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Hypoxia-inducible factor 1α cDNA cloning and its mRNA and protein tissue specific expression in domestic yak (*Bos grunniens*) from Qinghai-Tibetan plateau

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Abstract

Adaptation to hypoxia is regulated by hypoxia-inducible factor 1 (HIF-1), a heterodimeric transcription factor consisting of an oxygen-regulated α-subunit and a constitutively expressed β-subunit. How animals living on Qinghai-Tibetan plateau adapt to the extreme hypoxia environment is known indistinctly. In this study, the Qinghai yak which has been living at 3000–5000 m altitude for at least two millions of years was selected as the model of high hypoxia-tolerant adaptation species. The HIF-1α ORFs (open reading frames) encoding for two isoforms of HIF-1α have been cloned from the brain of the domestic yak. Its expression of HIF-1α was analyzed at both mRNA and protein levels in various tissues. Both its HIF-1α mRNA and protein are tissue specific expression. Its HIF-1α protein's high expression in the brain, lung, and kidney showed us that HIF-1α protein may play an important role in the adaptation to hypoxia environment.

Keywords: Qinghai-Tibetan plateau; Domestic yak; Hypoxia; HIF-1α; Isoform; mRNA; Protein

The Qinghai-Tibetan plateau is bound by the Himalayas in the southwest and the Kunlun and Aljin mountains in the northeast. It towers over southwestern China covering more than 2.5 million km^2 at an average altitude of 4000 m above sea level and is known as "the roof of the world". It is the highest and largest plateau in the world and it has a highland continental climate and a very complex topography with great variations. Yak are found extensively on the plateau of western China in alpine and subalpine regions at altitudes from 2000 to 5000 m with a cold, semi-humid climate, and hypoxia environment. The area extends from the southern slopes of the Himalayas in the south to the Altai in the north and from the Pamir in the west to the Minshan mountains in the east. The center of yak's distribution is the Qinghai-Tibetan plateau, which is interspersed with several mountain ranges. Yak expanded outward from that original area of domestication. At the present time, the total yak population is estimated to number around 14.2 million, of which 13.3 million are in Chinese territories, about 0.6 million in Mongolia, and the rest in other countries, notably those bordering the Himalayas and countries of the Commonwealth of Independent States. The present domestic yak is descended from wild yak. The plateau yak of Qinghai which used in our study looks similar to the wild yak in body conformation. Among domestic yak breeds it stands tall, having a relatively large body weight and big head. Both sexes are horned. Similar to wild yak, it has greyish-white hair down its back and around the muzzle and eye sockets. It adapts well to the cold and humid climate at high altitude [1].

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In general, oxygen is the single most important factor determining the distribution, stocking density and, indirectly, the growth rate of yak. The highest altitude where yak live normally is at 5500 m in the Tibetan Rongbusi region in the lower ranges of the Himalayas. Yak steers used as pack animals are quite capable of traversing terrain at 7200 m with not to 40% oxygen content with the sea level. Adaptation to low oxygen content of the air arises from yak having a large chest (14–15 pairs of thoracic ribs), large lungs, and a large heart relative to their overall body size. The hemoglobin content may not be exceptionally high relative to cattle at sea level, although the content increases with altitude, the hemoglobin of yak blood has a high affinity for oxygen. Also, anatomically, the yak is designed to be capable of breathing rapidly and take in large amounts of air [1,2].

For all organisms on earth, changes in O_2 concentration represent a fundamental physiologic stimulus. In animals, this stimulus elicits both acute (rapid-onset and short-term) and chronic (delayed-onset and long-term) responses. Intracellular O_2 concentrations are maintained within a narrow range due to the risk of oxidative damage from excess O_2 (hyperoxia) and of metabolic demise from insufficient O_2 (hypoxia). Whereas acute responses often entail changes in the activity of preexisting proteins, chronic responses invariably involve changes in gene expression. HIF-1 is specifically activated by decreases in tissue oxygen supplies, although some reports have suggested that HIF-1 activation can also be observed in response to stresses associated with energy deprivation [3]. HIF-1 is a heterodimer composed of a 120-kDa HIF-1α subunit and a 91–94 kDa HIF-1β/ARNT subunit, both of which are members of the basic helix–loop–helix (bHLH)-PAS family [4–7]. Hypoxia is the physiologic trigger that activates HIF1. Only HIF-1α responds to changes in oxygen tension, although HIF-1β, despite its apparent insensitivity to hypoxia, is required for HIF-1 activity [8]. In addition to its role in oxygen homeostasis, HIF-1 has been implicated as a critical factor in the pathogenesis of tumor vascularization, myocardial ischemia, and stroke by having a regulatory role in localized tissue hypoxia prevailing under these conditions. Also there is some study on the HIF-1α in the underground animal [9], fish [10,11], and hibernating animals [12]. However, there are very few researches about the HIF-1α of native plateau species which live under extreme hypoxia conditions [13]. The objective of our study was to characterize the HIF-1α cDNA of the domestic yak and to detect its mRNA and protein expression in various tissues (heart, liver, spleen, lung, kidney, brain, muscle, and testis) for further functional and ecological adaptation studies.

Materials and methods

Sample preparation. The domestic yak used in this study lived near the Haibei Alpine Meadow Research Station, the Chinese Academy of Sciences (3200 m in altitude). The station is located in the northeast of Qinghai-Tibet an plateau at latitude 37°29′N and longitude 101°12′E. The research site of the present study has a continental monsoon type climate, with long, cold winters and short, cool summers. Winds in the area are frequent and harsh, especially during winter and spring. The range of daily temperature change is great, and the average air temperature is −1.7 °C with extremes of maximus 27.6 °C and minimus −37.1 °C [14]. The barometric pressure at that area was approximately 508 mmHg. The domestic yak were bought from the indigene and killed outside the stable. The partial heart, liver, spleen, lung, kidney, brain, muscle, and testis were rapidly taken and frozen in liquid nitrogen, respectively. The metallic and vitreous instruments used in the process were warmed at 180 °C for 3 h. The plastic were marinated in 0.1% DEPC deionized water for 12 h. And then the plastic and the water were subjected to high-pressure disinfection at 15 lb (121 °C) for 60 min twice.

