Heterogeneity and Differential Expression under Hypoxia of Two-domain Hemoglobin Chains in the Water Flea, Daphnia magna*

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Hemoglobin (Hb) purified from the water flea, Daphnia magna, reared under hypoxia was analyzed by two-dimensional gel electrophoresis. The Hb was shown to be composed of six major subunit chain species (designated as DHbA to DHbF). The NH₂-terminal amino acid sequences of DHbA, DHbB, DHbC, and DHbF are different from one another, indicating that at least four Hb genes are present in D. magna. The NH₂-terminal amino acid sequences of DHbD and DHbE are the same as those of DHbA and DHbB, respectively. The six Hb chains were also found in the animal reared under normoxia in small amounts and with altered composition; the extent of decrease under normoxia was higher in the amounts of DHbC, DHbD, and DHbF than those of others. These results indicate that the Hb genes are differentially regulated by the ambient oxygen concentration. Four Hb genes constituting a cluster in the order, dhb4, dhb5, dhb1, and dhb2, were found on the chromosome of D. magna. The complete nucleotide sequences of the dhb1, dhb2, and dhb3 genes and their cDNAs showed that the genes have a seven-exon, six-intron structure. The structure consists of an intron separating an exon encoding a secretory signal sequence, two large repeated regions of a three-exon, two-intron structure that encode each a domain containing a heme-binding site, and an intron bridging the two repeated regions. The deduced amino acid sequences of the gene products showed higher than 79% identity to one another and showed unique features conserved in D. magna Hb chains. The analysis also suggested that DHbB (or DHbE), DHbF, and DHbC are encoded by the dhb1, dhb2, and dhb3 genes, respectively.

Hemoglobins (Hb) are widely distributed among eukaryotes and also in prokaryotes (1). Their amino acid sequences reveal that the globin fold has been conserved throughout evolution. Vertebrates have intracellular tetrameric Hb of very similar structures. Invertebrate Hb are more diverse in quaternary structure and oxygen binding properties. They are mostly large extracellular proteins classifiable by the number of chains and that of globin domains in each chain. They include single-domain chains organized as single or multiple subunits, two-domain chains arranged as multiple subunits, and multidomain chains, also organized as multiple subunits (2–4). Two-domain or multidomain Hb chains are only found in invertebrates, and their physiological roles and structures are interesting from the viewpoint of Hb evolution. cDNA clones encoding two-domain intracellular Hb chains from the clam Barbatia reeveana (5) and Barbatia lima (6) and two-domain extracellular Hb chain from nematode Pseudoterranova decipiens (7) have been isolated and their nucleotide sequences determined. The amino acid sequence of the two-domain extracellular Hb chain of nematode Ascaris suum has been determined by protein chemistry (8). The nematode Hb are very unusual: they show the affinity for oxygen 2 orders of magnitude higher than that of other Hb. Clam two-domain Hb are also unusual and have disadvantageous properties as oxygen carrier protein: their two oxygen-binding sites can bind oxygen but the resulting oxyhemoglobins undergo very rapid autoxidation and tend to precipitate (9). Physiological roles of these unusual Hb are not clear.

Extracellular Hb composed of multiple two-domain chains with relatively normal oxygen binding activity playing important roles in oxygen transport are found in hemolymph of Cladocera, such as water fleas Daphnia magna and Moina macrocopa (10). These animals show a drastic increase in Hb synthesis in response to a decrease in the ambient oxygen concentration, which results in a change in the body color from colorless to red (11), providing an excellent model system for studying the environmental control of gene expression. Although the physiological roles, oxygen binding properties, and quaternary structures of cladoceran Hb have been extensively investigated (11–13), information on their amino acid sequences has been scarce. Only the NH₂-terminal amino acid sequence of the second domain of the M. macrocopa Hb chain has been reported (9). No information about the cladoceran Hb gene was available until recently, when we cloned and analyzed a cDNA encoding a two-domain Hb chain of D. magna (14). D. magna Hb was reported to be composed of 16 polypeptide chains each carrying two heme-binding domains (12), whereas the purified Hb was separated into at least six multimeric species on nondenatured isoelectric focusing (15). These results suggest that D. magna contains several Hb subunit chains giving rise to several multimeric Hb species. No chemical analysis has been done on the subunit chains.

