The mammalian hypoxia-inducible factor-1 (HIF-1) is a heterodimeric transcription factor that controls the induction of several genes involved in glycolysis, erythropoiesis, and angiogenesis when cells are exposed to hypoxic conditions. Until now, the expression and function of HIF-1α have not been studied in fish, which experience wide fluctuations of oxygen tensions in their natural environment. Using electrophoretic mobility shift assay, we have ascertained that a hypoxia-inducible factor is present in rainbow trout cells. We have also cloned the full-length cDNA (3605 base pairs) of the HIF-1α from rainbow trout with a predicted protein sequence of 766 amino acids that showed a 61% similarity to human and mouse HIF-1α. Polyclonal antibodies against the N-terminal part (amino acids 12–363) and the C-terminal part (amino acids 330–730) of rainbow trout HIF-1α protein recognized rainbow trout and chinook salmon HIF-1α protein in Western blot analysis. Also, the human and mouse HIF-1α proteins were recognized by the N-terminal rainbow trout anti-HIF-1α antibody but not by the C-terminal HIF-1α antibody. The accumulation of HIF-1α was studied by incubating rainbow trout and chinook salmon cells at different oxygen concentrations from 20 to 0.2% O2 for 1 h. The greatest accumulation of HIF-1α protein occurred at 5% O2 (38 torr), a typical oxygen tension of venous blood in normoxic animals. The protein stability experiments in the absence or presence of a proteasome inhibitor, MG-132, demonstrated that the inhibitor is able to stabilize the protein, which normally is degraded via the proteasome pathway both in normoxia and hypoxia. Notably, the hypoxia response element of oxygen-dependent degradation domain is identical in mammalian, Xenopus, and rainbow trout HIF-1α proteins, suggesting a high degree of evolutionary conservation in degradation of HIF-1α protein.

The oxygen content, especially in freshwater environments, varies markedly daily, seasonally, and spatially. Due to the low oxygen capacitance of water, respiration of organisms and breakdown of organic material can cause large decreases in oxygen tensions especially during the night when oxygen-producing photosynthesis does not occur. It is thus not surprising that the large variations in environmental oxygen levels have played a significant role in the evolution of fishes. Therefore, fish have developed various physiological and biochemical adaptations to enable survival in hypoxic and anoxic environments, including air breathing organs (1), specialized metabolic pathways enabling long term anoxic survival (2), and modifications of the hemoglobin molecules to optimize oxygen transport (3).

Due to the variability of oxygen content in water and the pronounced role that oxygen has played in the evolution of structure and function of fishes, they present a unique opportunity to study the evolution, function, and regulation of oxygen-dependent genes and their role in the environmental adaptation. Up to the present, this possibility has been poorly utilized. In mammals, more than 40 hypoxically regulated genes have been characterized (4), including those for the glucose transporter, several enzymes of the glycolytic pathway, erythropoietin, transferrin, and the vascular endothelial growth factor. In contrast, although up-regulation of the synthesis of several proteins in hypoxic fish has been described (5), the identity of these hypoxia-inducible proteins is not known.

In mammals, oxygen-dependent gene expression is transcriptionally regulated by the hypoxia-inducible factor-1 (HIF-1), a heterodimer that consists of two subunits initially called HIF-1α and HIF-1β (6, 7). HIF-1α is unique for the hypoxia response, whereas HIF-1β turned out to be identical to the aryl hydrocarbon nuclear translocator (ARNT), which acts as a dimerization partner also for other transcription factors, among them the aryl hydrocarbon receptor (AhR, dioxin receptor (8)). Both HIF-1α and ARNT belong to the basic-helix-loop-helix (bHLH)-PAS family of proteins. All of these proteins have characteristic N-terminal bHLH and PAS domains. The bHLH domain is required for DNA binding and dimerization, whereas PAS domain is involved in heterodimerization, DNA binding, and transcriptional activation.
Characterization of HIF-1α Protein from Rainbow Trout

