expressed with xHIF1α, the GFP fluorescence was mainly detected in the nucleus of most cells (Fig. 3b). As a positive control, we co-expressed Myc-xArnt1-GFP with the xSim in similar conditions and also obtained a strong nuclear signal (Fig. 3c). This finding is consistent with our previous data (Coumailleau et al., 2003). This suggests that the xHIF1α contains enough information to allow the translocation of the heterodimeric complex xHIF1α/xArnt to the nucleus. This translocation may be mediated by the conserved nuclear localization signals in the bHLH (NLS-N) and C-terminal (NLS-C) regions of the xHIF1α (Fig. 1A,B). In addition, these results also confirmed that xHIF1α and xArnt1 were able to interact not only in vitro, but also inside cells. In normoxia conditions, mammalian HIF1α is mostly detected in the cytoplasm and under hypoxia it translocates into the nucleus [reviewed in Wenger, 2002; Kewley et al., 2004]. However, a nuclear accumulation of HIF1α with normal oxygen concentrations was also previously detected in various cell lines [Hofer et al., 2001; Zhong et al., 2002]. The nuclear accumulation observed in normoxia may be due to protein over-production, as is generally the case in cell cultures or, alternatively, to constitutive activation of HIF1α depending on the cell type used.

These results provide evidence that xHIF1α and xArnt1 are able to interact in *Xenopus*

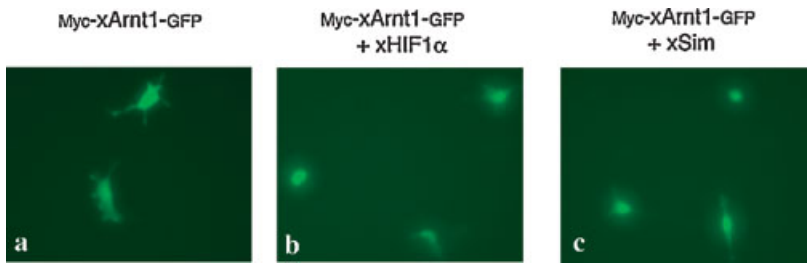

Fig. 3. Subcellular localization of xArnt1 in the absence (**a**) or in the presence of either xHIF1α (**b**) or xSim (**c**), another heterodimeric partner of xArnt1. Confocal analysis of the GFP fluorescence in unfixed *Xenopus* XTC cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

embryonic XTC cells, as strongly suggested by co-immunoprecipitations experiments, and subsequently translocate to the nucleus. xHIF1α appears to be a key factor in the nuclear translocation of the xHIF1α/xArnt1 transcriptional complex because xArnt1 is cytoplasmic in the absence of xHIF1α and nuclear in its presence.

xHIF1α Does Trans-Activate a HRE Reporter

We performed transfections in *Xenopus* XTC cells in normoxia conditions to investigate the transcriptional activity of the *Xenopus* xHIF1α and xArnt1 proteins, once the complex is translocated into the nuclear compartment. We used a pHRE/T81Luc reporter that contains three repeats of the consensus DNA binding site for HIF1α, also named HRE, upstream from the luciferase reporter (see "Cell Transient Expression and Luciferase Assay" Section; [Carrero et al., 2000]). As a positive control for this trans-activation assay, human HIF1α and human Arnt1 were used as they were efficient in trans-activating the same reporter under conditions of normoxia and in the COS-7 cell line [Carrero et al., 2000; Ruas et al., 2002]. A potent trans-activation (around 10-fold) was observed when the human HIF1α was co-tranfected with the human Arnt1 in XTC cells (Fig. 4A, comparing lanes 8 and 1). By contrast, a weak trans-activation was observed when the human Arnt1 was transfected alone (Fig. 4A, comparing lane 3 with lanes 1 and 8). Similarly, the xHIF1α protein strongly trans-activated the reporter gene when co-expressed with xArnt1 (Myc-xArnt1-GFP; Fig. 4A, lane 4). A weak trans-activation was detected when xArnt1 was expressed alone (Fig. 4A, lane 2). These results demonstrate that the xHIF1α/xArnt1 complex is transcriptionally active and targets the same DNA sequence element (HRE) as the mammalian HIF/Arnt complex. Moreover, these luciferase activities were in perfect agreement with the subcellular localization described above: the strongest reporter activity was observed when the xHIF/xArnt heterodimer was located in the nucleus, that is when both factors were over-produced in cells. The weak signals observed when xArnt1 (lane 2) or hArnt1 (lane 3) were expressed alone may be due to the presence of endogeneous xHIF1α. In addition, these results demonstrated that the presence of GFP and Myc in the *Xenopus* chimeric Arnt1 (Myc-xArnt1-GFP) did not affect trans-activation properties. As expected, the truncated xArnt1 version, Myc-xArnt1ΔbΔC-GFP, where both the DNA binding region (b, basic domain) and the trans-activation domain (C, C-terminal region) are deleted, was unable to transactivate the reporter gene efficiently when co-transfected with xHIF1α (Fig. 4A, lane 6). Efficient trans-activation was obtained when xHIF1α was combined with human Arnt1 (Fig. 4A, lane 7) or human HIF1α combined with *Xenopus* Arnt1 (Fig. 4A, lane 5). These results demonstrate that bHLH/PAS factors from different species are able to heterodimerize in amphibian cells and to trans-activate a reporter gene.

