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Cloning of hypoxia-inducible factor 1α cDNA from a high hypoxia tolerant mammal—plateau pika (*Ochotona curzoniae*)☆

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Abstract

Hypoxia-inducible factor 1 is a transcription factor composed of HIF-1α and HIF-1β. It plays an important role in the signal transduction of cell response to hypoxia. Plateau pika (*Ochotona curzoniae*) is a high hypoxia-tolerant and cold adaptation species living only at 3000–5000 m above sea level on the Qinghai-Tibet Plateau. In this study, HIF-1α cDNA of plateau pika was cloned and its expression in various tissues was studied. The results indicated that plateau pika HIF-1α cDNA was highly identical to those of the human (82%), bovine (89%), mouse (82%), and Norway rat (77%). The deduced amino acid sequence (822 bp) showed 90%, 92%, 86%, and 86% identities with those of the human, bovine, house mouse, and Norway rat, respectively. Northern blot analyses detected two isoforms named pLHIF-1α and pSHIF-1α. The HIF-1α mRNA was highly expressed in the brain and kidney, and much less in the heart, lung, liver, muscle, and spleen, which was quite different from the expression pattern of mouse mRNA. Meanwhile, a new variant of plateau pika HIF-1α mRNA was identified by RT-PCR and characterized. The deduced protein, composed of 536 amino acids, lacks a part of the oxygen-dependent degradation domain (ODD), both transactivation domains (TADs), and the nuclear localization signal motif (NLS). Our results suggest that HIF-1α may play an important role in the pika's adaptation to hypoxia, especially in brain and kidney, and pika HIF-1α function pattern may be different from that of mouse HIF-1α. Furthermore, for the high ratio of HIF-1α homology among the animals, the HIF-1α gene may be a good phylogenetic performer in recovering the true phylogenetic relationships among taxa.

Keywords: HIF-1α; Plateau pika; RT-PCR; RACE; Northern blotting; Qinghai-Tibet Plateau; Adaptation

Response to O_2 deprivation of organisms is a complex biological and physiological process. A number of studies have shown that two of the most significant hypoxia defense mechanisms exist in the animal society. These mechanisms are: (i) severe down-regulation of energy turnover [1,2]; and (ii) up-regulation of the energetic efficiency of ATP-producing pathways [3].

Plateau pika (*Ochotona curzoniae*) is a small (160 g), non-hibernating, rodent mammal that only inhabits meadows above 3000 m on the Qinghai-Tibetan Plateau. Plateau pika is sexually monomorphic in size and dimorphism in external sexual anatomy is minimal [4–9]. The family social unit is composed of a variable number and sex composition of adults and their young, inhabiting an interconnected series of burrows on continuous and generally flat meadow. Family members usually behave friendly with each other, but are aggressive towards individuals from other families [4,7,8].

The cold climate and hypoxia are the two most important ecological factors restricting plateau animals. The native plateau animals must have developed their own mechanisms in adaptation to harsh environmental stress during their long evolutional history. Plateau pika is a high hypoxia and low temperature tolerant mammal. The animal has an unusually high resting metabolic rate (RMR) and non-shivering thermogenesis (NST), and acts mainly via increasing NST to adapt to extreme

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cold of the Qinghai-Tibet Plateau [10]; high ratio of oxygen utilizing to cope with plateau hypoxia environment [11,12].

Hypoxia-inducible factor-1 is a heterodimer composed of two members of the basic-helix-loop-helix (bHLH)-containing PER-ARNT-SIM (PAS) domain family, HIF-1α and HIF-1β [13]. HIF-1β is also known as arylhydrocarbon receptor nuclear translocator (ARNT) [14], which constitutes a subunit of HIF-1 localized in the nucleus or non-nucleolar portion of the nucleus [14–18].