**TABLE I**

**PCR primers used in RT-PCR (ba1 and hi375) and 5’-3’ RACE**

| Primer  | Sequence                                      |
|---------|-----------------------------------------------|
| ba1     | 5’-TTG TGA AGC TCG AAA AGA AAA-3’             |
| hi375   | 5’-AGG ATG AGG CTC CTT CTT-3’                 |
| XSC-dT7 primers | 5’-GAC TCG AGT CAT CG-5’-3’               |
| RAC1    | 5’-AGT TCT TGT TGT TGT AGA TAA-3’            |
| XSC     | 5’-GAC TCG AGT CAT CG-3’                      |
| PAC3    | 5’-TGG AGT CTG ACA TGA CTG AGT TTA AAC TGG ACA TGG TGG AAA-3’ |
| RAT5    | 5’-CTT GTC GAT TTC AGC GAG ATA-3’            |
| ATF3    | 5’-TCA TGG TCT GTG CTG TGC AGC AGA TT-3’      |

**TABLE II**

A cDNA sequence comparison between two rainbow trout RT-PCR products (rb2a and rb5a) and human HIF-1α cDNA (nucleotides 345-1388, GenBank™ accession number U22431)

| hHIF-1α (cDNA 345-1388) | Identity | Similarity |
|-------------------------|----------|------------|
|                         | %        | %          |
| rb2 (RT-PCR)            | 66       | 66         |
| rb5 (RT-PCR)            | 67       | 67         |

- **transactivation**, and probably also in HSP90 ligand binding (9, 10). Several other members of bHLH-PAS family of proteins in mammals have recently been cloned that show a high homology to the HIF-1α such as HIF-2α, also termed EPAS (endothelial PAS domain protein (11)), HLF (HIF-1α-like factor (12)), HRF (HIF-related factor (13)), MOP2 (member of PAS superfamily 2 (14)), and HIF-3α (15). Hitherto, there are no reports on a hypoxia-inducible factor in fish. The previously characterized bHLH-PAS family proteins in fish play a role in transcriptional regulation induced by xenobiotics. To date, fish aryl hydrocarbon receptor (AhR) and at least two isoforms of its dimerization partner ARNT have been cloned (see Ref. 16).

- Since hypoxia-inducible transcription activators have not been characterized in fish, it is obvious that the mechanisms and conditions by which the up-regulation of protein synthesis occurs in hypoxic fish have not been elucidated. In mammals, the activation of hypoxic gene expression occurs at various levels. Although it appears that the mRNA for HIF-1α is constitutively expressed (17, 18), the levels of HIF-1α protein are markedly higher in hypoxic than in normoxic conditions. Under normoxic conditions, the HIF-1α protein is rapidly ubiquitinated and degraded by the 26 S proteasome, the half-life being less than 5 min (19, 20). However, a shift of cells to hypoxic conditions stabilizes and enables HIF-1α protein to translocate from the cytoplasm to the nucleus, where it heterodimerizes with HIF-1β (21). In addition, hypoxic conditions enhance the transactivating function of HIF-1α (22, 23). Although the stabilizing effects of hypoxia on the HIF-1α protein have been clearly demonstrated, the exact relationship between oxygen tension and the stability of the protein has not been elucidated. Furthermore, the possible differences in the oxygen thresholds of the hypoxia response between different cell types have not been investigated, and the possible effects on the HIF-1 response of previous acclimation to hypoxic conditions have remained unclarified. These questions are conveniently investigated using fish, which experience large variations in the ambient oxygen levels in their normal life. To enable such experiments, it is necessary to characterize the first fish HIF-1α protein from rainbow trout. Using recombinant fish HIF-1α protein, we have generated polyclonal antibodies recognizing fish HIF-1α protein. These antibodies have been utilized to investigate the oxygen tension dependence of HIF-1α protein levels in several types of fish cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Hypoxia Treatment**—Rainbow trout hepatocytes were isolated according to Räbergh et al. (24). The number of cells was calculated using a hemocytometer, and viability was tested with trypan blue solution (Sigma). The cells were diluted to a density of 5 × 10⁶ cells/ml. Ten ml of hepatocyte suspension was incubated overnight at 18 °C before hypoxia treatments. The human epithelial carcinoma cell line HeLa was cultured in Dulbecco’s modified Eagle’s medium (high glucose; Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The rainbow trout HIF-1α cDNA, called r33a, cloned into a Bluescript KS+ vector (Strategene) was cut with either SacI or HincII. The SacI fragment was ligated into the pGem-T vector (Promega) according to the manufacturer’s recommendations. This cDNA was used as a template to obtain a full-length cDNA, in which the primers were based on the mouse HIF-1α sequence. The primer pair of the ba1 primer within the basic region of HIF-1α and the hi375 primer distal to the PAS B region of HIF-1α sequence (Table I) produced two 1100-base pair RT-PCR fragments. The nucleotide sequence of these two products (termed rb2 and rb5a) differed only by 1%. Both PCR fragments were then cloned into a pGEM-T vector (Promega) according to the manufacturer’s instructions. The rb5a DNA fragment was used as a probe to screen a juvenile rainbow trout UNI-ZAP™ XR cDNA library (a kind gift of Dr. Thomas Chen, University of Connecticut). To find the correct 5’ and 3’ ends of the cDNAs, we used 5’-3’ rapid amplification of cDNA ends (RACE) according to Frohman et al. (28) using the primers listed in Table I. Briefly, 5 μg of total RNA from rainbow trout hepatocytes was reverse-transcribed either with the XSC-dT7 primer (for 5’ RACE) or the RAC1 primer (for 5’ RACE). In 3’ RACE, the cDNA was then amplified by PCR using the XSC-dT7 and PAC3 PCR primers. In 5’ RACE, the RAC1 primer was removed using 30-kDa Centricon concentrators (Millipore Inc.), and the cDNAs were polyadenylated using terminal deoxynucleotidyltransferase. The cDNAs were then amplified with the XSC-dT7, and RAT5 primer pair followed by a second round of amplification with the XSC and ATF3 primer pair.