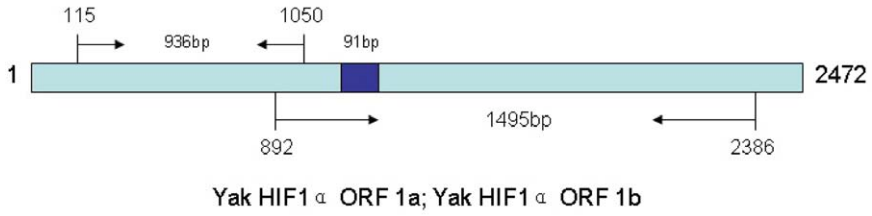
Preparation of primers. The domestic yak HIF-1α primers were designed according to the HIF-1α cDNA sequence of *Bos taurus* (GenBank Accession No. NM_174339), *Canis familiaris* (GenBank Accession No. AY455802), *Homo sapiens* (GenBank Accession No. BC012527), *Mus musculus* (GenBank Accession No. BC026139), *Rattus norvegicus* (GenBank Accession No. AF057308), and *Oryctolagus cuniculus* (GenBank Accession No. AY273790). The domestic yak β-actin primers were designed according to the β-actin cDNA sequence of *Bos taurus* (GenBank Accession No. AY141970), *Ovis aries* (GenBank Accession No. U39357), *Equus caballus* (GenBank Accession No. AF035774), *Homo sapiens* (GenBank Accession No. NM_001101), *Canis familiaris* (GenBank Accession No. NM_001003349), and *Cavia porcellus* (GenBank Accession No. AF508792). Sequence homology search and alignment was carried out with Vector NTI 7.0 (Invitrogen) (Fig. 1).

RNA isolation, reverse transcription, and PCR. Total RNA was extracted and purified from the domestic yak partial heart, liver, spleen, lung, kidney, brain, muscle, and testis using Trizol reagent (Invitrogen). The concentration of RNA samples was quantified with DU 800 (Beckman Coulter) for further analyses. The cDNA synthesis was performed with 5 μg total RNA after treated with DNase I (RNase free) using SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen). This cDNA was used as a template in reverse transcription-PCR. The PCR with 50 μl system (distilled deionized water 36.75 μl, 10× buffer 5 μl, dNTP mixture 4 μl, cDNA 2 μl, sense primer (10 pmol/μl) 1 μl, antisense primer (10 pmol/μl) 1 μl, and Ex *Taq* 0.25 μl). The expected fragment I (936 bp), fragment II (1495 bp), fragment III (2472 bp), fragment IV (2595 bp), and fragment V (1128 bp) were extracted from the low-melt agarose gels with DNA Gel Extraction Kit (V-gene). The fragments were cloned into pGEM T-Easy Vector with the pGEM^R T-Easy Vector System I (Promega). The sequence of those PCR products was determined by Invitrogen Biotechnology Co., Ltd.

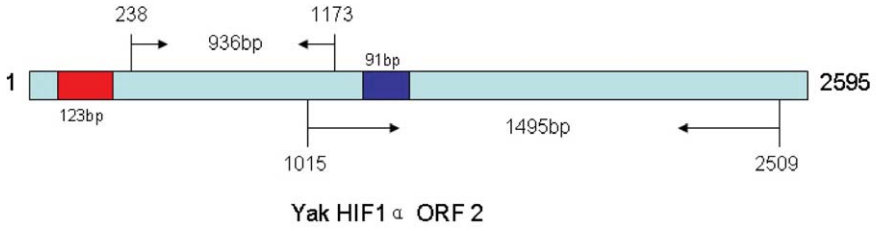
Northern blot analysis. About 20 μg of total RNA from various tissue was loaded onto 1% agarose gel containing 0.22 M formaldehyde and electrophoresed within 1× MOP running buffer [0.02 M 3-(*N*-morpholino)propanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA] for 1 h 40 min at 70 V. After soaking the gel at 10× SSC for 20 min, RNAs were transferred to nylon membrane by using Zeta-Probe membrane (Bio-Rad) following the manual description. Transferred RNAs were immobilized with 254 nm of UV light for 4 min and then baked at 80 °C for 1 h. About 2×10^5 cpm of [^{32}P]dCTP-labeled cDNA (fragment II 1495 bp) probe was added to Church (0.5 M Na_2HPO_4, pH 7.2, 7% SDS, 1 mM EDTA, 1% BSA) [15]. At the end of hybridization, the filters were washed sequentially with the final stringent wash in Wash Solution I (2× SSC/0.1% SDS) for 5 min at 68 °C with shaking two times, Wash Solution II (1× SSC/0.1% SDS) for 10 min at 68 °C with shaking, and Wash Solution III (0.1× SSC/0.1% SDS) for 10 min at room temperature with shaking. Then the filter was exposed to the film at −70 °C for 72 h.