In this work, we analyzed the heterogeneity and properties of D. magna Hb chains by separating the chains with the aid of...
two-dimensional gel electrophoresis and by determining the nucleotide sequences of their genes and DNAs.

**EXPERIMENTAL PROCEDURES**

**Preparation of Hb-containing Crude Extracts and Purification of Hb from D. magna—**The D. magna strain isolated in Matsuyama, Japan (16) was used. Hb-rich (red) and Hb-poor (pale) D. magna were obtained by rearing animals under hypoxia (1.2 ml of O2/liter of water) and normoxia (4.8 ml of O2/liter of water), respectively, as described previously (17). Frozen animals were homogenized on ice in 50 ml Tris- HCl (pH 7.4) containing 1 mM CaCl2 and 1 mM PMSF. After filtration through MIRACLOTH (Calbiochem), the homogenate was centrifuged at 10,000 g for 30 min at 4 °C. The supernatant was bubbled with carbon monoxide gas to convert Hb into the carboxyform and then applied on a DE52 column (Whatman) equilibrated with 25 mM Tris-HCl (pH 7.4). The adsorbed Hb was washed with 25 mM Tris-HCl (pH 7.4) supplemented with 1 mM PMSF and bubbled with carbon monoxide gas and then eluted with 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl. The eluted fractions with a red color containing Hb were collected and combined (Hb-containing crude extract). For the purification of Hb, the crude extract prepared from 10.7 g wet weight of red animals was subjected to gel filtration with 50 mM Tris-HCl (pH 7.4) containing 1 mM CaCl2 (5 cm diameter × 80 cm length) of Sepharose 6B (Amersham Pharmacia Biotech). Fractions with A280/A293 values higher than 3 were collected. The collected fractions were dialyzed with an equal volume of water and then applied to a column (1.4 cm diameter × 21 cm length) of DE52. The adsorbed Hb was washed with 25 mM Tris-HCl (pH 7.4) and then eluted from the column with a linear gradient of 0–100 mM NaCl in 25 mM Tris-HCl (pH 7.4) containing 1 mM CaCl2. The fractions eluted at about 40 mM NaCl with A280 values higher than 4 were collected and combined. The combined fractions (30 ml) contained 0.087 mg/ml Hb with a purity higher than 95%, as judged from the profile on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie Brilliant Blue. It was concentrated with the aid of a spin column, UFC3TGC00 (Nihon Millipore, Japan) and used as a sample for electrophoresis.

**Chromosomal Walking Analysis—**The genomic DNA fragments, a to e shown in Fig. 5, were amplified by PCR using a TaKaRa LA PCR Kit Version 2 (Takara Shuzo, Tokyo, Japan). 250 ng of D. magna DNA was used as a template. The nucleotide sequences of the oligonucleotide primers used were 5′-GAGAACGTCGCCAAATGCT-3′ and 5′-CAAATTGTTTGTCTACGAC-3′ (for amplification of fragment a), 5′-GCCGCG-TTGGACGTTGCT-3′ and 5′-TTGGAGTCCAATTCG- TACC-3′ (for amplification of fragment b), 5′-GAGAACGTCGCCAAATGCT-3′ and 5′-CGGGGATTGCAGTGGCCGATC-3′ (for amplification of fragment c), and 5′-TTTGGACGTTGGTCAATTCGAT-3′ (for amplification of fragment d), and 5′-ATATTGAATGGCCGCAATTTT-3′ and 5′-CCATTGCGGAGTTGAAAT-3′ (for amplification of fragment e). The nucleotide sequences of the amplified fragments were directly determined with a Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems).