HIF-1 is a transcription factor expressed in most cells and plays key roles in oxygen sensing and conduction [19]. The α subunit of HIF-1 is a functional part. Some early studies have demonstrated that HIF-1α mRNA is highly inducible by hypoxia in human hepatoma cells [20]. Other studies, however, revealed that HIF-1α mRNA did not change after 0.1–4 h of exposure to 0.5% O_2 in a variety of cell lines including hepatoma cells [21]. Furthermore, the expression of HIF-1α mRNA was increased following cardiac ischemia in human [22]. The HIF-1α is subjected to ubiquitination and proteasomal degradation under normal oxygen conditions, while the degradation pathway is blocked under hypoxia, allowing HIF-1α to accumulate and migrate to the nucleus [13]. However, there is no research about the HIF-1α of natural plateau species, which live under hypoxia and cold conditions. The objective of the present study was (1) to characterize the HIF-1α cDNA of plateau pika and (2) to detect the expression of HIF-1α in various pika tissues (heart, liver, spleen, kidney, brain, skeleton muscle, and lung) for further functional and ecological adaptation studies.

Materials and methods

Sample preparation. Plateau pikas were captured near the Haibei Alpine Meadow Research Station, the Chinese Academy of Sciences (3200 m in elevation). The station is located in the northeast of Qinghai-Tibet Plateau at latitude 37°42′N and longitude 101°35′E. The average air temperature is −1.7 °C in that area [23]. The barometric pressure at that area was approximate 508 mmHg determined by the logarithmic relationship of Zuntz et al. [24]. The pikas were killed by cervical dislocation and dissected on the spot. The whole heart, liver, spleen, kidney, lung, brain, and skeleton muscle (gaskin) were rapidly taken and frozen with liquid nitrogen, respectively, for storage. Mice used in this experiment were provided by Tsinghua Institute of Genome Research (Beijing, about sea level), and their tissues were also collected in the same way. All of the instruments were treated with DEPC water.

All procedures involved in the handling and care of animals were in accordance with the China Practice for the Care and Use of Laboratory Animals and were approved by China Zoological Society.

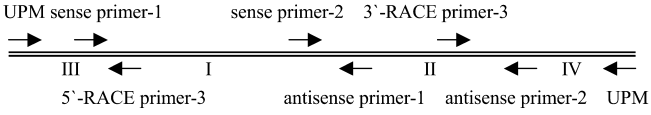
RNA isolation. Total RNA was extracted and purified from the plateau pika and mouse whole heart, liver, spleen, kidney, brain, lung, testicle, stomach, and skeleton muscle (gaskin) using TRIZOL reagent (Gibco-BRL). The concentrations of RNA samples were quantified with an Ultrospec 3000 for further analyses.

Preparation of primers. The PCR primers designed are shown in Fig. 1 according to the HIF-1α cDNA sequence of mouse, human, bovine, and Norway rat in GenBank. All of the primers were produced by Shanghai Biotechnology Corporation.

RT-PCR. Reverse-transcription polymerase chain reaction (RT-PCR) was performed with Access RT-PCR System (Promega) according to the manual. Aliquots (0.8 μg) of the total RNA samples isolated from the plateau pika and mouse brains were reverse-transcribed for 90 min at 48 °C with *AMV* reverse transcriptase. The reverse-transcription products were heated for 2 min at 94 °C for initial denaturation and activation of *TfI* DNA polymerase before amplification. For amplification of the pika HIF-1α cDNA, a three temperature PCR of 30 cycles was carried out, where the samples were denatured for 40 s at 95 °C, annealed for 40 s at 50.3 °C for expected fragment I (51 °C for expected fragment II, 55 °C for mouse), and extended for 2 min at 72 °C [25]. These amplification cycles were then followed by a final extension for 7 min at 72 °C. Bands of similar sizes to those of the expected products (expected fragment I about 1.3 kb and II of about 1.1 kb for the pika HIF-1α, 1.3 kb for mouse) were purified with low-melt agarose gels and cloned into the pMD18-T vector with Ligation Solution I supplied by Takara (Japan). The sequences of those PCR products were determined with an automated sequencer. Sequence homology search was carried out with an ALIGNMENT program of BIOEDIT.

