Hypoxic stress tolerance of the blind subterranean mole rat: Expression of erythropoietin and hypoxia-inducible factor 1α

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Blind subterranean mole rats (*Spalax*, Spalacidae) evolved adaptive strategies to cope with hypoxia that climaxes during winter floods in their burrows. By using real-time PCR, we compared gene expression of *erythropoietin* (*Epo*), a key regulator of circulating erythrocytes, and *hypoxia-inducible factor 1*α (*HIF-1*α), *Epo* expression inducer, in the kidneys of *Spalax* and white rats, *Rattus norvegicus*. Our results show significantly higher, quicker, and longer responses to different O_2 levels in *Spalax* compared with *Rattus*. (*i*) In normoxia, both *Spalax* and *Rattus* kidneys produce small amounts of *Epo*. Maximal expression of *Rattus Epo* is noticed after a 4-h hypoxia at 6% O_2. Under these conditions, *Spalax Epo* levels are 3-fold higher than in *Rattus*. After 24 h of 10% O_2, *Spalax Epo* reaches its maximal expression, remarkably 6-fold higher than the maximum in *Rattus*; (*ii*) the *HIF-1*α level in normoxia is 2-fold higher in *Spalax* than in *Rattus*. *Spalax HIF-1*α achieves maximal expression after 4-h hypoxia at 3% O_2, a 2-fold increase compared with normoxia, whereas no significant change was detected in *Rattus HIF-1*α at any of the conditions studied; (*iii*) at 6% O_2 for 10 h, in which *Rattus* cannot survive, *Epo* and *HIF-1*α levels in *Spalax galili*, living in heavily flooded soils, are higher than in *Spalax judaei*, residing in light aerated soil. We suggest that this pattern of *Epo* and *HIF-1*α expression is a substantial contribution to the adaptive strategy of hypoxia tolerance in *Spalax*, evolved during 40 million years of evolution to cope with underground hypoxic stress.

Spalax is a unique model of hypoxic tolerance. The blind subterranean mole rat (*Spalax ehrenbergi* superspecies) is a wild group of species of rodents belonging to the family Spalacidae that originated some 40 million years ago (1). *Spalax* spends its entire life underground (1), exposed to fluctuating O_2 and CO_2 levels (1). It consists of at least 12 allospecies in the Near East. The four allospecies in Israel were intensively studied interdisciplinarily across all organizational levels (2). They adapted to four different climatic regimes: *Spalax galili* ($2n = 52$) inhabits the cool-humid Upper Galilee Mountains; *Spalax golani* ($2n = 54$), the cool-semihumid Golan Heights; *Spalax carmeli* ($2n = 58$), warm-humid central Israel, and *Spalax Judaei* ($2n = 60$) the warm-dry southern regions (2). Hypoxia climaxes, especially in the northern *S. galili* and *S. golani*, during winter rains and flooding when gas solubility and permeability are restricted and the animal is occupied in tunnel rebuilding (1). We measured 7% O_2 inside the *Spalax* burrows after rains (I.S., A.A., and E.N., unpublished work) and in the laboratory it can survive at least 14 h under 3% O_2, whereas *Rattus* dies after 2–4 h. *Spalax* displays a record among mammals for hypoxia tolerance.

During 40 million years of life underground, *Spalax* evolved physiological strategies underlying their respiratory and cardiovascular systems to cope with hypoxia (1–3). *Spalax* is dramatically superior to *Rattus* in *vascular endothelial growth factor (VEGF)* activity (4) and myocardial performance (5), and harbors much higher capillary and mitochondrial density than does *Rattus* (6). It has a higher erythrocyte count (7), increased lung diffusion capacity, and different structure of hemoglobin (8), haptoglobin (9), myoglobin (10), neuroglobin, and cytoglobin. Furthermore, there are differences among the four Israeli *Spalax* allospecies in their s.c. gas tension (7), hemoglobin, and hematocrit concentrations, with higher values in the northern *S. galili* compared with the southern *S. judaei* (7).

Erythropietin and its expression inducer hypoxia-inducible factor-1 (HIF-1). Erythropoietin (Epo) is the main factor involved in erythropoiesis, regulating the level of circulating red blood cells. Epo is expressed mainly in fetus liver and adult kidneys (11, 12) and is regulated by hypoxia stimulus at the RNA level (13). Epo has also angiogenic activity that stimulates neovascularization *in vivo* (14), similar to VEGF angiogenic potential (15). Epo also participates in neuron protection from ischemic damage and cell death (16, 17). The *Epo* gene was fully or partially cloned from a variety of mammals (18), including the intron1/exon 2 fragment from the *dolphin* that also experiences hypoxic environment. All mammals display high homology as well as interspecies biological and immunological crossreactivity (18).

To counteract the possible deleterious effects of hypoxia, an immediate molecular response is initiated. This response is mediated by HIF-1, which is the mammalian master regulator of O_2 homeostasis (19, 20). HIF-1 consists of HIF-1α and HIF-1β subunits (20, 21). HIF-α is the hypoxia-inducible subunit, and its expression is regulated at both the transcription (22) and post-transcription stages (23, 24). HIF-1 is the transcription factor of many genes related to erythropoiesis (*Epo*, *transferrin*), vascularization, vasodilatation (*VEGF* and *nitric oxide synthase*), and genes involved in the glycolysis process (25). Under hypoxia, HIF-1 protein binds to a specific site in the 3′ enhancer of the *Epo* gene, which induces its transcription (13).

We cloned *Spalax Epo* and *HIF-1*α genes and showed dramatic differences in their expression compared with *Rattus* under normoxic and hypoxic conditions. *Spalax Epo* and *HIF-1*α expression pattern under hypoxia contribute to its unique mammalian record of hypoxia tolerance.

Materials and Methods

Animals. *Spalax* was captured in the field and housed in individual cages in the animal house of the Institute of Evolution. To test for hypoxic stress, animals were placed in a $70 \times 70 \times 50$ cm chamber divided into separate cells. The gas mixture was delivered at 3.5 liters/min. All experiments were conducted on adults of similar weight (100–150 g). A total of 25 *Spalax*, from *S. judaei*, the Anza population, and from *S. galili* from the Kerem Ben

Abbreviations: Epo, erythropoietin; HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AJ715791–AJ715795).