**Generation of Polyclonal Antibodies against Rainbow Trout HIF-1α**—Two different peptides were constructed to produce antibodies against both the N-terminal and the C-terminal part of HIF-1α. The rainbow trout HIF-1α cDNA, called r33a, cloned into a Bluescript KS+ vector (Strategene) was cut with either SacI or HincII. The SacI fragment was ligated into the pGem-T vector (Promega) according to the manufacturer’s instructions. The rb5a DNA fragment was used as a probe to screen a juvenile rainbow trout UNI-ZAP™ XR cDNA library (a kind gift of Dr. Thomas Chen, University of Connecticut). To find the correct 5’ and 3’ ends of the cDNAs, we used 5’-3’ rapid amplification of cDNA ends (RACE) according to Frohman et al. (28) using the primers listed in Table I. Briefly, 5 μg of total RNA from rainbow trout hepatocytes was reverse-transcribed either with the XSC-dT7, primer (for 3’ RACE) or the RAC1 primer (for 5’ RACE). In 3’ RACE, the cDNA was then amplified by PCR using the XSC-dT7 and PAC3 PCR primers. In 5’ RACE, the RAC1 primer was removed using 30-kDa Centricon concentrators (Millipore Inc.), and the cDNAs were polyadenylated using terminal deoxynucleotidyltransferase. The cDNAs were then amplified with the XSC-dT7, and RAT5 primer pair followed by a second round of amplification with the XSC and ATF3 primer pair.

**Cloning of Rainbow Trout HIF-1α cDNA**—Total RNA was isolated from RTG-2, CHSE-214 cells, and freshly isolated rainbow trout hepatocytes using the RNAzol™B method (TEL-TEST Inc.) based on the method described by Chomczynski and Sacchi (27). cDNA synthesis was performed with 5 μg of total RNA using avian myeloblastosis virus reverse transcriptase (Promega) and oligo(dT) primers (Invitrogen) according to the manufacturer’s recommendations. This cDNA was used as a template in reverse transcription-PCR, in which the primers were based on the mouse HIF-1α sequence. The primer pair of the ba1 primer within the basic region of HIF-1α and the hi375 primer distal to the PAS B region of HIF-1α sequence (Table I) produced two 1100-base pair RT-PCR fragments. The nucleotide sequence of these two products (termed rb2 and rb5a) differed only by 1%. Both PCR fragments were then cloned into a pGEM-T vector (Promega) according to the manufacturer’s instructions. The rb5a DNA fragment was used as a probe to screen a juvenile rainbow trout UNI-ZAP™ XR cDNA library (a kind gift of Dr. Thomas Chen, University of Connecticut). To find the correct 5’ and 3’ ends of the cDNAs, we used 5’-3’ rapid amplification of cDNA ends (RACE) according to Frohman et al. (28) using the primers listed in Table I. Briefly, 5 μg of total RNA from rainbow trout hepatocytes was reverse-transcribed either with the XSC-dT7 primer (for 3’ RACE) or the RAC1 primer (for 5’ RACE). In 3’ RACE, the cDNA was then amplified by PCR using the XSC-dT7 and PAC3 PCR primers. In 5’ RACE, the RAC1 primer was removed using 30-kDa Centricon concentrators (Millipore Inc.), and the cDNAs were polyadenylated using terminal deoxynucleotidyltransferase. The cDNAs were then amplified with the XSC-dT7, and RAT5 primer pair followed by a second round of amplification with the XSC and ATF3 primer pair.
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A