Rapid amplification of the cDNA ends. The 5′- and 3′-rapid amplification of the cDNA end (RACE) reactions were performed with

A Primers and location of expected fragments



B Primer sequences and the annealing temperatures

Primers	sequences	Annealing temperature
Sense primer-1	agt tct gaa cgt cga aaa g	50.3°C
Antisense primer-1	cag g(a/g)t cag cac tac ttc (g/t)	50.3°C
Sense primer-2	ttg cct gct tct gaa tct cc	51°C
Antisense primer-2	gtt aac ttg atc caa agc tc	51°C
3`-RACE primer-3	aca gca gcc aga tga tcg tgc cac tac	68°C
5`-RACE primer-3	cca gca tcc aga agt ttc ctg aca cgc	68°C

Fig. 1. Primers used for amplification. (A) Six primers were designed for four plateau pika HIF-1α cDNA fragments cloning (I, II, III, and IV). The clone I was with sense primer-1 and antisense primer-1; II with sense primer-2 and antisense primer-2; III with 5′-RACE primer-3 and UPM supplied with the kit; and IV with 3′-RACE primer-3 and UPM supplied with the kit. (B) Primer sequences and the annealing temperatures.

SMART RACE cDNA Amplification kit (Clontech) according to the manufacturer's instructions. Briefly, aliquots of total RNA (0.8 μg) obtained from the plateau pika brain were reverse transcribed with SuperScriptII reverse transcriptase (Gibco-BRL) and 5′-RACE cDNA antisense primer or 3′-RACE cDNA sense primer for 2 h at 42 °C. The 5′-RACE and 3′-RACE products were amplified from the specific cDNA products with primers (one of the following gene specific primers shown in Fig. 1 and the Universal primer supplied with the kit) and Advantage 2 PCR Enzyme Mix (Clontech). The touch down PCR conditions used were as follows: five cycles consisting of 94 °C for 5 s and 72 °C for 3 min; five cycles of 94 °C for 5 s, 70 °C for 10 s, and 72 °C for 3 min; and 25 cycles consisting of 94 °C for 5 s, 68 °C for 10 s, and 72 °C for 3 min. The RACE products were cloned into the pMD-18T vector with Ligation Solution I (Takara) and sequenced as described above. Homology search was also done as mentioned above.

Northern blot analysis. Aliquots (20 μg) of the plateau pika and mouse total RNA samples were size-fractionated by electrophoresis through 1% agarose/3% formaldehyde gels and transferred to nylon membrane (Hybond-N, Amersham Pharmcia Biotech) in 20× SSC (3 M NaCl/0.3 M sodium citrate, pH 7.0), respectively. The filters were prehybridized in 7 ml ExpressHyb Hybridization Solution (Clontech) with continuous shaking at 68 °C for 45 min to replace the ExpressHyb Solution with the fresh solution containing the denatured radiolabeled HIF-1α cDNA probe and incubate with continuous shaking at 68 °C for 2 h. The probes used in this study were the *Hin*dIII fragments about 900 bp of the pika and mouse brain HIF-1α cDNA. These cDNA fragments were labeled by random priming method with reagent provided by Prime-a-Gene Labelling System kit (Promega) and [^{32}P]dCTP, [^{32}P]dATP for 3 h at room temperature. We used approximately 2×10^7 cpm/ml of these probes for hybridization. At the end of hybridization, the filters were washed sequentially with the final stringent wash in Wash Solution 1 (2× SSC/0.05% SDS) for 20 min at 25 °C with shaking two times and Wash Solution 2 (0.1× SSC/0.1% SDS) for 20 min at 50 °C with shaking two times, and then exposed to phosphor screen for 72 h. The radioactivity counts of the mRNA signals were determined directly with a Storm (Molecular Dynamics, Amersham Pharmacia Biotech).