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Zimra population (2), and 21 laboratory *Rattus norvegicus* were used for *Epo* and *HIF-1α* gene quantification.

The Ethics Committee of the University of Haifa approved all experiments.

Tissues. Animals were killed by injection with Ketaset CIII (Fort Dodge, IA), 5 mg/kg of body weight. Whole organs were taken out and immediately frozen in liquid nitrogen.

RNA and cDNA Preparation. Total RNA was extracted from tissues by using TRI Reagent (Molecular Research Center, Cincinnati) following manufacturer's instructions. RNA samples were treated with DNase I (DNA-free, Ambion), and 1 μg was taken for first-strand cDNA synthesis (iScript, Bio-Rad) in a 20-μl volume. Aliquots of 1 μl of undiluted cDNA were used per PCR or real-time PCR.

Cloning of *Spalax Epo* (*sEpo*) and *HIF-1α*. The *sEpo* gene and *sHIF-1α* ORF were isolated by PCR using *Taq*DNA polymerase (Qbiogene, Illkrich, France) and oligonucleotides (Sigma Genosys, Rehovot, Israel) designed according to published human, mouse, and rat sequences. For cloning of the *sEpo* gene, 100 ng of genomic DNA samples from each of the four Israeli *Spalax* allospecies (2) was used as a template. The promoter region was amplified from two additional individuals of each species. The ORF of *sHIF-1α* was cloned from brain total RNA from *S. galili* and *S. judaei*. The PCR products of both genes were subcloned into pGEM T-easy vector (Promega) and transformed by electroporation into *Escherichia coli* XL1-blue. The sequence was determined on both DNA strands (Technion, Haifa, Israel), and analyzed by using GCG software.

Gene Quantification. Absolute gene quantification was performed by using ABI PRISM 7000 (Applied Biosystems). Both *HIF-1α* and *Epo* gene expression were normalized to *cyclophilin* (26) as an internal housekeeping gene control. We quantified *cyclophilin*, *18-s rRNA*, and *β-actin* in α-^{32}P-labeled cDNA from normoxic and hypoxic kidneys from *Rattus* and *Spalax* to ensure an equal amount of template in the real-time PCR. Both *Spalax* and *Rattus* expressed similar amounts of *cyclophilin*, which did not respond to hypoxia. For *Spalax* and *Rattus* gene expression comparison, we designed primers in the conserved regions of the genes by using PRIMER EXPRESS 2 software (Applied Biosystems). *Cyclophilin* and *HIF-1α* primers were identical for *Spalax* and *Rattus*. The *Epo* primers were not completely homologous, but amplified the same amplicon in *Spalax* and *Rattus*.

For the standard curves, dilutions of plasmid-DNA constructs containing the amplicons of each gene were used. Gene expression rates are given in copies of cDNA starting from 50 ng of total RNA. Reactions were performed by using SYBR green PCR Master Mix (Applied Biosystems) in a total volume of 25 μl. Dilutions of cDNA were used to verify efficiency of the PCR (slope = 3.3 ± 0.1, $R^2 \approx 1$). 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 were isolated from individual animals, and each sample was tested in duplicate or triplicate. Unless otherwise specified, the expression of *Epo* and *HIF-1α* in *Spalax* represents the results obtained from both *S. galili* and *S. judaei*.

Epo Promoter Constructs. The pX2 luciferase constructs Δ18, containing 152 bp of the human *Epo* promoter and ΔEPΔ18, where the element named EP17 was deleted, were provided by Drs. E. Goldwasser and M. Gupta (University of Chicago, Chicago; ref. 27). To insert the *Spalax*-specific nucleotide substitutions in the EP17 element, point mutations were inserted by PCR, using primers with the requested substitution. In addition, *Spalax* promoter fragment corresponding to the human 152 bp (positions 15–167 in the *Spalax* gene) was amplified and inserted into pXP2 upstream to the luciferase reporter.

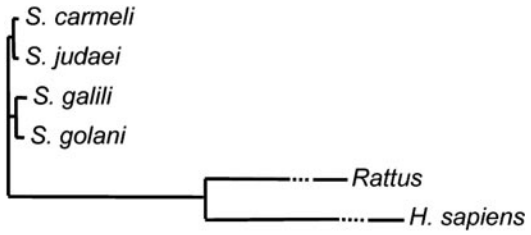

Fig. 1. Phylogenetic tree based on the *Epo* gene sequence reconstructed by using the neighbor-joining method (28).

Transfections and Luciferase Assays. Hep3b cells were grown in DMEM (Biological Industries, Beit Haemek, Israel) containing 10% FCS. Cells were plated in six-well plates at ≈60% confluence 1 day before the transient transfection. Transfection was performed by using the following: 6 μl of FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis) in 100 μl of serum-free media, 3.5 μg of pX2, and 0.7 μg of *cmv-β*-galactosidase. The reaction mix was incubated at room temperature for 20 min and added to 35-mm plates already containing adherent culture. Duplicate plates were tested; one was incubated at 37°C for 48 h under hypoxic conditions (5% CO_2/1% O_2/94% N_2), and the other under normoxic conditions (5% CO_2/95% air). For cultured cell lysis, we used M-Per mammalian protein extraction reagent (Pierce) following the manufacturer's instructions. Luciferase activity was measured by Anthos Lucy-1 (Anthos Labtech Instruments, Wals, Austria) using the Promega luciferase assay system. For β-galactosidase activity assay, we used 50 μl of cell lysis in 200 μl of 70 mM Na-phosphate buffer with 1 mM $MgCl_2$. The reaction mixture was incubated for 15 min in room temperature and activity was measured by Lucy-1 at OD_{405}. All experiments were performed in triplicate.

Data Analysis. Values are presented as mean ± SEM for $n = 3$–6 animals, depending on the experiment. Student's t test was used for comparison between groups ($P < 0.05$ considered significant).