ATG bHLH PAS A/B TGA PolyA (33)
+1 301 2601 3572

r3α (327) (3605)
r5α (580) (3605)
r3α (582) (2980)

B

rtHIF-1α bHLH PAS A PAS B TAD-N TAD-C ODD

hHIF-1α 81% 72% 83% 37% (ins 7) 70% (del 4)

xHIF-1α 70% 74% 77% 37% (ins 18) 67% (del 5)

hHIF-2α 81% 58% (ins 2) 69% 25% (ins 49) 58% (del 4)

C

rtHIF-1α, human (h) HIF-1α, Xenopus (x) HIF-1α, and human HIF-2α. Amino acid identities are presented as percentages. The number of amino acid changes by insertions (ins) or deletions (del) compared with rtHIF-1α is indicated below the amino acid identities. C. A comparison of the predicted amino acid sequences of rainbow trout HIF-1α (GenBank accession number AF304864) to Xenopus laevis HIF-1α (GenBank accession number CAB96628), human HIF-1α (GenBank accession number AAC68568), rat (r) HIF-1α (GenBank accession number AA024443), and mouse (m) HIF-1α (GenBank accession number AA024443), human (h) HIF-1α (GenBank accession number AA024443), mouse (m) HIF-1α (GenBank accession number AA024443), and mouse (m) HIF-1α (GenBank accession number AA024443). Nuclear localization signals (NLS-N, NLS-C), bHLH and PAS A/B regions, transactivation domains (TAD-N, TAD-C), and the oxygen-dependent degradation domain (ODD) are indicated above the sequence comparisons. The comparison is made using ClustalW multiple sequence alignment and BOX SHADE programs (BCM).

Fig. 1. cDNA cloning of rainbow trout HIF-1α. A, schematic presentation of different rainbow trout HIF-1α cDNA clones. The length of different cDNAs is related to the length of HIF-1α mRNA, where +1 corresponds to the 5′ end of the transcript. B, comparison of the amino acid identities between rtHIF-1α, human (h) HIF-1α, Xenopus (x) HIF-1α, and human HIF-2α. Amino acid identities are presented as percentages. The number of amino acid changes by insertions (ins) or deletions (del) compared with rtHIF-1α is indicated below the amino acid identities. C. A comparison of the predicted amino acid sequences of rainbow trout HIF-1α (GenBank accession number AF304864) to Xenopus laevis HIF-1α (GenBank accession number CAB96628), human HIF-1α (GenBank accession number AAC68568), rat (r) HIF-1α (GenBank accession number AA024443), and mouse (m) HIF-1α (GenBank accession number AA024443), human (h) HIF-1α (GenBank accession number AA024443), mouse (m) HIF-1α (GenBank accession number AA024443), and mouse (m) HIF-1α (GenBank accession number AA024443). Nuclear localization signals (NLS-N, NLS-C), bHLH and PAS A/B regions, transactivation domains (TAD-N, TAD-C), and the oxygen-dependent degradation domain (ODD) are indicated above the sequence comparisons. The comparison is made using ClustalW multiple sequence alignment and BOX SHADE programs (BCM).
HindII DNA fragment of rainbow trout HIF-1α cDNA using standard methods (33).