Results

Cloning HIF-1α cDNA from plateau pika

Using RNA isolated from the plateau pika brain, the core regions of plateau pika HIF-1α were amplified by RT-PCR and sequenced. The 5′ and 3′ ends were obtained by the 5′ and 3′ RACE technique. A total of three independent 5′ RACE and three independent 3′ RACE products were sequenced and merged to the core sequence resulting in the full-length plateau pika HIF-1α cDNA (3569 bp) as shown in Fig. 2A. The translation initiation sequence (nucleotides 287–296, GTCGCC ATGG) of the pika HIF-1α gene was in agreement with the Kozak consensus sequence [26] except the bold T. Two stop codons (2759 TGA, 2771 TAA) were found downstream of the initiation sequence.

Characterization of plateau pika HIF-1α

The plateau pika HIF-1α cDNA covered an open reading frame of 822 amino acids, whose molecular weight was estimated as 93.7 kDa (Fig. 3). The deduced amino acid sequence of the pika HIF-1α showed high identities with the human (90%), bovine (92%), mouse (86%), Norway rat (86%), chicken (76%), African claw flow (53%), and rainbow trout (40%) HIF-1α cDNAs.

The most intriguing difference was found in the RT-PCR (II). The plateau pika sequence was similar in all of the three sequenced RT-PCR (II) products, differing only in the length of the extensions. One of the extensions lacks a 133 bp fragment in the middle of plateau pika HIF-1α cDNA (Fig. 2B), and the fragment lacking is neither starting with GT nor ending with AG. Consequently, this alternative splicing introduces eight new amino acids, G–P–S–I–S–C–H–L, as a new C-terminus following V_{528} of the 822-amino acid HIF-1α protein. This novel form of HIF-1α mRNA translates into a 536-amino-acid protein (Fig. 3).

The plateau pika HIF-1α 5′ untranslated region (UTR) is GC-rich (72.4%) and 3′ untranslated region (UTR) contains eight AUUUA mRNA instability elements [27] compared to the mouse sequence which contains seven such elements [28]. Six of these elements are conserved between plateau pika and mouse HIF-1α, while five of these elements are the same between plateau pika and human HIF-1α.

Tissue-specific expression patterns of the plateau pika HIF-1α mRNA

Two transcripts of the HIF-1α mRNA in plateau pika were detected by Northern blot. The levels of the HIF-1α mRNA expression differed markedly between the two transcriptions. The levels of the shorter transcript were higher than the longer in all tissues examined (Fig. 4A). The shorter HIF-1α mRNA of plateau pika, named as pSHIF-1α, was expressed most abundantly in the kidney and brain, followed by heart, lung, spleen, skeleton muscle, and liver. However, there is no connection detected between pSHIF and pLHIF to the two isoforms found in the RT-PCR. The HIF-1α mRNA expression patterns were also detected in mouse and showed distinct differences from those of the plateau pika (Fig. 4).

Discussion

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 maximum 27.6 °C and minimum −37.1 °C. Most precipitation falls within the growing season from May to September, and the average annual precipitation ranges from 426 to 860 mm [23]. The alpine meadow soil at the