Results

Cloning. *Epo*. An *Epo* (*sEpo*) genomic fragment of ≈4.5 kb of the four Israeli *Spalax* allospecies was amplified and sequenced, starting at −366 bp upstream to the translation start site and ending at +1009 downstream of the translation termination site. Sequences are published in GenBank: *S. galili* (GenBank accession no. AJ715795), *S. golani* (GenBank accession no. AJ715792), *S. carmeli* (GenBank accession no. AJ715793), and *S. judaei* (GenBank accession no. AJ715794). All four *Spalax* species exhibit identical *Epo* putative peptide, apart from one nonsynonymous substitution that was found in *S. golani* at base pair 1912, which leads to amino acid substitution (R to Q) at position 102 of the putative peptide. A phylogenetic tree (28) of *sEpo* subdivided the four *Spalax* allospecies into two groups: the northern *S. galili* and *S. golani*, and the southern *S. carmeli* and *S. judaei* (Fig. 1). The same subdivision was found by allozymes (29), *cytochrome b* (30), mitochondrial DNA (31, 32), and random amplified polymorphic DNAs (33).

***HIF-1α*.** The isolated ORF of *S. judaei* and *S. galili HIF-1α* (*sHIF-1α*) is identical and contains 2,475 bp, coding a predicted peptide of 825 aa (GenBank accession no. AJ715791). It is highly conserved, and in comparison with the human, mouse, and rat

EVOLUTION

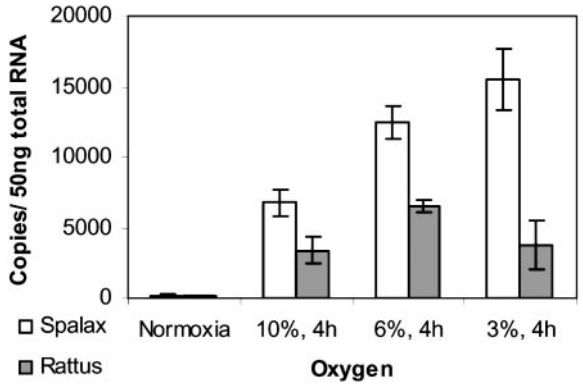

Fig. 2. *Epo* gene expression in *Spalax* and *Rattus* kidneys in normoxia and after 4 h of increasing hypoxia. Values in *Spalax* were 190 ± 57, 6,805 ± 946, 12,449 ± 1,173, and 15,519 ± 2,208; and values in *Rattus* were 130 ± 53, 3,398 ± 898, 6,501 ± 425, and 3,784 ± 1,793, under normoxia and 10%, 6%, and 3% O_2, respectively.

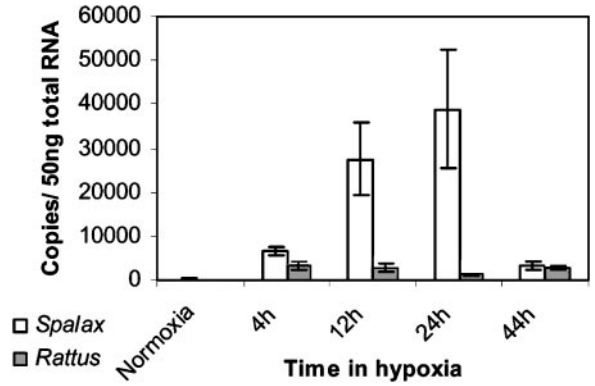

Fig. 3. Time course of *Epo* gene expression in *Spalax* and *Rattus* kidneys in normoxia and 10% hypoxia. The numbers of copies in 50 ng of total RNA in *Spalax* were 190 ± 57, 6,805 ± 946, 27,485 ± 8,322, 38,898 ± 13,548, and 3,177 ± 877; and the numbers in *Rattus* were 130 ± 53, 3,398 ± 898, 3,040 ± 963, 1,355 ± 209, and 2,691 ± 523 under normoxia and after 4, 12, 24, and 44 h of hypoxia, respectively.

showed ≈91% identity at the nucleotide level, and 93% identity at the amino acid level, for all three species.

Regulatory Domains in *Spalax Epo*. Most of the known regulatory domains in both the promoter and the enhancer of the *Epo* gene, including the HIF-binding site, steroid hormone receptors (DR2), and GATA site were highly conserved in *Spalax* compared with the human (13), mouse (34), and rabbit (35). Promising substitutions were found in the *Spalax* EP17 site known as a hypoxia-sensitive element (27). In humans and mice, the sequence is completely conserved: 5′-CCCCCACCCCCAC-3′. However, in *Spalax*, at least some of the individuals of *S. judaei* and *S. carmeli* have C-to-T substitution in position 10 in the EP17, and some of the *S. golani* are heterozygotes and have an A or C (M) in position 9 of the EP17. The *S. galili* EP17 element is identical to that of the human and mice.

Contradictory to the findings in the human modified EP17, which caused a loss of the hypoxic response (27), no significant differences were found in the hypoxic response of any of the *Spalax* EP17 compared with the authentic human EP17. Moreover, we did not find any difference in hypoxic activity of the *Spalax* promoter region compared with the equivalent human promoter. As was published (27), we found an ≈4-fold increase in luciferase reporter activity by hypoxia, suggesting that the differences in the sequence of the *Spalax* EP17 element or in its promoter did not affect its hypoxic inducibility.

Epo Expression. In normoxia, the levels of *Epo* transcript were very low, and varied between individuals in both rodents. *Spalax* expressed 190 ± 57 copies of *Epo* compared with 130 ± 53 copies of *Epo* in *Rattus*, although the differences are not statistically significant (Figs. 2 and 3).

Remarkably, however, in lower oxygen concentrations for 4 h (Fig. 2) and compared with normoxia, *sEpo* increased 82-fold ($P < 0.01$), significantly higher ($P < 0.05$) than *Rattus Epo*, which increased 50-fold ($P < 0.05$). Moreover, *sEpo* response is significantly and positively correlated with hypoxic stress, unlike *Rattus Epo*.