**RESULTS**

**Separation by Two-dimensional Gel Electrophoresis and Determination of the NH2-terminal Amino Acid Sequences of Hb Chains—**Hb was purified from D. magna reared under hypoxia (red). Total amount of Hb contained in red animals was determined to be 3 mg/g wet weight of animals by Western blotting analysis of the crude extract using the purified Hb as a standard. To determine whether D. magna contains heterogeneous Hb chains or not, the purified Hb was subjected to two-dimensional gel electrophoresis followed by staining with Coomassie Brilliant Blue. Six predominant spots were found on the gel (Fig. 1A, denoted as A–F) (see Methods). The six spots were designated as DHbA to DHbF, respectively, and further analyzed. Although minor spots were found in these truncated forms of a, e, and f, they have not been characterized. Composition of the six major Hb chains was determined by a densitometric analysis of Fig. 1A and then the content of each chain in red animals was calculated. The amounts of DHbA to DHbD were about two times higher than those of DHbE and DHbF (Table I). The NH2-terminal amino acids of these chains were determined by a proteolytic digestion of the purified Hb and the partial amino acid sequences were directly determined by a high-sensitivity gas phase sequencer as described by Matsudaira (21) using a protein sequencing system, Applied Biosystems model 477A-120A. For the detection of Cys residues, the pyridylethylthion method described by Cavins and Friedmann (22) was used. Insulin B chain (Wako Pure Chemical Industries, Osaka, Japan) containing Cys at the 7th position from its NH2 terminus was used in a control experiment.

**Isolation of cDNA Clones and Determination of Their Nucleotide Sequences—**The construction of a cDNA expression library and the isolation of cDNA clones carrying cDNA for Hb chains were described previously (14). Because the 5′ region of the DHb2 cDNA was sequenced, the sequence was extended by 5′ rapid amplification of cDNA ends using a Marathon cDNA Amplification Kit (CLONTECH). Total cDNA prepared from red D. magna and oligonucleotides, 5′-GGTCTTTTAAAGGCCTTTGAT-3′ and 5′-GAGAATGCGGTGGTCTCTC-3′, were used as a template and a specific primers, respectively. Various restriction fragments obtained from the cDNA clones were subcloned into the M13 mp10, mp11, and pUC119 vectors (23) and then their nucleotide sequences were determined by the dideoxy chain termination method using an ABI-377 DNA sequencer (PE Applied Biosystems). The sequencing was performed for the entire lengths of both strands, and all the ends of the restriction fragments used overlapped with one another.

**Preparation of Total DNA from D. magna—**Manipulation of DNA was carried out as described by Sambrook et al. (24) except where otherwise noted. 4.2 g wet weight of animals frozen in liquid nitrogen was ground in a prechilled mortar with a pestle and then suspended in 15 ml of extraction buffer (10 mM Tris-HCl (pH 9.4), 300 mM EDTA, 1% SDS). The suspension was supplemented with protease K (Wako Pure Chemical Industries) to 50 μg/ml and then incubated for 9 h at 50 °C (proteinase K was added at a final concentration of 100 μg/ml). After the incubation, the suspension was centrifuged at 3000 g for 10 min at 25 °C. The supernatant was extracted three times with an equal volume of phenol at room temperature (30 min, overnight, and 30 min, respectively), and then extracted once with a mixture of chloroform, phenol, and isooamyl alcohol (25:24:1). DNA was precipitated with ethanol and then dissolved in 5 ml of 10 mM Tris-HCl (pH 8.0) containing 10 mM EDTA and 1% SDS. The solution was supplemented with protease K to 100 μg/ml, incubated for 6 h at 50 °C, and then extracted with phenol and a mixture of chloroform, phenol, and isooamyl alcohol as described above. DNA was precipitated with ethanol, dissolved in 2.5 ml of 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, and then purified by equilibrium centrifugation in a CsCl gradient. The yield was 12 μg/ml DNA was performed using 8% separating and 4.5% stacking gels as described by Laemmli (18). Western blot analysis was performed as described by Burnette (19). For this analysis, rabbit antiserum produced against M. macrocopa Hb and goat anti-rabbit IgG conjugated with peroxidase were used for detection. Two-dimensional gel electrophoresis was performed by the method of O’Farrell (20) with minor modifications. 12.5 μl of the sample solution containing D. magna Hb was mixed with 15 μl of urea, 3 μl of Nonidet P-40 (Nacalai Tesque, Kyoto, Japan), and 1.5 μl each of 40% Biolyte (pH 3–10, Bio-Rad) and 2-mercaptoethanol and then loaded onto an 11-cm isoelectric focusing tube gel consisting of 8 x urea, 4% polyacrylamide, 2% Nonidet P-40, 0.4% Biolyte (pH 3–10), and 1.6% Biolyte (pH 5–8). The bottom buffer (anode) consisted of 10 mM phosphophosphate buffer (cathode) and 0.5 M sodium chloride. Proteins were fixed at 400 V for 12 h, and then at 800 V for 1 h, after which the gel was layered perpendicularly over an SDS-polyacrylamide slab gel consisting of 4.5% stacking and 8% separating gels.