Real-time reverse transcription-PCR analysis. Total RNAs from 100 mg tissues (eight different tissues) were extracted with 1 ml of Trizol (Invitrogen). RNA pellet was resuspended in sterile water treated with DEPC. Twenty micrograms of total RNA was treated with 2 μl DNase I (RNase-free, TaKaRa) at 37 °C for 30 min. Through PCR detection the genome DNA was proved to have been completely eliminated [16,17]. Absolute gene quantification was performed by using ABI PRISM 7000

(1) A



(1) B



(1) C

(2)

PCR products size	Primers Name	Primers composition
936bp	Yak senseprimer-1	5'-GAG CTT GCT CAT CAG TTG CC-3'
	Yak antisenseprimer-1	5'-CAA GTC GTG CTG AAT AAT ACC-3'
1495bp	Yak senseprimer-2	5'-GGA CAA GTC ACC/A ACA GGA CAG-3'
	Yak antisenseprimer-2	5'-CA/GT AAC TGG TCA GC/TT GTG G-3'
2472bp 2595bp	Yak 3	5'-ATG GAG GGC GCC GGC/G GGC G-3'
	Yak ORF terminate	5'-TCA GTT AAC TTG ATC CAA AGC-3'
	Yak H1a ORF 2S	5'-ATG GAG GGC GCC GGC/G GGC GCG AAC G-3'
	Yak H1a ORF 2AS	5'-TCA GTT AAC TTG ATC CAA AGC TCT GAG-3'
1128bp	Yak bactin S1	5'-ATG GAT GAT GAT ATT/C GCT/C GCG CTC G-3'
	Yak bactin AS1	5'-CTA GAA GCA TTT GCG GTG GAC GAT G-3'
124bp	Yak bactin realtime S	5'-TCA CGA AAC TAC CTT CAA TTC CAT C-3'
	Yak bactin realtime AS	5'-TTT CTG CAT CCT GTT TGC GAT-3'
91bp	Yak H1a realtime S	5'-GAT AAA CTT AAG AAG GAG CCT GAT GCT-3'
	Yak H1a realtime AS	5'-TGT CAT TGC TGC CAA AAT CTA AAG-3'

Fig. 1. PCR primers used in the RT-PCR and real-time RT-PCR. (1) (A) The domestic yak HIF-1α ORF 1a and the domestic yak HIF-1α ORF 1b, (B) the domestic yak HIF-1α ORF 2, and (C) the domestic yak β-actin ORF. The blue part is the real-time RT-PCR products of the whole ORFs. The red part is the 123 bp of the domestic yak HIF-1α ORF 2 excessive than the domestic yak HIF-1α 1a and the domestic yak HIF-1α 1b. (2) The primers used in the RT-PCR and real-time RT-PCR. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

(Applied Biosystems). HIF-1α gene expression was normalized to β-actin as an internal housekeeping gene control. For comparison of HIF-1α and β-actin gene expression, we designed identical primers on the sequence of the genes by using PRIMER EXPRESS 2 software (Applied Biosystems). Five micrograms of total RNA extracted from different tissues of domestic yak which had been treated with DNase I (RNase-free) was used in the reverse transcription with SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen). The plasmid–DNA constructs containing the amplicons of each gene (HIF-1α ORF 1a+pGEM T-Vector; β-actin ORF+pGEM T-Vector) were diluted to 3.2×10^{-7} followed by 5-fold dilutions from 10^{-3} ng/μl to 3.2×10^{-7} ng/μl which were used for the standard curves. Gene expression rates were given in copies of cDNA followed by 10-fold dilutions from 10^{-1} to 10^{-4} of the reverse transcription products. Reactions were performed by using SYBR green PCR Master Mix (Applied Biosystems) in a total volume of 25 μl. The PCR plate was incubated at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Samples isolated from individual animals and different tissues were tested in triplicate. The expression of HIF-1α and β-actin represents the results obtained from the domestic yak.

Generation of polyclonal antibodies against domestic yak HIF-1α. One peptide was constructed to produce antibodies against the C-terminal of

HIF-1α with added *Nde*I enzyme digesting site on the 5′ and *Xho*I enzyme digesting site on the 3′ of the peptide [18]. The domestic yak HIF-1α cDNA spanning the C-terminal amino acids 539–823, called HIF-1α 0.8 K, cloned into the pET30a (Novagen) was cut with *Nde*I and *Xho*I. The bacterial strain BL21 (DE3) (Novagen) was transformed with the plasmids. Cells were cultured in the presence of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside at 30 °C for 3 h and collected by centrifugation. The overexpressed proteins were extracted. The His_6-tagged protein was then purified using metal (Ni^{2+}) chelate affinity chromatography under 20 mM imidazole according to the manufacturer's recommendations with HiTrap Chelating HP (Amersham Biosciences) on FPLC. The purified protein was finally desalted and lyophilized. The protein was dissolved in PBS and injected subcutaneously into mouse for fourth in 45 days. The collected serum was tested with Western blot analysis. Sera recognizing domestic yak HIF-1α protein were further purified by affinity purification of antibodies using CNBr-activated Sepharose 4B (Amersham Biosciences).

Western blot analysis on protein expression. Cells and tissues were lysed in 20 mM Tris–Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM Na_3VO_4, 2.5 mM sodium PP_I, 1 mM β-glycerol-phosphate, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 5 μg/ml leupeptin (pH 7.5) [19]. Tissues lysate including 50 μg total protein and cell lysate including 5 μg total protein were separated onto 10% SDS–PAGE and electro-transferred to membrane filters cellulose nitrate (Micro Filtration Systems) at 300 mA for 1.5 h (Bio-RAD). The transferred membrane was blocked with 7% nonfat milk in Tris-buffered saline Tween 20 (TBST) at 37 °C for 1 h, then incubated with antibodies (Actin (I-19) polyclonal antibody was purchased from Santa Cruz; His-Tag monoclonal antibody was purchased from Merck), followed by horseradish peroxidase-conjugated secondary antibodies, and developed by enhanced chemiluminescence according to the manual's description (SuperSignal West Pico Chemiluminescent Substrate, PIERCE). The protein extracted from the 293T cells transfected with the domestic yak HIF-1α ORF 1a (823 amino acids) and pcDNA3.1/myc-His A was used as the positive (P) control, and the protein extracted from the 293T cells transfected with the plasmid pcDNA3.1/myc-His A was used as the negative (N) control.