Because *Rattus* does not survive in very low oxygen concentrations (3% or 6%) for >2–6 h, we studied *Epo* mRNA production in 10% O_2 for different periods (Fig. 3). *sEpo* levels increased up to 205-fold after 24 h ($P < 0.01$) compared with normoxia whereas *Rattus Epo* levels, although significantly higher than in normoxia ($P < 0.05$), did not increase further after 4 h.

The Northern *S. galili* Is Better Adjusted to Hypoxia than the Southern S. judaei. Under 6% or 3% O_2 for 4 h or at 10% O_2 for up to 44 h, we found no differences in *Epo* expression among Israeli *Spalax* species. We then exposed *S. galili* (the northern species), living in heavy soil and high rainfall, and *S. judaei* (the southern species), living in light soil and lower rainfall, to severe hypoxia (6% O_2) for a relatively long period (10 h), conditions in which *Rattus* did not survive. *S. galili's Epo* level (Fig. 4) is 3-fold higher than in *S. judaei* under normoxic conditions, as well as after exposure to the hypoxic stress ($P < 0.05$, $n = 5$).

HIF-1α Expression. We also quantified *HIF-1α* expression in the same cDNA kidney samples isolated from *Spalax* and *Rattus*. Under normoxic conditions, *sHIF-1α* levels were two times higher compared with *rHIF-1α* ($P < 0.05$; Fig. 5). Moreover, *rHIF-1α* does not respond to hypoxia, whereas *sHIF-1α* does significantly. A dramatic increase ($P < 0.01$) of *sHIF-1α* followed increased hypoxia, contrasting with *rHIF-1α*.

At a relatively mild hypoxic stress of 10% O_2 and up to a 12-h exposure, *sHIF-1α* expression is similar to its levels under normoxia (Fig. 6). After exposure for 24 h or more to 10% O_2, *sHIF-1α* levels in kidneys decreased to one half-fold ($P < 0.01$) compared with normoxia, whereas *rHIF-1α* levels fluctuated insignificantly compared with normoxia (Fig. 6).

In most of the experiments, we did not find significant

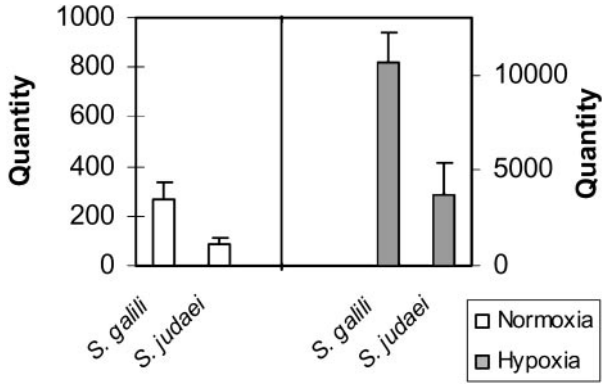

Fig. 4. Comparative *Epo* gene expression in *S. galili* and *S. judaei* under normoxia and 6% O_2 for 10 h. *S. galili* values were 269 ± 65 and 10,687 ± 1,506; and *S. judaei* values were 85 ± 30 and 3,739 ± 1,620, under normoxia and hypoxia, respectively.

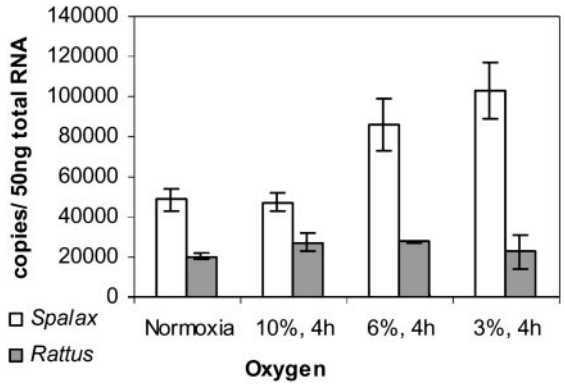

Fig. 5. *HIF-1α* gene expression in *Spalax* and *Rattus* kidneys in normoxia and after 4 h of increased hypoxia. *Spalax* values were 48,644 ± 5,407, 47,288 ± 4,357, 85,858 ± 12,745, and 102,890 ± 13,797; and *Rattus* values were 20,043 ± 1,519, 27,212 ± 4,590, 27,631 ± 479, and 22,797 ± 8,465, under normoxia and 10%, 6%, and 3% O_2, respectively.

differences in *HIF-1α* expression among the *Spalax* species that were tested. Only at extreme conditions, after 10 h at 6% O_2, *HIF-1α* mRNA levels were 40% higher in the northern species *S. galili* compared with the southern species *S. judaei*.

Discussion

General Overview. All higher organisms developed adaptive mechanisms to detect and respond to hypoxia. However, animals that chronically inhabit hypoxic ecological niches developed uniquely effective strategies to survive under hypoxia. High-altitude mammals (36), diving mammals (37), and particularly *Spalax* (38) that represent an extreme case among subterranean mammals (1), faced hypoxia and developed adaptations to tolerate it. Higher muscle myoglobin, larger blood buffer capacity, and hemoglobin properties allow these animals better O_2 utilization (8, 38). Nevertheless, *Spalax* differs dramatically from all diving mammals that are exposed intermittently to hypoxia, recharging O_2 breathing (38). High-altitude mammals are often limited in their vertical ascent and hypoxic exposure. *Spalax*, by contrast, spends its life mostly underground in sealed burrows (1, 2), and experiences hypoxia down to 7% O_2 (I.S., A.A., and E.N., unpublished work), and probably much lower when the burrows are flooded by rainwater. Moreover, at least three of the allospecies of *Spalax* studied here, the *S. galili*, *S. golani*, and *S. carmeli* (2) inhabit heavy soil and high rainfall, drastically limiting soil ventilation and gas permeability (1). Consequently, *Spalax* is confronting the hypoxia primarily during its winter activities (1) under persistent hypoxia.