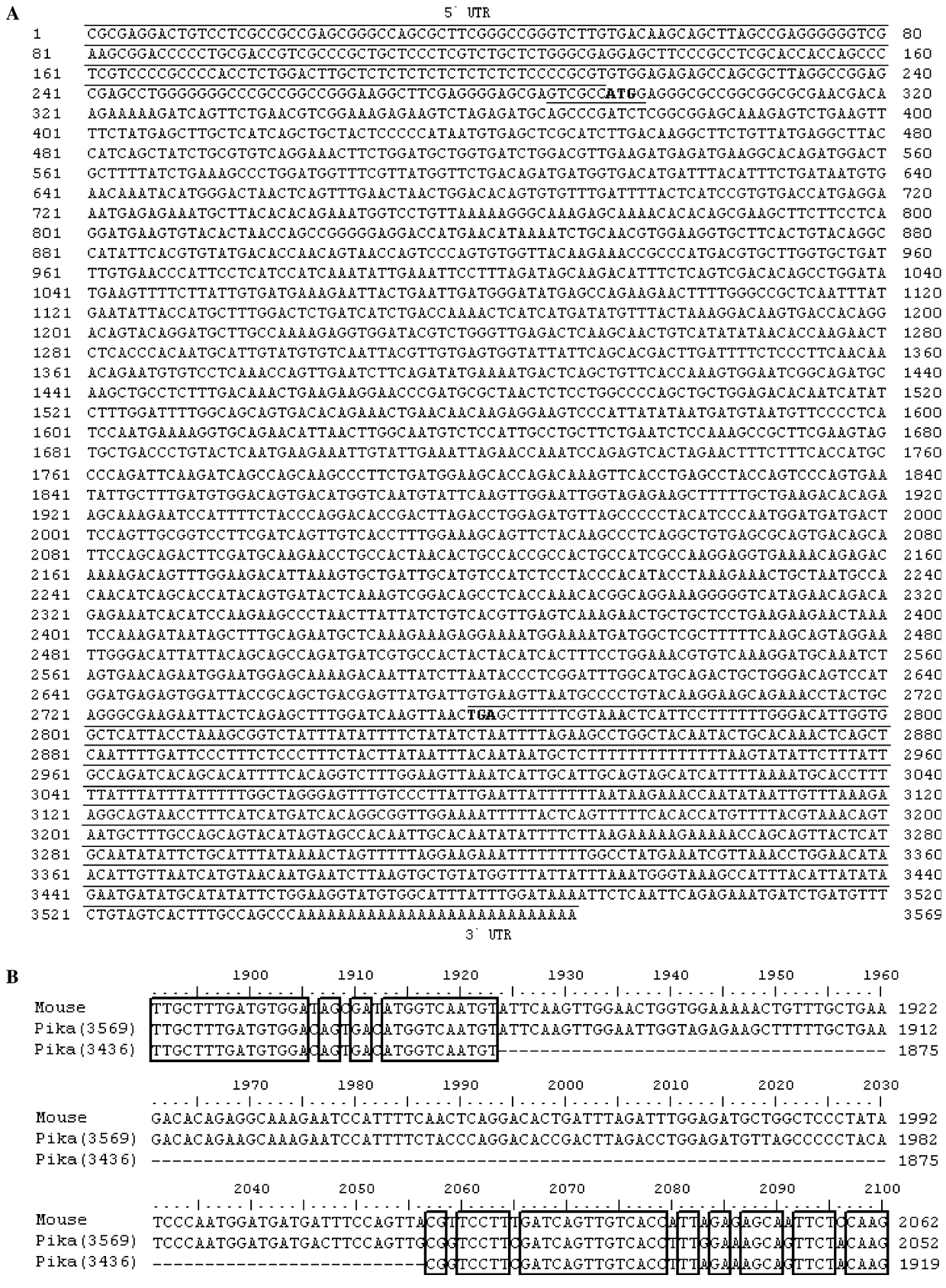


Fig. 2. Nucleic acid sequences of plateau pika HIF-1α. (A) The HIF-1α cDNA is 3569 bp in length. Start codon and termination codon are bolded. Solid lines indicate the Kozak sequence, 5′ UTR and 3′ UTR. (B) The 133 bp reduction (nucleotides 1875–2008) results in a new isoform of plateau pika with an earlier stop codon-TAG (nucleotide 1900).