Results

Cloning and characterization of HIF-1α ORF and β-actin ORF from domestic yak

Using the primers derived from the consensus sequence of HIF-1α and β-actin from other mammalian species, five RT-PCR products were retrieved from the total RNA prepared from the brain of domestic yak. Using the method described under Materials and methods, six clones of the PCR products were obtained. Two clones with insertion of 936 bp fragment and 1495 bp fragment were partial HIF-1α ORF. The clone containing 1128 bp insert was β-actin ORF (GenBank Accession No. DQ838049). While two clones containing 2472 bp fragments were different and named as yak HIF-1α ORF 1a (GenBank Accession No. DQ838046) and yak HIF-1α ORF 1b (GenBank Accession No. DQ838047), respectively. The clone containing 2595 bp fragment was named as yak HIF-1α ORF 2 (GenBank Accession No. DQ838048). The yak HIF-1α ORF 1a and yak HIF-1α ORF 1b encoded for 823 amino acids and their molecular weight was estimated as 95 kDa. The yak HIF-1α ORF 2 encoded for 864 amino acids and its molecular weight was estimated as 100 kDa. In comparison with HIF-1α ORF 2, the yak HIF-1α ORF 1a and HIF-1α ORF 1b lost their coding region for 41 amino acid residues between 13 and 53 at the N-terminal of the protein encoded by HIF-1α ORF 2. These lost amino acids did not contribute to the bHLH (basic helix–loop–helix) domain, thus we speculated that they might play an important role in the domestic yak [20,21]. However, their function remains unknown at this stage (Fig. 2).

The nucleic acid sequences of the yak HIF-1α ORF 1a and yak HIF-1α ORF 1b exhibit 87%, 87.8%, 91.2%, 98.7%, 90%, 93.7%, 93.9%, and 99.6% identities with the mouse, Norway rat, rabbit, Tibetan antelope, dog, human, the yak HIF-1α ORF 2, and cow HIF-1α ORFs, respectively. The nucleic acid sequences of the yak HIF-1α ORF 2 exhibit 82.2%, 83%, 86.2%, 93.2%, 85.8%, 88.6%, and 94% identities with the mouse, Norway rat, rabbit, Tibetan antelope, dog, human, and cow HIF-1α ORFs, respectively. The amino acid sequences encoded by HIF-1α ORF 1a and yak HIF-1α ORF 1b were slightly smaller in size than the corresponding HIF-1α in human (826 amino acids) and mouse (836 amino acids), while larger in size than the rabbit (819 amino acids). Also, they showed the same size with the cow, Norway rat, and Tibetan antelope. The size of amino acids sequence encoded by HIF-1α ORF 2 was larger than the corresponding HIF-1α in all of the animals. The predicted amino acids of the yak HIF-1α ORF 1a and the yak HIF-1α ORF 1b had about 99% similarity. The bHLH (basic helix–loop–helix), PAS (Per-Arnt-Sim), TAD-C (carboxy-terminal transactivation domain), and TAD-N (amino-terminal transactivation domain) in HIF-1α 2, HIF-1α 1a, and HIF-1α 1b were almost identical. The predicted amino acids of the yak HIF-1α ORF 1a and yak HIF-1α ORF 1b had about 93.8% similarity to that of HIF-1α ORF 2, 91.5% similarity to the dog, 94.6% similarity to the human, 98.8% similarity to the Tibetan antelope, 99.2% similarity to the cow, 88% similarity to the mouse, 89.6% similarity to the Norway rat, and 92.6% similarity to the rabbit HIF-1α. The predicted amino acids of the yak HIF-1α ORF 2 had 83.8% similarity to the mouse, 85.3% similarity to the Norway rat, 88.1% similarity to the rabbit, 87.3% similarity to the dog, 90.2% similarity to the human, 94% similarity to the Tibetan antelope, and 94.3% similarity to the cow HIF-1α.

HIF-1α mRNA expression in domestic yak

Using Northern blot, we found that HIF-1α mRNA was widely expressed in different tissues (heart, liver, spleen, lung, kidney, brain, muscle, and testis) of the domestic yak. Transcript levels of HIF-1α were exactly assessed in various tissues using real-time RT-PCR. Through real-time RT-PCR we found that HIF-1α mRNA was highly expressed in the testis and spleen of the domestic yak. Its mRNA expression in the testis was about twice as much as in the spleen. Its mRNA expression in the testis was about quintuple as much as

in the heart, lung, kidney, and brain. And its mRNA expression in the testis was about octuple as much as in the liver and also it was about decuple as much as in the muscle (Figs. 3 and 4).

HIF-1α protein expression in domestic yak

Maintaining oxygen homeostasis is essential to the tissues. Through Western blot on the total protein of eight