A common adaptive strategy of mountaintop and diving mammals is the down-regulation of metabolism (39), i.e., myocardial and skeletal muscle contractile activity (36–40). However, permanent activity under hypoxia necessitates constant O_2 delivery to tissues, especially muscles used in burrowing. Erythrocyte supply is critical in hypoxia, depending on Epo expression (41) and its regulation by HIF-1 (25); hence, our analysis of these two genes in *Spalax*.

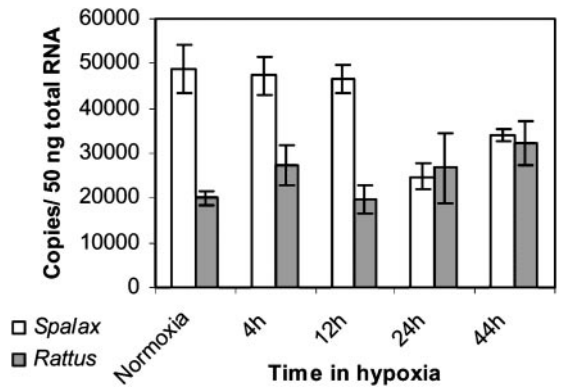

Fig. 6. Time course of *HIF-1α* expression in *Spalax* and *Rattus* kidneys in normoxia and 10% hypoxia. *Spalax* values were 48,644 ± 5,407, 47,288 ± 4,357, 46,599 ± 3,302, 24,832 ± 2,910, and 34,041 ± 1,248 and *Rattus* values were 20,043 ± 1,519, 27,212 ± 4,590, 19,767 ± 3,286, 26,743 ± 7,746, and 32,067 ± 4,974 under normoxia and 4, 12, 24, and 44 h of hypoxia, respectively.

Epo Is a Key Factor to Confront Hypoxia. Only partial cloning of dolphin *Epo* was heretofore reported in mammals living under hypoxia (18). Therefore, we present here the first comprehensive study, to our knowledge, on *Epo* and *HIF-1* gene expression in an animal chronically exposed to hypoxic stress.

Erythropoesis is regulated by several cytokines, initiated by bone marrow pluripotent stem cells, and terminating in mature erythrocytes. Epo acts primarily on colony-forming unit erythroid proliferating into mature erythrocetes (41, 42). Likewise, Epo participates in angiogenesis (14, 15), and stimulates postnatal neovascularization (43). Epo is synthesized mainly in fetal liver (11), shifting to the kidneys shortly after birth (12). Nevertheless, *Epo* expression has been detected also in the lung, spleen, brain, and testis (44).

Epo synthesis in hypoxia is regulated at the mRNA level (45, 46) and is controlled by both transcription and posttranscriptional mechanisms (47). Decreased oxygen tension in tissues (in anemia or hypoxia) induces glycolytic gene activity, growth factors, and hormones (20). Transcription of most of these genes is induced by HIF-1, the main regulator of oxygen homeostasis in mammals (19). In *Epo*, HIF-1 binds to a specific site in the enhancer at the 3′ end of the gene (25). HIF-1 is a heterodimer composed of two subunits, both of which are basic helix–loop–helix proteins in the PAS (Per-AHR-ARNT-SIM) family of transcription factors. The *HIF-1α* subunit responds to hypoxia, and HIF-1 activation is correlated with oxygen-dependent accumulation of HIF-1α protein (20).

Spalax Epo Expression Is Adapted to Oxygen Fluctuations. The *Epo* gene of all Israeli *Spalax* contains all of its known regulatory sites (13). The EP17 element is involved in hypoxia-regulated *Epo* gene expression (27). Transfections of Hep3B cells with the minimal active fragment of the human promoter, containing the EP17 element, conferred a 4-fold induction in luciferase-reporter activity due to hypoxia. Internal deletion or multiple point mutations in the EP17 resulted in loss of response to hypoxia, suggesting that factors binding to the EP17 element activate *Epo* transcription in cells under hypoxia. We have found that the EP17 element in three of the four Israeli *Spalax* species contain one nucleotide substitution compared with the human (27) and mouse (12). However, these changes do not affect *Spalax* EP17 response to hypoxia, which resembles the human's. Notably, demolishing the hypoxic response of the human EP17 element (27) involved more than one nucleotide substitution, and the sequence of the rabbit EP17 element (35) is different from that of the human, mouse, and *Spalax*, yet is still active. Furthermore, the *Spalax* authentic equivalent promoter fragment also induced hypoxic responses similar to the human homolog. Therefore, we conclude that any changes in *sEpo* expression under hypoxic stress cannot be attributed to the changes found in its promoter as compared to other species.

Rattus kidney responds to hypoxia by increasing *Epo* production (45, 48). We found that maximum *Epo* expression in the *Rattus* kidney is 50-fold higher compared with its normoxic levels after 4 h at 6% O_2. *Spalax*, by contrast, shows a unique, extreme ability to respond to long-term hypoxia, and maximal expression,

found at 10% O_2 after 24 h, is five times higher than the maximal expression of *Rattus Epo* (Figs. 2 and 3). However, after 44-h hypoxia at 10% O_2, *Epo* levels in *Spalax* kidneys decreased and were similar to the *Rattus Epo* levels under identical conditions. It has been shown that the injection of recombinant human Epo to *Rattus* did not affect *Epo* production under hypoxia; thus, this decline is not due to feedback inhibition (49). Nevertheless, others reported (50) that after 24 h of hypoxia, Epo protein level reaches its maximum, then significantly decreases, and returns to normoxic values after 14 days. However, the number of colony-forming unit erythroid cells, the target cells of Epo (41), reach a peak on day 14 and then progressively decrease. Therefore, it can be suggested that the increase in Epo during the first 24 h of hypoxia, similar to what we noticed in *Spalax Epo* mRNA, is sufficient to induce a satisfactory rate of erythropoiesis for the maintenance of a steady state under longer hypoxic conditions.