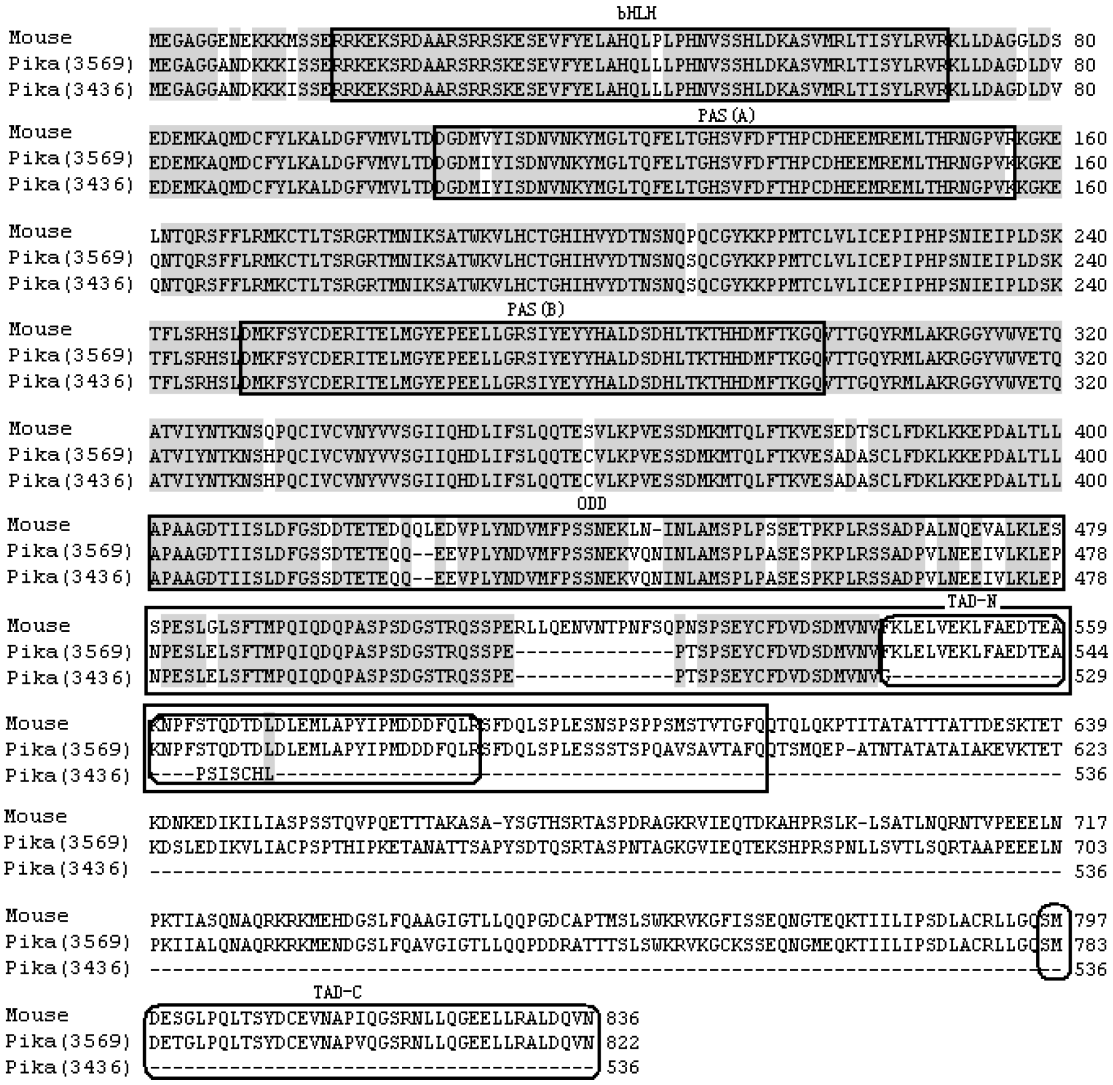


Fig. 3. Comparison of the deduced amino acid sequence of the mouse and plateau pika HIF-1α cDNAs. The mouse HIF-1α cDNA covered an open reading frame of 836 amino acids (mouse). The plateau pika HIF-1α cDNA covered an open reading frame of 822 amino acids (Pika 3569). The new variant of plateau pika translated into a 536-amino-acid protein (Pika 3436), with abridged ODD and no both TADs (TAD-N and TAD-C). Identical amino acid residues are indicated by shading. Conserved regions of the bHLH, PAS (A), PAS (B), ODD, TAD-N, and TAD-C are surrounded by boxes.