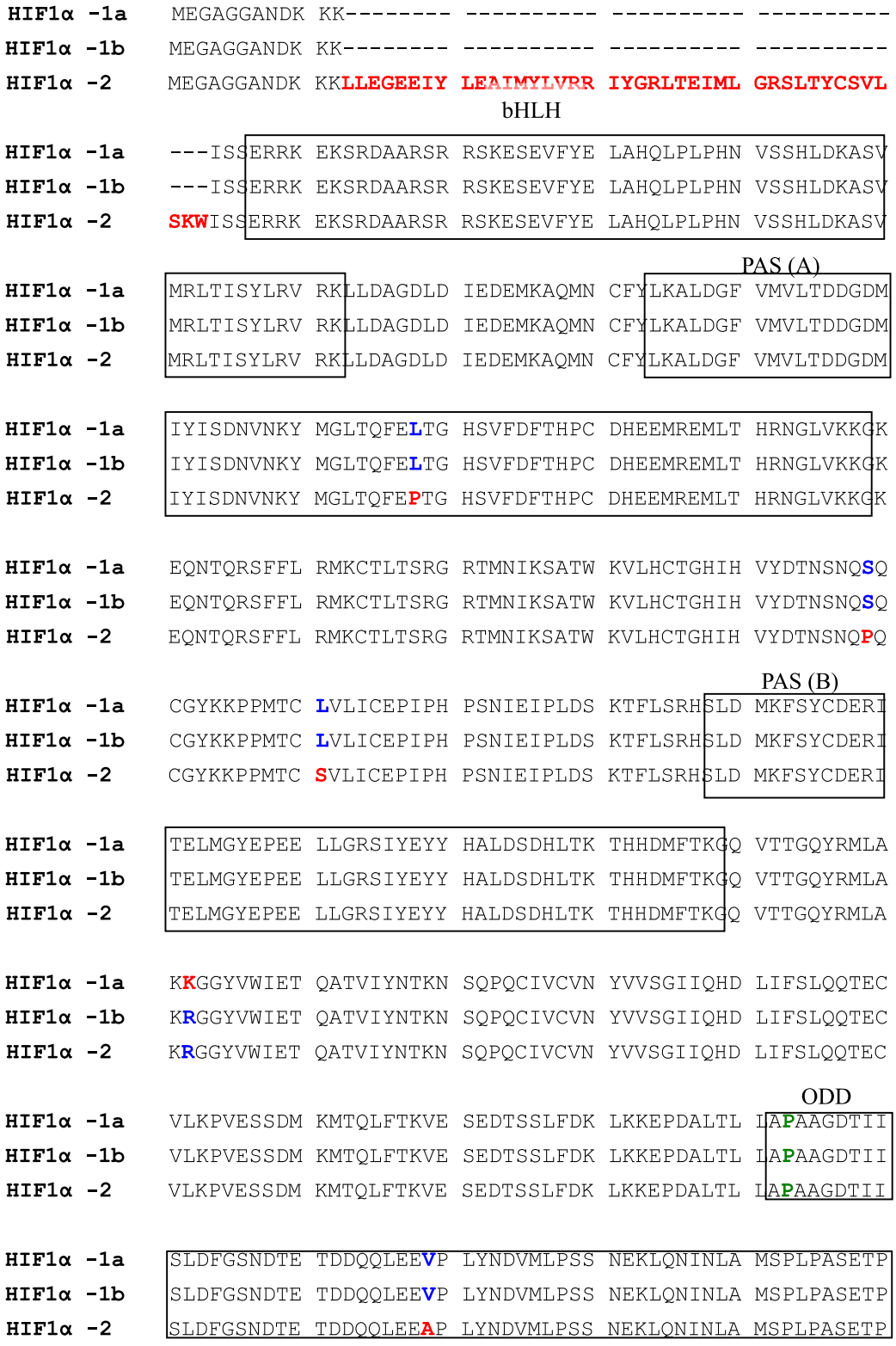


Fig. 2. Comparison of the deduced amino acid sequences of the three different HIF-1α ORFs of the domestic yak. The red is the different amino acids of the three sequences. The green is deductive O_2-dependent hydroxylation position. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