Because *Rattus* did not survive at lower oxygen concentrations for more than 4–6 h, we exposed animals to different oxygen concentrations (10%, 6%, and 3% O_2) for 4 h. It should be reemphasized that *Spalax*, in contrast to *Rattus*, demonstrated no deleterious effects and no changes of behavior under 6% or 3% O_2 for up to 14 h when the observations were terminated. The data suggest that *Rattus Epo* does not respond significantly to different O_2 concentrations. Hence, *Rattus* cannot adapt to increased hypoxic stress by increasing *Epo* production. *Spalax*, in contrast, responds according to the stress in an oxygen-concentration-dependent manner, and *Epo* levels after 4 h are maximal at the lowest O_2 supply studied (3%); i.e., it is adapted to live in a wide range of oxygen levels and responds appropriately to variable oxygen stresses. The quick and stress-dependent elevation in *Epo* mRNA levels in *Spalax* support its exposure to abrupt, drastic falls in oxygen supply, down to a low % O_2, in its sealed tunnels while flooded during rain. In this situation, when the animal is provoked into heavy work, exhausting the already limited supply of oxygen present, erythrocyte production is induced to better use the limited levels of available oxygen.

Epo was shown to have nonerythropoietic functions as well (reviewed in ref. 51). It protects brain neurons and the spinal cord from ischemic damage in animal models; it plays a role in the modulation of the ventilatory acclimatization to hypoxic exposure; it can reduce the infarct size in patients after ischemic brain stroke; and it protects the retina from experimentally light-induced retinal degeneration. Therefore, considering the fact that Epo-dependent erythropoiesis takes a few weeks to elevate the hematocrit (52), and as *Spalax* hematocrit is higher than in other rodents (7), the *Epo* mRNA pattern of expression in *Spalax* might have nonerythropoietic protective functions to meet its hypoxic environment.

Moreover, we observed higher levels of *Epo* and *HIF-1α* mRNA in the northern allospecies *S. galili* compared with the southern *S. judaei* species under both normoxic conditions and after long periods (10 h) of severe hypoxia (6% O_2). These results, reflected also in the phylogentic tree of *Epo* that separates the northern from the southern species, complement previous observations on higher hematocrit and Hb levels in the northern species (7) and its higher genetic tolerance to lower O_2 levels (3). This finding supports our hypothesis of adaptive respiratory variations associated with climatic and consequent hypoxic severity, also within the *Spalax ehrenbergi* superspecies.

***sEpo* Response to Hypoxia Is Different from Its *VEGF* Response.** The *Epo*-adaptive expression patterns in *Spalax* are different from those of *VEGF* (4). In *Spalax* muscle, the most energy consuming tissue, *VEGF* is maximal in normoxia with no further increase in hypoxia. In *Rattus*, *VEGF* is significantly lower than *Spalax* in normoxia, but increases 2.2-fold in hypoxia (4). *VEGF* is needed for relatively longer processes, such as neovascularization and angiogenesis, whereas *Epo* is mainly used for relatively shorter processes of maintaining the erythrocyte level and urgent increases of erythrocyte production in hypoxia. Therefore, in an animal like the *Spalax*, which spends most of its life underground exposed to drastic fluctuations in oxygen levels, *VEGF* constitutively supports long-term neovascularization, whereas *Epo* levels respond quickly to the hypoxic stress.

***HIF-1α* Levels in *Spalax* Rise Under Severe Hypoxia.** Previous reports (22) on the *in vivo* regulation of *HIF-1α* expression by hypoxia are contradictory. *HIF-1α* mRNA concentrations of mice exposed to 0.1% carbon monoxide for 4 h were not up-regulated despite a strong serum Epo protein induction. However, a nonquantitative Northern hybridization study on rats exposed to 7% O_2 for 60 min before sacrifice, indicated a modest increase of *HIF-1α* mRNA levels, but returned to baseline after 4 h of (23). Our results show that the levels of *HIF-1α* in *Rattus* kidneys did not change significantly under different hypoxic conditions compared with its normoxic levels. It seems that *HIF-1α* mRNA (22) and protein (24) response to hypoxia is transient and short. Our findings indicate that the induction of *Rattus Epo* expression due to hypoxia is probably not dependent on an increase of *HIF-1α* mRNA level in kidneys, but induced either by the existing HIF-1 present in the kidney under normoxia or secreted by other organs and/or by posttranscriptional mechanisms (47). The inability of the *Rattus* kidney to respond to hypoxia by elevated *HIF-1α* mRNA level (Figs. 5 and 6) may be related to our findings that *Rattus* kidney levels of *Epo* are significantly lower compared with *Spalax* under different hypoxic stresses and do not change significantly with time length or the degree of the hypoxic stress (Figs. 2 and 3). We can assume that the inability of Rattus *HIF-1α* to respond to the hypoxic stress for periods >60 min (23), and its *Epo* unresponsiveness to the duration or the degree of the hypoxic stress, contribute to its death under severe hypoxia and after a relatively short time.

Spalax kidneys, in contrast to *Rattus*, contribute to *HIF-1α* gene expression, first by maintaining a high level in normoxia, and second, by stimulating its mRNA synthesis in severe hypoxia. Thus, *Spalax* seems to have more than one mechanism of *HIF-1α* activity that supports its tolerance to hypoxia: (*i*) *Spalax* has a 2-fold higher level of kidney *HIF-1α* in normoxia compared with *Rattus*; (*ii*) the up-regulation of *Spalax Epo* levels under a long period of mild hypoxic conditions (10% O_2) is controlled through the induction of high levels of HIF-1 present at basic normoxic conditions and/or by posttranscriptional processes in *Epo* (48) and *HIF-1α* (20, 21); (*iii*) under severe hypoxia, *Spalax HIF-1α* expression in the kidneys, the main *Epo* synthesis organ, is elevated in a stress-related mode, although the increase at O_2 lower than 6% O_2 is not significant (Fig. 5). We hypothesize that at 6% O_2 *Spalax HIF-1α* reaches its maximal efficiency and/or capacity to respond to the hypoxic stress. Nevertheless, this occurrence does not affect its ability to survive at 3% O_2.