site is wet and fertile. The vegetation is classified as alpine steppe meadow; the dominant plant species include *Kobresia* spp., *Carex* spp., *Stipa* spp., and *Achanatherum splendens*. *Buteo hemilasius*, *Eremophila alpestris*, *Myosplax baileyi*, and *Mustela eversmanni*. Domestic yaks and sheep, root voles (*Microtus oeconomus*), upland buzzard (*Buteo hemilasius*), steppe polecats (*Mustela eversmanni*), weasels (*Mustela altaica*), and plateau pikas are the main vertebrates in the meadow ecosystem. The unique alpine environment may be one of the major factors determining the HIF-1α specific function of the plateau pika.

The deduced amino acid sequence of the pika HIF-1α shares high identity with both mouse (86%) and Norway rat (86%) HIF-1α cDNAs. The high degree of sequence homology suggests that molecular evolution of HIF-1α may be relatively slow. For the high ratio of HIF-1α homology among the animals, the HIF-1α gene may be a good phylogenetic performer in recovering the true phylogenetic relationships among taxa. Homology of the deduced amino acid sequences was particularly high in the bHLH region and the PAS domains (Fig. 3). The bHLH region is known to be responsible for DNA binding and dimerization, and the PAS domains are suggested to be involved in ligand binding, dimerization, and other biological activities [29]. In comparison to human, mouse, and bovine, the PAS (B) domain was served with 100% amino acid identity. Interestingly, a leucine instead of proline (L45) was found in the plateau pika HIF-1α bHLH region, which is different from any other animals compared. However, its biological significance is unclear.

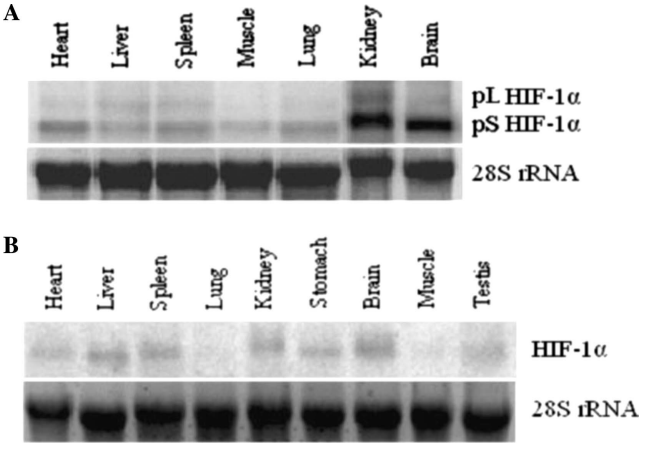


Fig. 4. Tissue specific expression of HIF-1α in plateau pika and mouse. (A) The expression of HIF-1α mRNA in different plateau pika tissues is performed by Northern blot analysis with ^{32}P-labeled specific cDNA probes. Two isoformates were detected and named as pSHIF-1α and pLHIF-1α. (B) Northern blots containing 20 μg RNA from mouse whole heart, liver, spleen, kidney, lung, stomach, brain, testicle, and skeleton muscle were hybridized to mouse HIF-1α cDNA probes. Pictures of ethidium bromide-stained 28S rRNAs are shown as internal controls.