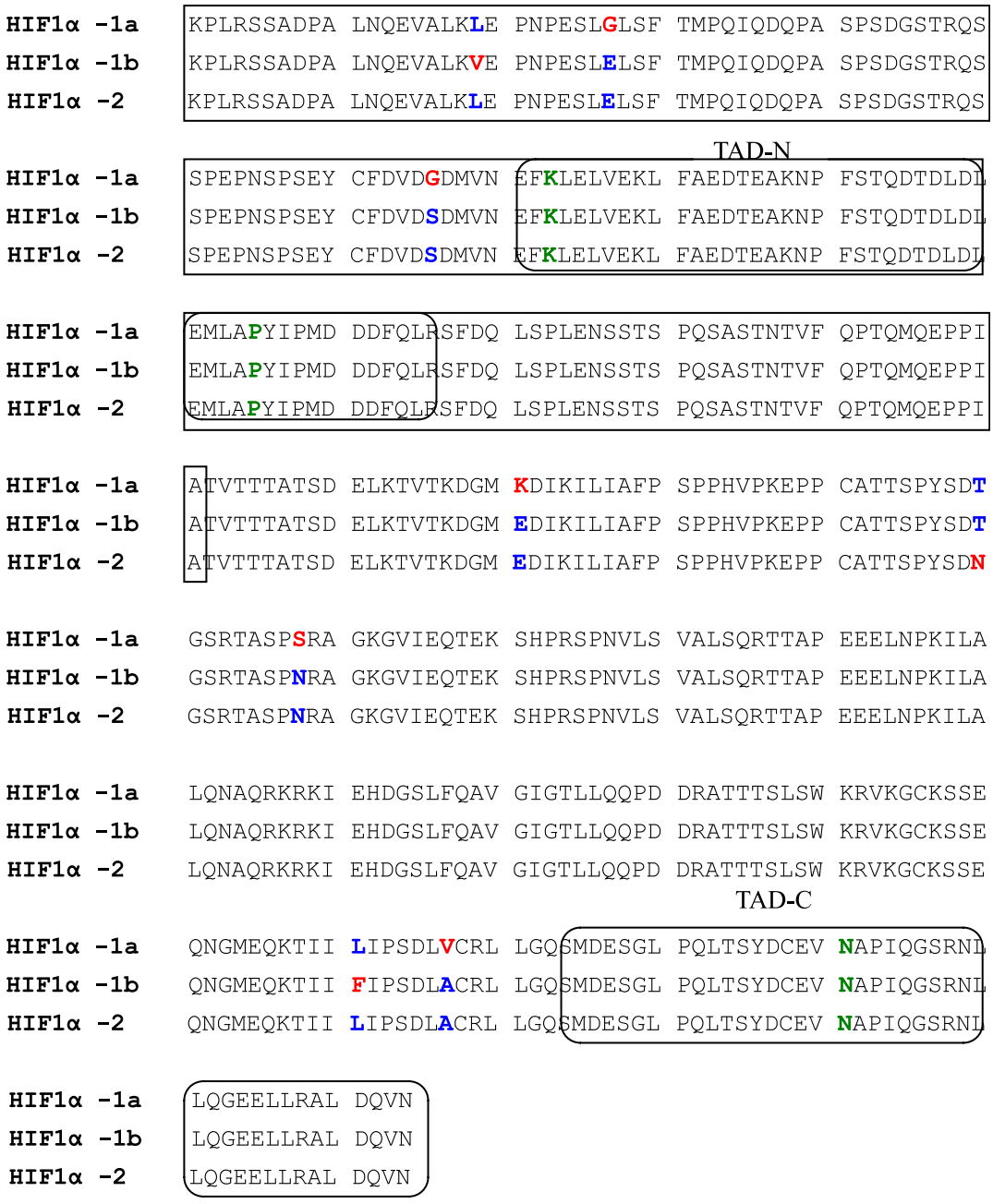


Fig. 2 (*continued*)

tissues, we found that HIF-1α was widely expressed in them only differing in quantity. To our knowledge this is the first report on HIF-1α protein's wide expression in the native plateau animals' different tissues. HIF-1α protein was highly expressed in the brain, spleen, and kidney of the domestic yak. Its protein expression in the brain was about decuple as much as in the muscle and it was about sextuple as much as in the heart. And its protein expression in the brain was about double as much as in the testis. Its protein expression in the spleen and kidney was about quadruple as much as in the heart. The domestic yak HIF-1α protein showed lower expression in the muscle and heart than in other tissues (Fig. 5).

Discussion

Our results differ from those of other studies greatly because our model is the native plateau animals. The high altitude which has extremely low oxygen concentration and the long-term evolution of the domestic yak to adapt to the hypoxia are the two major factors. The domestic yak HIF-1α protein expression had a few differences with its mRNA expression. As known, HIF-1α plays the pivotal role on the post-transcriptional level as the protein [4–7], so its mRNA expression has no direct connection with its action [22]. Thus we can understand that HIF-1α mRNA expression differing from its protein expression is possible and natural.

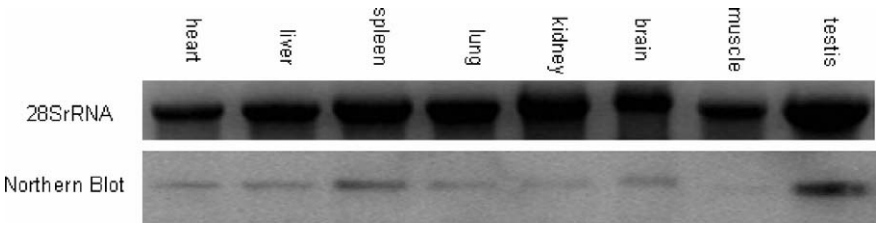


Fig. 3. Expression of HIF-1α mRNA in different tissues of the domestic yak (Northern blot results). The expression of HIF-1α mRNA is performed by Northern blot analysis containing 20 μg total RNA isolated from different tissues of domestic yak and it was hybridized with ^{32}P-labeled specific cDNA probes of partial HIF-1α ORFs.

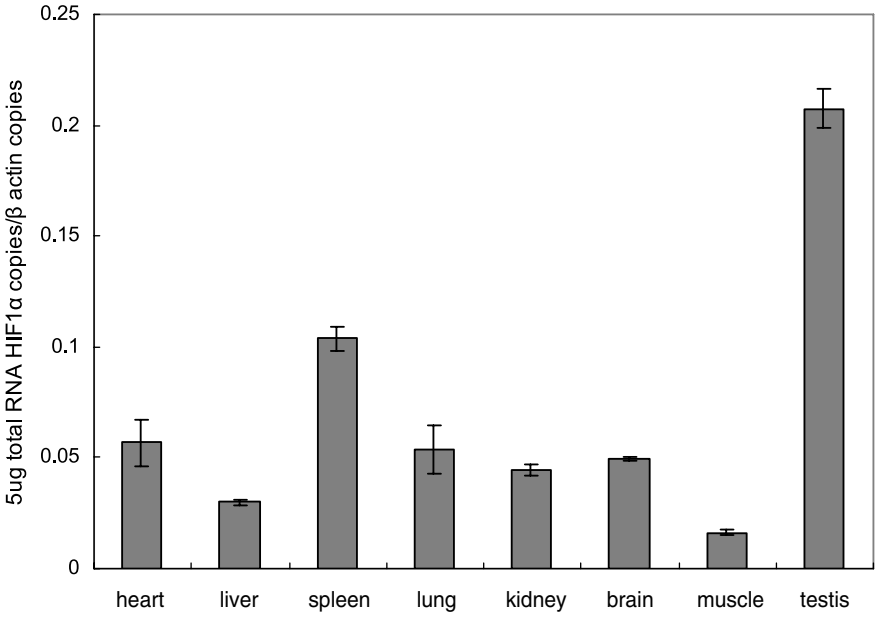


Fig. 4. Expression of HIF-1α mRNA in different tissues of the domestic yak (real-time RT-PCR results). The figure is the ratio of different tissues HIF-1α gene copies to β-actin gene copies in 5 μg total RNA of the domestic yak. Representative results from three independent experiments in triplicate on the same mRNA of different domestic yak are presented as means ± standard errors.