It should be mentioned that *in vitro* (24) HIF-1α protein was not detected in the nuclei of HeLaS3 cells under normoxic conditions. However, exposure of the cells to very low O_2 (0–5%) for periods of 2 min to 4 h revealed synthesis of nuclear HIF-1α protein that was observed after 2 min and continued to accumulate for up to 60 min. At 60 min, it reached a maximum level that was maintained for up to 4 h of hypoxic exposure. Noteworthy, we must emphasize that we have noticed (I.S., A.A., and E.N., unpublished work) significant differences in the pattern of expression of *HIF-1α* as well as *VEGF* mRNAs *ex vivo* compared with *in vivo*. Furthermore, a special emphasis in our future studies should be addressed to the expression of HIF-2α. This emphasis is in light of results (53) that indicate a selective hypoxic induction of HIF-1α and -2α and their possible dependent target gene activation in different parts of the kidney. Accordingly, peritubular cortical fibroblasts were identified as

the site of both Epo and HIF-2α production, suggesting a possible role for HIF-2α in Epo regulation.

Accumulation of *sHIF-1*α and *sEpo* shown here and in erythrocyte maturation (50, 54), may indicate a cascade-like kinetics: *HIF-1*α, the hypoxia-sensitive subunit of HIF-1 that induces *Epo* production, reaches its maximal expression in *Spalax* kidney at 12 h. *Epo* expression, regulated by the binding of HIF-1 protein to its promoter, lags and achieves maximal level in *Spalax* kidney at 24 h. Maximal erythrocyte maturation, driven by Epo protein, occurred when Epo levels decreased (50, 54).

Prospects. A few questions are raised in this study and need further investigation: (*i*) what is the role of *Spalax Epo* mRNA stability in the observed high levels after long-term hypoxia? Northern blot analyses showed that the steady state of *Epo* mRNA levels in Hep3B cells increase >50-fold in response to hypoxia, but nuclear runoff experiments demonstrated only a 10-fold increase in *Epo* gene transcription under identical conditions. This finding suggests that the stability of *Epo* mRNA and its steady-state levels are modulated by both transcriptional and posttranscriptional events (42); (*ii*) is the *HIF-1*α promoter also induced by hypoxia and does it contribute to the *HIF-1*α gene expression in *Spalax*? At present, there is no evidence for *HIF-1*α promoter's response to hypoxia (24). It is of great interest to test *Spalax* Epo and HIF-1α and -2α expression, at both mRNA and protein levels, in fluctuating and severe hypoxia and in hypoxia followed by reoxygenation, because *Spalax* lives in a greatly fluctuating oxygen atmosphere, ranging from very low to normal oxygen levels.

Clearly, *Epo* and *HIF-1*α are only two of the genes in *Spalax*'s gene battery of hypoxic tolerance adaptation that evolved during its 40 million years of underground evolution (1). *Spalax* is undoubtedly an extreme wild mammal model exposed sporadically to extreme levels of hypoxia, particularly during heavy winter rains. The adaptive trajectory of *Spalax* evolved toward ever-increasing hypoxia tolerance rather than the alternative mammalian channel of hibernation.

Future studies in our laboratory aim at probing the extensive adaptive battery of genetic responses of *Spalax* to variable hypoxic stress through comprehensive functional genomic studies and to unravel the numerous genes collaborating in *Spalax* hypoxia tolerance. These studies could later be used in human gene therapy in the fight against ischemia (55) and cancer and contribute to life in environmental extremes such as outer space flights and deep sea diving.