Western Blot Analysis—Protein samples (10–30 μg/well) were resolved by denaturing electrophoresis on 7.5% SDS-polyacrylamide slab gels (34) and transferred to a nitrocellulose membrane (Schleicher & Schuell) according to Sambrook et al. (33). The membrane was blocked for 2 h in 3% nonfat dry milk in PBS, 0.3% Tween 20, rinsed, and subsequently incubated with the primary antibody diluted in 1% bovine serum albumin, PBS ranging from 1:100 to 1:1000 for 1 h at room temperature. The membrane was washed with PBS, 0.3% Tween 20 and incubated with the secondary antibody (horseradish peroxidase-conjugated anti-rabbit antibody, Amersham Pharmacia Biotech) diluted 1:10,000 in 3% nonfat milk in PBS, 0.3% Tween 20. After washing the membrane, the rainbow trout HIF-1α protein was detected using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

RESULTS AND DISCUSSION

Cloning of Rainbow Trout HIF-1α cDNA—RT-PCR was used to generate rainbow trout-specific HIF-1α gene fragments. Two isoformic cDNAs (rb2a and rb5) with sizes of 1100 base pairs were identified (Table II). These cDNA fragments had 66 and 67% nucleotide sequence identity to human HIF-1α, respectively, and were therefore considered to be the first fish HIF-1α sequences. The rb5 DNA fragment that had slightly higher sequence homology to human HIF-1α cDNA was used as a probe in screening a cDNA library from juvenile rainbow trout. As shown in Fig. 1A, four cDNA clones were obtained that had similar and overlapping cDNA sequences. These cDNA sequences were missing the 5′ end of the cDNA, including ATG, the start codon. The missing 5′ end of cDNA sequence was isolated by 5′-3′ RACE. Furthermore, the previously found 3′ end of the cDNA could be verified. The 3′ end had an atypical polyadenylation signal (CATAAA) 5′ to the normal site of polyadenylation. In addition, the 3′-untranslated region contained only three AUUU intron instability elements in rainbow trout HIF-1α, whereas human HIF-1α has been shown to contain eight such elements (7).

Total cDNA of rainbow trout (rt) HIF-1α was 3605 base pairs long, with an open reading frame of 766 amino acids. Thus, the rainbow trout HIF-1α was slightly smaller in size than the corresponding HIF-1α in human (826 amino acids), mouse, or rat (810 amino acids). The predicted rtHIF-1α amino acid sequence (Table III) had a 61% similarity to the human, mouse, and rat HIF-1α, about 52% similarity to the human and mouse HIF-2α, and 46% similarity to the human and mouse HIF-3α.

Although the overall similarity of the predicted amino acid sequence of rtHIF-1α was much closer to human HIF-1α than HIF-2α, the sequence corresponding to the bHLH domain had a similar homology to both human HIF-1α and to HIF-2α (Fig. 1B). Interestingly, the homology to the human proteins was greater than to the HIF-1α from the amphibian Xenopus laevis. However, the bHLH/PAS A/B regions appear to be relatively well conserved, with 70–90% similarity between the different proteins. Another conserved sequence was the hypoxia response element of the oxygen-dependent degradation domain, which comprises of amino acids 557–571 in human HIF-1α protein (35); it was identical in all the HIF-1α proteins examined. In contrast, the transactivation domains (TAD), especially the TAD-N (20, 22) sequence, varied considerably between the different proteins; there was less than 40% identity in the TAD-N sequence between the proteins (Figs. 1, B and C).

The cloning of rtHIF-1α indicates that the encoding gene is present in rainbow trout genome. Its presence was further ascertained using Southern blot analysis (Fig. 2). The cleavage of rainbow trout genomic DNA with either EcoRI or HindIII restriction enzymes produced a band of 8-kilobase pairs in the rainbow trout genome (Fig. 2).

Rainbow Trout HIF-1α mRNA Is Constitutively Expressed—Using Northern blot analysis, the expression pattern of HIF-1α was determined in rainbow trout gonad (RTG-2) and chinook salmon embryonic (CHSE-214) cells exposed to hypoxia for 2 and 4 h (Fig. 3). The levels of HIF-1α mRNA, normalized to 18 S rRNA levels, showed no change during a 2-h hypoxia treatment and only a slight decrease after a 4-h hypoxia treatment. This is in agreement with the notion that HIF-1 activation during hypoxia is due to posttranslational mechanisms (17, 18, 36). Increased mRNA levels have also been reported (37–39), possibly as a result of increased stability of mRNA in hypoxia (4).