With the altitude increasing and the PO_2 (partial pressure of oxygen) decreasing, the yak blood oxygen content decreases. Thus, promotes the RBC (red blood cell) numbers, Hb (hemoglobin) content, and HCT (hemotocrit) increase to meet the body oxygen demand. The RBC numbers, Hb contents, and HCT of Tibetan Naqu yak which live at 4500 m higher than those of the Tibetan Linzhi yak which lived at 3000 m. But the MCV (mean corpuscular volume) is lower and the MCHC (mean corpuscular hemoglobin concentration) is higher in the blood of the Tibetan Naqu yak than the Tibetan Linzhi yak. The increasing of the RBC numbers and the reducing of the MCV can increase the total superficial area of the RBC. Thus O_2 and CO_2 exchanging quantities through RBC are enhanced. And at the same time it can decrease the blood sticky degree to accelerate the flow rate [23]. The yak Hb components exhibit higher oxygen affinities than cow Hb. The yak β-chain differs from that of the cow in three residue positions, none of which are likely to be involved in the heme or subunit contacts. The yak β-chain differs from those of most mammalian Hbs in that alanine at position 135 (H13) is replaced by valine, introducing a bulkier hydrophobic side chain in the vicinity of the heme, which might cause a small change in the H-helix and alter oxygen affinity [24,25]. Since normal tissue function in mammals depends on adequate supply of oxygen through blood vessels, a discrepancy between oxygen supply and consumption (hypoxia) induces a variety of specific adaptation mechanisms at the cellular, local, and systemic levels. These mechanisms are in part governed by the activation of hypoxia-inducible transcription factors (HIF-1), which in turn modulate expression of hypoxically regulated genes such as those encoding vascular endothelial growth factor (VEGF) and erythropoietin (EPO). Released into circulation, EPO makes its way to the bone marrow, where it regulates red cell production by preventing apoptosis of erythroid progenitor cells [26]. More RBC (red blood cell) numbers and more hemoglobin (HB) concentration are the hematological character of many high altitude animals. They increase the ability to transport oxygen of the blood. In general, plasma EPO protein concentrations show an inverse log/linear relationship with O_2 capacity of the

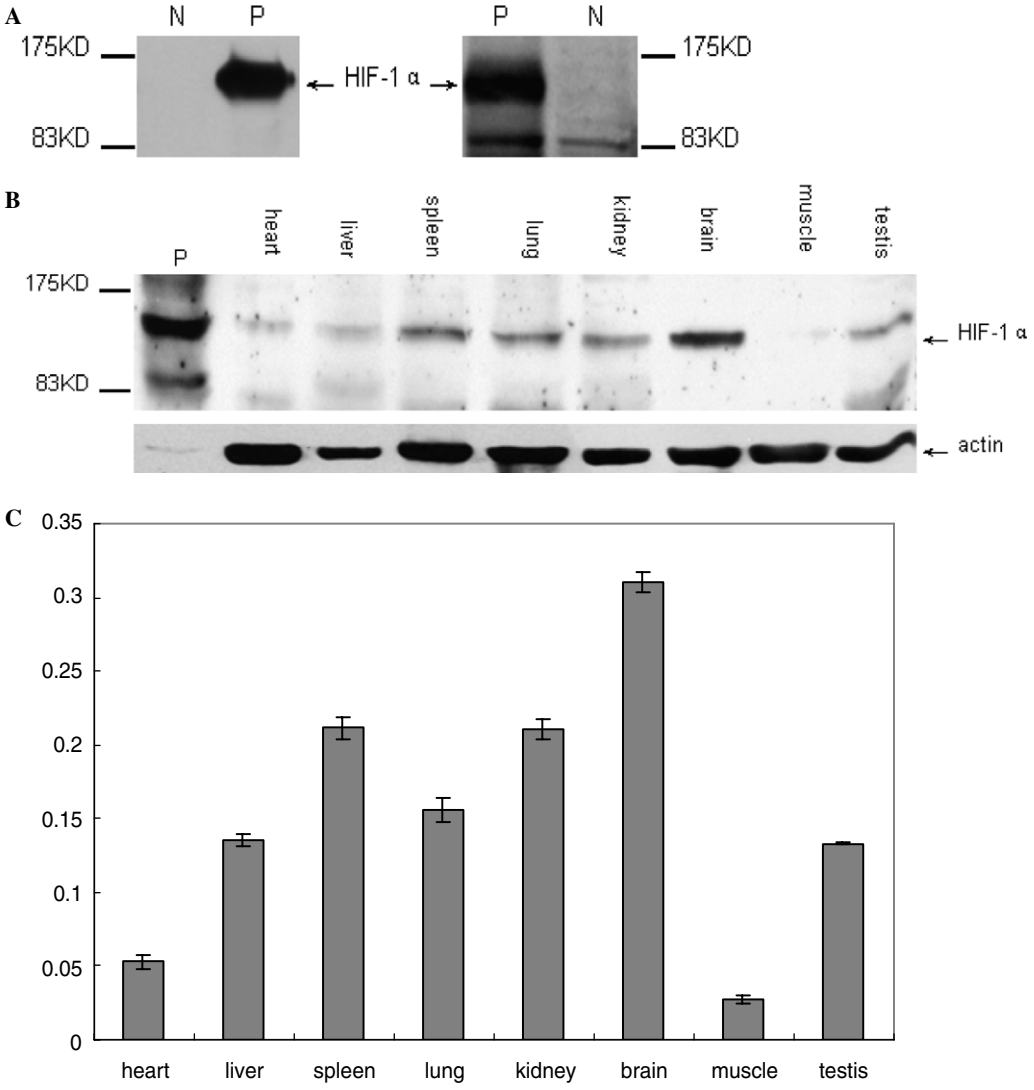


Fig. 5. Expression of HIF-1α protein in different tissues of the domestic yak. The actin protein was used as the standard to determine the quantity of the total protein. The left picture in (A) is the His-Tag antibody result. The right picture in (A) is the polyclonal antibody result. The over picture in (B) is the HIF-1α polyclonal antibody result and its molecular weight is about 120 kDa. The under picture in (B) is the actin polyclonal antibody result and its molecular weight is about 43 kDa. (C) The ratio of HIF-1α protein expressed quantity to actin protein expressed quantity calculated by the software ImageJ. Representative results from three independent experiments on the same protein of different domestic yak are presented as means ± standard errors.

blood, and EPO is the main factor involved in erythropoiesis, regulating the level of circulating red blood cells which have hemoglobin that is the oxygen transport protein used in the blood of vertebrates. Because EPO is transcriptionally activated by HIF-1, this phenomenon suggests that HIF-1 may play a very specific role in controlling the behavior of kidney producing red blood cells to transport oxygen. But it was necessary to understand that it is not the RBC number itself that is sensed, but the blood O_2 content that in turn determines the oxygenation of EPO-producing tissues and thus EPO production.