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1. Nevo, E. (1999) *Regression, Progression, and Global Convergence Mosaic Evolution of Subterranean Mammals* (Oxford Univ. Press, Oxford).
2. Nevo, E., Ivanitskaya, E. & Beiles, A. (2001) *Adaptive Radiation of Blind Subterranean Mole Rat* (Backhuys, Leiden, The Netherlands).
3. Arieli, R. & Nevo, E. (1991) *Comp. Biochem. Physiol.* **3,** 543–545.
4. Avivi, A., Resnik, M. B., Joel, A., Nevo, E. & Levi, A. P. (1999) *FEBS Lett.* **452,** 133–140.
5. Edoute, Y., Arieli, R. & Nevo, E. (1988) *J. Comp. Physiol.* **158,** 575–582.
6. Widmer, H. P., Hoppeler, H., Nevo, E., Taylor, C. R. & Weibel, E. W. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 2062–2267.
7. Arieli, R., Heth, G., Nevo, E. & Hoch, D. (1986) *Experientia* **42,** 441–443.
8. Kleinschmidt, T., Nevo, E. & Braunitzer, G. (1984) *Hoppe-Seyler's Z. Physiol. Chem.* **365,** 531–536.
9. Nevo, E., Ben-Shlomo, R. & Maeda, N. (1989) *Heredity* **62,** 85–90.
10. Gurnett, A. M., O'Connell, J., Harris, D. E., Lehmann, H., Joysey, K. A. & Nevo, E. (1984) *J. Protein Chem.* **3,** 445–454.
11. Zanjani, E. D., Poster, J., Burlington, H., Mann, L. I. & Wasserman, L. R. (1977) *J. Lab. Clin. Med.* **89,** 640–644.
12. Jacobson, L. O., Goldwasser, E., Fried, W. & Plzak, L. (1957) *Nature* **179,** 633–634.
13. Ebert, B. L. & Bunn, H. F. (1999) *Blood* **94,** 1864–1877.
14. Ribatti, D., Presta, M., Vacca, A., Ria, R., Giuliani, R., Dell'Era, P., Nico, B., Roncali, L. & Dammacco, F. (1999) *Blood* **93,** 2627–2636.
15. Jaquet, K., Krause, K., Tawakol-Khodai, M., Geidel, S. & Kuck, K.-H. (2002) *Microvasc. Res.* **64,** 326–333.
16. Sakanaka, M., Wen, T. C., Matsuda, S., Masuda, S., Morishita, E., Nagao, M. & Sasaki, R. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 4635–4640.
17. Celik, M., Gokmen, N., Erbayraktar, S., Akhisaroglu, M., Konakc, S., Ulukus, C., Genc, S., Genc, K., Sagiroglu, E., Cerami, A. & Brines, M. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 2258–2263.
18. Wen, D., Boissel, J. P., Tracy, T. E., Gruninger, R. H., Mulcahy, L. S., Czelusniak, J., Goodman, M. & Bunn, H. F. (1993) *Blood* **82,** 1507–1516.
19. Hopfl, G., Ogunshola, O. & Gassmann, M. (2004) *Am. J. Physiol.* **286,** R608–R623.
20. Wang, G. L. & Semenza, G. L. (1995) *J. Biol. Chem.* **270,** 1230–1237.
21. Iyer, N. V., Leung, S. W. & Semenza, G. L. (1998) *Genomics* **52,** 159–165.
22. Wiener, C. M., Booth, G. & Semenza, G.L. (1996) *Biochem. Biophys. Res. Commun.* **225,** 485–488.
23. Wenger, R. H., Rolfs, A., Marti, H. H., Guenet, J. L. & Gassmann, M. (1996) *Biochem. Biophys. Res. Commun.* **223,** 54–59.
24. Jewell, U. R., Kvietikova, I., Scheid, A., Bauer, C., Wenger, R. H. & Gassmann, M. (2001) *FASEB J.* **15,** 1312–1314.
25. Semenza, G. L. (1998) *Curr. Opin. Genet. Dev.* **8,** 588–594.
26. Weisinger, G., Gavish, M., Mazurika, C. & Zinder, O. (1999) *Biochim. Biophys. Acta* **1446,** 225–232.
27. Gupta, M. & Goldwasser, E. (1996) *Nucleic Acids Res.* **24,** 4768–4774.
28. Saitou, N. & Nei, M. (1987) *Mol. Biol. Evol.* **4,** 406–425.
29. Nevo, E., Filippucci, M. G. & Beiles, A. (1994) *Heredity* **72,** 465–487.
30. Nevo, E., Beiles, A. & Spradling, T. (1999) *J. Mol. Evol.* **49,** 215–226.
31. Nevo, E., Honeycutt, R. L., Yonekawa, H., Nelson, K. & Hanazawa, N. (1993) *Mol. Biol. Evol.* **10,** 590–604.
32. Reyes, A., Nevo, E. & Saccone, C. (2003) *Mol. Biol. Evol.* **20,** 622–632.
33. Ben-Shlomo, R., Fahima, T. & Nevo, E. (1996) *Isr. J. Zool.* **42,** 317–326.
34. Shoemaker, C. B. & Mitsock, L. D. (1986) *Mol. Cell. Biol.* **6,** 849–858.
35. Vilalta, A., Wu, D., Margalith, M. & Hobart, P. (2001) *Biochem. Biophys. Res. Commun.* **284,** 823–827.
36. Burlington, R. F. & Maher, J. T. (1968) *Nature* **219,** 1370–1371.
37. Kerem, D., Hammond, D. D. & Elsner, R. (1973) *Comp. Biochem. Physiol.* **45,** 731–736.
38. Arieli, R. (1989) in *Evolution of Subterranean Mammals at the Organismal and Molecular Levels*, eds. Nevo, E. & Reig, O.A. (Wiley-Liss, New York), pp. 251–268.
39. Hochachka, P. W., Buck, L. T., Doll, C. J. & Land, S. C. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 9493–9498.
40. Hochachka, P. W., Clark, C. M., Monge, C., Stanley, C., Brown, W. D., Stone, C. K., Nickles, R. J. & Holden, J. E. (1996) *J. Appl. Physiol.* **81,** 1355–1361.
41. Gregory, C. J. & Eaves, A. C. (1978) *Blood* **51,** 527–537.
42. Wu, H., Klingmuller, U., Besmer, P. & Lodish, H. F. (1995) *Nature* **377,** 242–246.
43. Heeschen, C., Aicher, A., Lehmann, R., Fichtlscherer, S., Vasa, M., Urbich, C., Mildner-Rihm, C., Martin, H., Zeiher, A. M. & Dimmeler, S. (2003) *Blood* **102,** 1340–1346.
44. Tan, C. C., Eckardt, K. U. & Ratcliffe, P. J. (1991) *Kidney Int.* **40,** 69–76.
45. Goldberg, M. A., Glass, G. A., Cunningham, J. M. & Bunn, H. F. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 7972–7976.
46. Goldberg, M. A., Dunning, S. P. & Bunn, H. F. (1998) *Science* **242,** 1412–1415.
47. Goldberg, M. A., Gaut, C. C. & Bunn, H. F. (1991) *Blood* **77,** 271–277.
48. Bondurant, M. C. & Koury, M. J. (1986) *Mol. Cell. Biol.* **6,** 2731–2733.
49. Eckardt, K. U., Dittmer, J., Neumann, R., Bauer, C. & Kurtz, A. (1990) *Am. J. Physiol.* **258,** 1432–1437.
50. Biljanovic-Paunovic, L., Clemons, G. K., Ivanovic, Z. & Pavlovic-Kentera, V. (1996) *Indian J. Med. Res.* **104,** 304–310.
51. Gassmann, M., Heinicke, K., Soliz, J., Ogunshola, O. O., Marti, H. H., Hofer, T., Grimm, C., Heinicke, I. & Egli, B. (2003) *Adv. Exp. Med. Biol.* **543,** 323–330.
52. Koury, S. T., Koury, M. J., Bondurant, M. C., Caro, J. & Graber, S. E. (1989) *Blood* **74,** 645–651.
53. Rosenberger, C., Mandriota, S., Jurgensen, J. S., Wiesener, M. S., Horstrup, J. H., Frei, U., Ratcliffe, P. J., Maxwell, P. H., Bachmann, S. & Eckardt, K.U. (2002) *J. Am. Soc. Nephrol.* **13,** 1721–1732.
54. Veng-Pedersen, P., Chapel, S., Schmidt, R. L., Al-Huniti, N. H., Cook, R. T. & Widness, J. A. (2002) *Pharm. Res.* **19,** 1630–1635.
55. Roguin, A., Avivi, A., Nitecki, S., Rubinstein, I., Levy, N. S., Abassi, Z. A., Resnick. M. B., Lache, O., Melamed-Frank, M., Joel, A. *et al.* (2003) *Proc. Natl. Acad. Sci. USA* **100,** 4644–4648.