The HIF-α DNA Binding Complex in Rainbow Trout Is Smaller than in Mammals—Since the hypoxia response elements (HREs) of hypoxia-inducible genes have not been characterized in rainbow trout, we performed electrophoretic mobility shift assay using the HRE of the human erythropoietin gene (Fig. 4A). As a control for HIF-1α DNA binding, we used nuclear extracts from hypoxia-treated HeLa cells (Figs. 4, A and C). In fish RTG-2 cells, the induction of the HIF-1α complex in nuclear extracts was detected after 2 h of hypoxia treatment (1% O2), and the amount of HIF-1α complex was further increased after a 4-h treatment (Fig. 4A). Interestingly, the HIF-1α complex of nuclear extracts isolated from RTG-2 cells migrated faster in a 4% nondenaturing polyacrylamide gel than that isolated from HeLa cells, suggesting a lower molecular weight of the HIF-1 complex in rainbow trout. This is in agreement with data obtained from the HIF-1α cDNA cloning, suggesting that HIF-1α protein in rainbow trout has smaller molecular weight than the corresponding human HIF-1α protein. Both isoforms of HIF-1α (ARNT) in rainbow trout are also smaller (70 and 79 kDa (40)) than the corresponding mammalian proteins (91 to 94 kDa (6)). The formation of the HIF-1α-HRE complex was also observed after 4 h of hypoxia in primary-cultured rainbow trout hepatocytes (Fig. 4B). The specificity of the HIF-1α DNA binding was demonstrated for the hepatocytes by adding a 500 μ excess of unlabeled eryth-

| TABLE III | Amino acid comparison between different HIFs |
|------------|---------------------------------------------|
| rtHIF-1α (amino acids, 1–766) | Identity | Similarity |
| hHIF-1α | 47 | 61 |
| mHIF-1α | 47 | 61 |
| rHIF-1α | 47 | 61 |
| xHIF-1α | 43 | 57 |
| hHIF-2α | 37 | 52 |
| mHIF-2α | 37 | 51 |
| rHIF-2α | 37 | 50 |
| hHIF-3α | 32 | 46 |
| mHIF-3α | 32 | 46 |

**Fig. 2.** HIF-1α gene is present in rainbow trout genomic DNA. A, Southern blot analysis was performed on genomic DNA isolated from rainbow trout using the SacI fragment of rainbow trout cDNA as a probe. The genomic DNA was cleaved using two restriction enzymes, EcoRI and HindIII. The DNA marker indicates the size of genomic DNA fragments in kilo base pairs (kbp).
Characterization of HIF-1α Protein from Rainbow Trout

Fig. 3. HIF-1α mRNA is constitutively expressed. The amount of HIF-1α mRNA was quantified using Northern blot analysis after treatment of rainbow trout gonad (RTG-2) and chinook salmon embryonic (CHSE-214) cells under normoxia (0 h) and hypoxia (1% O2) for 2 and 4 h. The level of HIF-1α mRNA was normalized to the ribosomal 18 S rRNA (lower panel). The level of HIF-1α mRNA under hypoxia was compared with the level of HIF-1α mRNA under hypoxia for 2 and 4 h. The level of HIF-1α mRNA under hypoxia was used as control (defined as 1.0). Numerical data are based on four independent experiments.

Characterization of Antibodies Produced against Rainbow Trout HIF-1α Protein—Two polyclonal antibodies were generated against the rainbow trout HIF-1α protein, one against the N-terminal part and another against the C-terminal part. Initial characterization of these antibodies using crude serum indicated that the antibody produced against the N-terminal part of the recombinant rainbow trout HIF-1α protein recognized a hypoxia-inducible protein both in HeLa cells and in rainbow trout hepatocytes (Fig. 5A). In contrast, the antibody produced against the C-terminal part only recognized fish proteins in a hypoxia-dependent manner (Figs. 4B and 5A). This result is expected, since the N-terminal part of the protein shows more homology across the animal groups than the C-terminal part.