**Quantitation of Hb and Hb Chains—**A crude extract prepared from red D. magna was subjected to SDS-PAGE and Western blotting as described above. Total Hb content was determined by densitometric analysis of the intensity of the detected bands, using DuoWave Densitometer, Shimadzu Corp., Japan). Hb purified from red D. magna was used as a control. The intensity was proportional to the amount of Hb within the range of amounts used. Hb chain composition was determined also by densitometric analysis of Hb chains after separation of them by two-dimensional electrophoresis of the purified Hb and staining with Coomassie Brilliant Blue. Content of each Hb chain in red animals was calculated from the total Hb content and the Hb chain composition thus determined. Content of each Hb chain in pale animals was calculated from the determined content in red animals and the ratio of intensity of each chain detected after Western blotting of the crude extract prepared from pale animals to that detected after Western blotting of the crude extract prepared from red animals.

**Determination of NH2-terminal Amino Acid Sequences—**Hb chains separated by two-dimensional gel electrophoresis were transferred to a PVDF membrane and then their NH2-terminal amino acid sequences were determined as described by Matsudaira (21) using a protein sequence analyzer, Applied Biosystems model 477A-120A. For the detection of Cys residues, the pyridylethylthion method described by Cavins and Friedmann (22) was used. Insulin B chain (Wako Pure Chemical Industries, Osaka, Japan) containing Cys at the 7th position from its NH2 terminus was used in a control experiment.
Heterogeneity of *D. magna* Hemoglobin Chains

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**FIG. 1.** *D. magna* Hb chains separated by two-dimensional gel electrophoresis and their NH$_2$-terminal amino acid sequences.

A. 32 μg of Hb purified from *D. magna* reared under hypoxia was subjected to two-dimensional gel electrophoresis and then stained with Coomassie Brilliant Blue. The six major Hb chains were designated as DHBA to DHBF and denoted as A–F, respectively. B, the determined NH$_2$-terminal amino acid sequences of the Hb chains, DHBA to DHBF.

**TABLE I**

| Amounts (μg)/g wet weight of animals reared under: | DHBA | DHBB | DHBC | DHBD | DHBE | DHBF |
|--------------------------------------------------|------|------|------|------|------|------|
| Hypoxia*                                          | 570  | 570  | 610  | 640  | 330  | 280  |
| Normoxia*                                         | 60   | 110  | 30   | 30   | 60   | 20   |

Rate of induction

9.5 5.2 20 21 5.5 14

*Calculated from total Hb content determined by Western blotting of the crude extract and Hb chain composition determined by densitometric analysis of Hb chains stained with Coomassie Brilliant Blue after separation by two-dimensional electrophoresis of the purified Hb (see “Experimental Procedures”).

Calculated from the relative intensity of each spot detected by Western blotting of Hb chains after separation by two-dimensional electrophoresis of the crude extract based on Hb chain contents in red animals determined as above (see “Experimental Procedures”).

Hypoxia values divided by normoxia values.