We tested the inducibility of xHIF1α activity by performing trans-activation experiments in hypoxia conditions. We used various chemicals that mimic hypoxia, for example CoCl2, the Fe-chelator desferrioxamine and 2,2′ dipyridyl. We report the results obtained in an XTC cell line over-producing xHIF1α and exposed to 2,2′ dipyridyl (Fig. 4B). Under hypoxia conditions, transactivation was about twofold that in normoxia conditions (compare lane 2 and lane 3). Similar results were obtained using other chemicals and/or COS7 cells. No difference was observed when cells were not transfected with exogeneous xHIF1α protein (data not shown). These data clearly show that the xHIF1α activity can be regulated by hypoxia conditions, like its vertebrate orthologs.

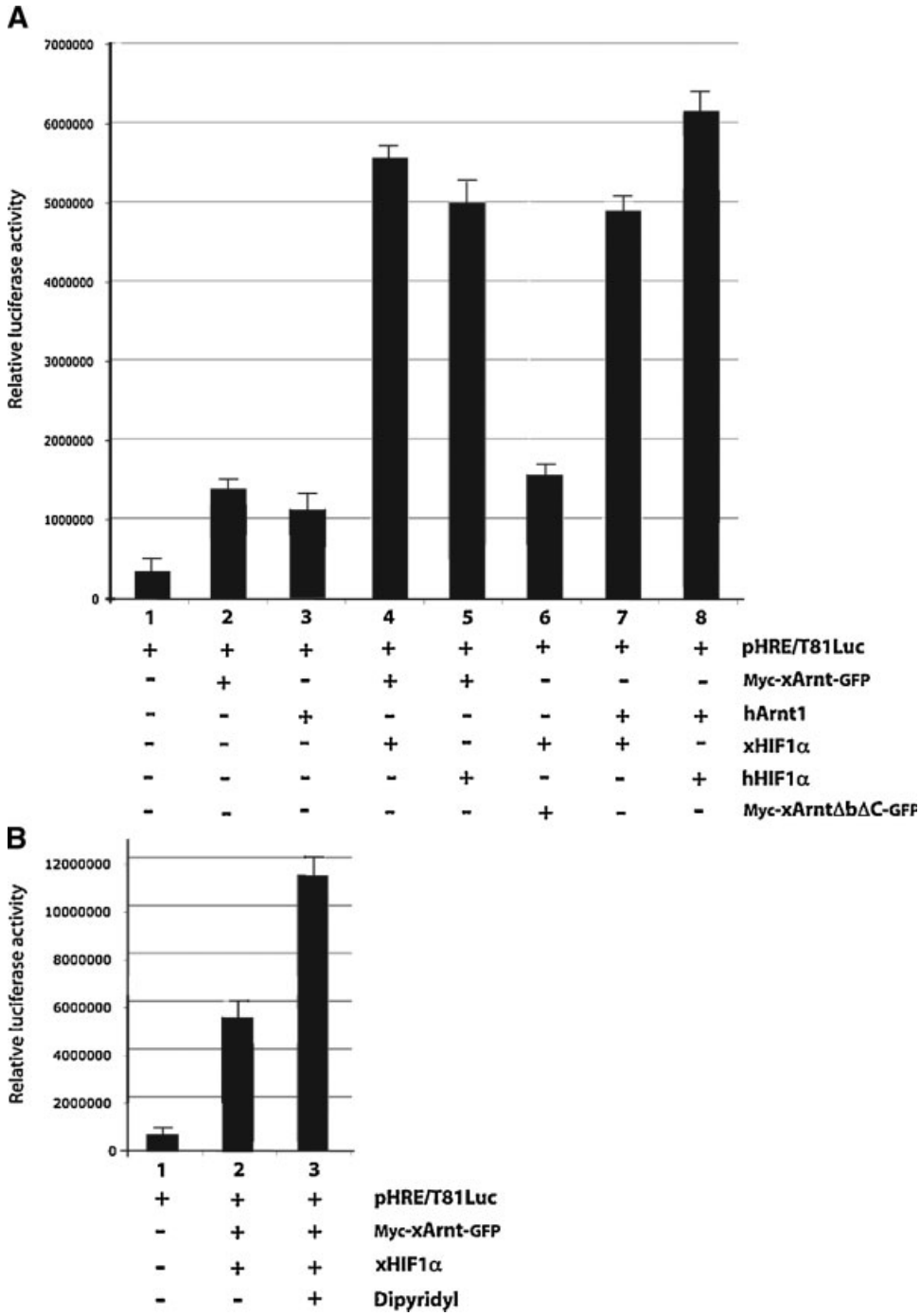

Fig. 4. **A**: Trans-regulation properties of the *Xenopus* HIF1α using an HRE luciferase reporter (pHRE/T81Luc) and XTC cells in normoxia. Human HIF1α and Arnt1 (hHIF1α, hArnt1) were used as controls. **B**: Trans-regulation properties of the xHIF1α in normoxia and hypoxia-mimicking conditions (dipyrydil). Each transfection was repeated at least twice and the error bars represent standard error.

The conserved HIF1α amino acid sequence, the dimerization of xHIF1α with xArnt1, the subcellular localization and trans-activation properties of the xHIF1α/xArnt1 complex in both normoxia and hypoxia conditions strongly suggest that the HIF1α transcriptional pathway is conserved in the amphibian *X. laevis*. Thus, HIF1α is an evolutionarily conserved transcriptional regulator found in most, if not all, multicellular animals. Further investigations of the function of xHIF1α will be necessary to determine if the *Xenopus* HIF(s) ortholog(s),

like mammalian HIF(s), play a central role in the regulation of gene expression during hypoxia in the adult, and also in vascular development during embryogenesis.

ACKNOWLEDGMENTS

We are grateful to Professor Kintner for his generous gifts of the neurula *Xenopus* library.

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