To investigate whether the C-terminal rainbow trout anti-HIF-1α antibody recognized a HIF-1α protein in fish cell lines, two trout cell lines were exposed to hypoxia for 4 h. The antibody recognized a hypoxia-inducible protein of 95 kDa in both rainbow trout gonad (RTG-2) and chinook salmon embryonic (CHSE-214) cells (Fig. 5B) based on calculation of the mobility of HIF-1α protein to the standard molecular weights.

Stabilization of HIF-1α Protein Occurs under Physiological Oxygen Concentrations—Since the initial screening indicated that the crude serum containing antibodies against both N- and C-terminal parts of the HIF-1α recombinant protein reacted with hypoxia-inducible proteins in fish cells, the antisera were further purified in order to study the behavior of HIF-1α protein in normoxic and hypoxic conditions (for details, see "Experimental Procedures"). After the purification, the specificity of antibodies was greatly increased, and apart from the hypoxia-specific recognition, only some unspecific bands remained that could be used as markers for equal loading in Western blot analysis after normoxic and hypoxic treatments (Fig. 6).

Consequently, the antibodies were used to investigate how oxygen levels affect the amount of HIF-1α protein in rainbow trout gonad (RTG-2) and chinook salmon embryonic (CHSE-214) cells. Since the mRNA levels were not affected by hypoxia (Fig. 3), changes in the HIF-1α protein levels would indicate increased stability of the protein. In CHSE cells, the HIF-1α protein accumulated already at 10% oxygen (76 torr) (Fig. 6). The accumulation of HIF-1α protein was maximal at 5% O2 (38 torr) in both cell types. Below 5% oxygen (38 torr), the HIF-1α protein levels tended to decrease, and there was a pronounced drop in the level of HIF-1α at the lowest oxygen level (0.2% = 1.5 torr). Thus, the fish HIF-1α appears to accumulate at much higher oxygen levels than mammalian HIF-1α, which shows a half-maximal response between 1.5 and 2% O2 and maximal response at 0.5–1% O2 (41, 42). Ambient oxygen levels of 55–60
torr are easily tolerated by the active rainbow trout, resulting in arterial oxygen tension of 35–45 torr and venous oxygen tensions of 15–25 torr (43). In normoxic conditions (140–150 torr), the arterial and venous oxygen tensions are around 100 and 30–40 torr, respectively (43). Thus, oxygen levels under which HIF-1α protein accumulates during the in vitro incubation are routinely experienced by fish cells in vivo. Consequently, it is possible that oxygen-dependent gene regulation forms an important component of regulation of gene expression in fishes, not only in extreme conditions and during environmental hypoxia but also in more or less normoxic conditions.

In mammals, the rapid degradation of HIF-1α protein occurs by a ubiquitin-proteasome pathway that is inhibited by hypoxia (19, 20, 44). To study whether this applies also to fish, we carried out experiments with proteasome inhibitor MG-132. Treatment with MG-132 slowed down the degradation of fish HIF-1α protein under normoxia, hypoxia, and during re-oxygenation (Fig. 7). The mechanism of degradation and stabilization of HIF-1α protein is therefore most likely the same in man and fish. Interestingly, although the oxygen-dependent degradation domain of HIF-1α protein generally shows only 47% similarity between rainbow trout and man, a critical hypoxia response element of oxygen-dependent degradation domain (35) is identical. This element appears to bind von Hippel-Lindau tumor repressor protein (VHL), which functions as a ubiquitin ligase, directing HIF-1α protein degradation (45–47).

In conclusion, our results show that HIF-1α is present in fish. The predicted amino acid sequence shows high level of conservation at the bHLH/PAS A/B region, whereas there are large variations in the transactivation domains among vertebrates. The HIF-1α levels in fish cells are regulated via the ubiquitin-proteasome pathway as has been shown in mammals, and the protein shows similar oxygen-dependent DNA binding as other known hypoxia-inducible transcription factors. However, the HIF-1α of rainbow trout and chinook salmon cells is stabilized at much higher oxygen levels than previously reported for mammals, suggesting a role for oxygen-regulated gene expression in the normal physiology of these fish.

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