EPO has also emerged as a multifunctional growth factor that plays a significant role in the nervous system. EPO is expressed throughout the brain in glial cells, neurons, and endothelial cells. Hypoxia has been recognized as an important driving force of EPO expression in the brain. EPO has potent neuroprotective properties in vivo and in vitro, and appears to act in a dual way by directly protecting neurons from hypoxia damage and by stimulating endothelial cells and thus supporting the angiogenic effect of VEGF in the nervous system. Thus, hypoxia-induced gene products such as VEGF and EPO might be part of a self-regulated physiological protection mechanism to prevent neuronal injury. Depending on the severity of hypoxia, EPO mRNA levels increase between 3- and 20-fold in the brain compared with up to 200-fold induction in the kidney. Thus, hypoxic EPO gene activation in the brain appears to occur in a very similar way in the kidney, albeit induction levels are lower [27]. Because the brain is the most important neural system, HIF-1α high expression in it probably is to protect it, preventing from damage.

It is well known that when mammalian species that live at sea level are exposed to high altitude, certain species develop pulmonary hypertension with increased muscularization of the pulmonary arterioles [28,29]. The degree of pulmonary hypertension varies among species and is related to the duration of high altitude residence and magnitude of the hypoxic stimulus. The high altitude native yak has successfully adapted to chronic hypoxia despite being in the same genus as domestic cows, which are known for their great hypoxic pulmonary vasoconstrictor responses, muscular pulmonary arteries, and development of severe pulmonary hypertension on exposure to chronic hypoxia. Yak small pulmonary arteries have medial thickness, with vessels devoid of smooth muscle. Yak pulmonary artery endothelial cells are much longer, wider, and rounder in appearance than domestic cows. Thus the yak has successfully adapted to high altitude conditions by maintaining both a blunted hypoxic pulmonary vasoconstrictor response and thin-walled pulmonary vessels. Yak adaptation to high altitude may include changes both in the amount of pulmonary vascular smooth muscle and in endothelial cell function and structure [30–32]. Endogenous NO is enzymatically produced from conversion of the amino acid L-arginine to L-citrulline, a reaction catalyzed by the enzyme NO synthase (NOS). NO is a potent systemic and pulmonary vasodilator that inhibits smooth muscle cell proliferation, migration, and inflammation, and matrix protein production [33–35]. Hence enhancement in NO production in the lung may inhibit with the development of pulmonary hypertension by both decreasing vascular tone and preventing vascular remodeling. Although the exact mechanism remains to be determined, it is interesting that exhaled NO is elevated in Tibetans and Bolivian Aymara living at high altitude [36]. We speculate in the domestic yak lung that HIF-1α protein high expression increases NOS expression thus producing more NO [37]. Thus NO prevents building up of pulmonary hypertension. We thought this is the main reason that HIF-1α protein is highly expressed in the lung.

Formal research had demonstrated in normal mouse brain, kidney, liver, and heart that HIF-1α protein is present in the nucleus under normoxic, hypoxia, and anoxic conditions [22,38]. However, our results were different from their outcomes especially as regards the lung. This is the most interesting finding in our study. Because our model was the native plateau animals that lived well in the Qinghai-Tibetan plateau which has the extreme low oxygen concentration, whereas their models were the manual hypoxia or anoxic treated animals which needed to live well under normal oxygen concentrations and the hypoxia and anoxic environment would disturb their physiological metabolism and inherent microenvironment [39,40]. The manual action on the animals cannot find the HIF-1α's real function exactly. But our model will let us get the most actual data on the HIF-1α expression and its true function in the native plateau living animals under the true physiological conditions. Those are the most excited things that we felt. First, the data showed us the high altitude animal domestic yak had the complicated and perfect mechanisms to adapt to the hypoxia conditions. Thus they can survive and move with heavy goods in the extreme environment such as 7200 m altitude [1]. Second, we thought that before the domestic yaks were killed on the plateau, they ran quickly that resulted in consuming abundant oxygen and struggled for some time until they died. The data indicated that HIF-1α protein may play an important role in the domestic yak response to external injury. Third, the EPO and NOS may play an important role in the domestic yak to adapt to hypoxia. Of course this will entail more work to be done by us to prove it.

In conclusion, in this study we obtained two different HIF-1α ORFs of domestic yak. The predicted amino acid sequences show high level of conservation at the bHLH, PAS A/B region, whereas there are a few variations in the ODD, TAD-N, and TAD-C region among vertebrates. Meanwhile, we identified and characterized a new alternatively spliced variant of domestic yak HIF-1α ORF which had excessive 123 base pairs in the 5′ terminal and which can translate 41 amino acids. But the incremental 41 amino acids were not on the bHLH domain. We found that both HIF-1α mRNA and protein of the domestic yak are tissue specific expression. Especially HIF-1α protein tissue's specific expression may give us the useful information to understand and further study HIF-1α protein's important role in the adaptation to the high altitude hypoxia of the domestic yak on long-term evolution.

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