The full-length plateau pika HIF-1α cDNA exhibits 82%, 89%, 82%, 77%, 68%, 62%, and 30% identities with the human, bovine, mouse, Norway rat, chicken, African claw flow, and rainbow trout HIF-1α cDNAs, respectively. The plateau pika 292 bp 5′ UTR shares 73% identity with that of mouse 257 bp 5′ UTR. A remarkable difference was found at the beginning of the 3′ UTR, where the plateau pika sequence lacks a 35 bp long GT-rich insertion present in the mouse sequence. Meanwhile, the plateau pika 811 bp 3′ UTR shares 76% identity with the mouse 1208 bp 3′ UTR and 86% identity with the human 1172 bp 3′ UTR.

Several studies have shown that HIF-1α mRNA in mammalian species consists of single major transcripts [14,30–33]. Recently, two major HIF-1α mRNAs in various tissues were discovered in 10-day-old chick embryonic ventricular myocytes [29], and several alternatively spliced variants of HIF-1α mRNA have been found in mouse, rat, and human cells [34,35]. Chun et al. [36] reported a new alternatively spliced variant of human HIF-1α mRNA and demonstrated it functions as a dominant-negative isoform to inhibit endogenous HIF-1α mRNA.

The plateau pika HIF-1α cDNA sequence was similar in all of the three sequenced RT-PCR (II) products, differing only in the length of the extensions. One of the extensions lacks a 133 bp fragment in the middle of plateau pika HIF-1α cDNA (Fig. 2B). The shorter fragment was identified as having lost part of exons 11 (70 bp) and 12 (63 bp), representing a new variant of HIF-1α cDNA. Lack of this 133 bp results in a new open reading frame of 536 amino acids, with abridged oxygen-dependent degradation domain (ODD), no both transactivation domains (TADs), and nuclear localization signal motif (NLS) because of the deletion of part of exons 11 (70 bp) and 12 (63 bp), respectively (Fig. 3). Based on its structure, we inferred that it was regulated partly by oxygen tension and presents no transactivation activity and hypoxia-induced translocation into the nucleus [36]. Associated with the high hypoxia tolerance of plateau pika, we speculate that this protein may play important roles in the plateau pika adaptation to hypoxia environment.

The distinctive tissue-specific differences in the plateau pika HIF-1α mRNA expression, especially in brain and kidney, are a new finding because early studies have shown that HIF-1α mRNA appeared to be abundantly expressed in most murine tissues [28,31], which is consistent with our results (Fig. 4B). Comparison of HIF-1α mRNA expression between plateau pika and mouse may help us to further understand its functional and ecological significance. The tissue-specific expression of plateau pika HIF-1α, especially in brain and kidney, may be responsible for its roles in adaptation to hypoxia.

The appropriate choice of an internal standard is indispensable for quantitative RNA analyses. The housekeeping genes β-actin, GAPDH, cyclophilin, and 28S rRNA are four common internal controls used to standardize for uneven loading among samples. However, the 28S rRNA was suggested as the most reliable internal standard for comparative analyses of transcription under hypoxia [37]. So the 28S rRNA was used for internal control in our study.

Conclusions

We have cloned the plateau pika HIF-1α cDNA and predicted its amino acid sequence. To our knowledge, this is the first cloning of HIF-1α cDNA from a natural plateau species. Meanwhile, we identified and characterized a new alternatively spliced variant of pika HIF-1α mRNA. Comparison of the plateau pika HIF-1α with various species indicates that the molecular structure of this protein is highly conserved throughout evolution. The results suggest to us that the HIF-1α gene may be a good phylogenetic performer in recovering the true phylogenetic relationships among taxa. In contrast to HIF-1α expression in other animals, the HIF-1α mRNA was highly expressed in the plateau pika brain and kidney. This result may provide a new mechanism to explain the survival of plateau pika over hypoxia. Comparison of HIF-1α mRNA expression between plateau pika and mouse may help us to further understand its functional and ecological significance.

Acknowledgments

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