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Acid sequences of the six Hb chains were determined after blotted them onto a PVDF membrane (Fig. 1B). All of the determined sequences contained unusual repeats of Thr clusters with a Val in between. The sequences of DHBA and DHBB were the same as those of DHBD and DHBE, respectively. The sequences of DHBA (or DHBD), DHBB (or DHBE), DHBC, and DHBF were different from one another, indicating that at least four Hb chain species produced from different genes are present in *D. magna*. The first residues of DHBB, DHBE, and DHBF could not be determined by the phenylisothiocyanate method of Edman (25) employed here, suggesting that they are Cys. These NH$_2$-terminal Cys were confirmed by comparison of the amino acid sequences with the nucleotide sequences of cDNAs, as described below.

**Differential Expression of Hb Chains under Hypoxia and Normoxia**—Hb-containing crude extracts prepared from *D. magna* reared under hypoxia (red) and normoxia (pale) were subjected to two-dimensional gel electrophoresis and then the Hb chains separated on the gel were detected by Western blot analysis. The pattern of spots obtained with the extract prepared from red animals (Fig. 2A) was essentially the same as that shown in Fig. 1A. This indicates that the antiserum used in this analysis recognized all of the heterogeneous *D. magna* Hb chains. The efficiencies of recognition seemed to differ depending on Hb chain species. For example, the intensity of spot E seems to be nearly equal to those of spots A–D in Fig. 2A, while its intensity was about half of the spots A–D when the spots were stained with Coomassie Brilliant Blue (Fig. 1A). The six Hb chains were detected with altered composition when the extract prepared from pale animals was analyzed (Fig. 2B), clearly showing that the Hb chain composition was affected by the ambient oxygen concentration. It must be noted that the extract used in the analysis shown in Fig. 2B corresponds to seven times larger amount of animals compared with that used in the analysis shown in Fig. 2A. Although the efficiency of detection in Western blotting analysis differs depending on Hb chain species, the amounts in pale animals of each Hb chain could be roughly calculated from the ratio of intensity of each spot compared between Fig. 2A and B, on the basis of the amount of each chain in red animals determined by staining with Coomassie Brilliant Blue (see “Experimental Procedures” and Table I). As shown in Table I, all Hb chains were up-regulated by hypoxia. The amounts of the three Hb chains, DHBC, DHBD, and DHBF, were relatively smaller than those of other chains under normoxia and highly increase (14–21-fold) under hypoxia, while other chains show 5–10-fold increase under hypoxia. Total amount of Hb chains in pale animals was 1/10 of that in red animals (0.3 mg/g wet weight of animals).

**cDNA Analysis**—We previously isolated 11 clones that carry cDNAs of about 1.2 kb length encoding *D. magna* Hb chains, one of which was analyzed (λ-DHb1) (14). On analyzing the other 10 clones, we found two clones containing new species of Hb cDNAs. The entire nucleotide sequences of the cDNAs contained by the two clones, designated as λ-DHb2 and λ-DHb3, have been determined. The nucleotide sequences of the λ-DHb2 and λ-DHb3 cDNAs showed 79.4% and 86.6% identity, respectively, with that of λ-DHb1 cDNA. Fig. 3 compares the deduced amino acid sequences of the Hb chains, DHb1, DHb2, and DHb3, encoded by the three cDNAs. Both DHb2 and DHb3 had a signal peptide-like sequence at their NH$_2$ terminus and a two-domain structure similar to DHb1. The NH$_2$-terminal amino acid sequences of the predicted mature forms of DHb1, DHb2, and DHb3 exactly matched the above described NH$_2$-terminal sequences of DHbB (or DHbE), DHbF, and DHbC, respectively. The calculated molecular weights of the mature forms of DHb2 and DHb3 were 36,177 and 36,217, respectively, which were very close to that of DHb1 (36,228). Several key amino acids that are invariant in all or most Hb from other organisms and required for functional heme binding are conserved in each of the two domains. The identities of the amino acids that are invariant in all or most Hb from other organisms and required for functional heme binding are conserved in each of the two domains.
acid sequences between DH-b1 and DH-b2 and DH-b1 and DH-b3 are 83.1 and 92.4%, respectively. Fig. 4 shows the identities of the amino acid sequences among the Hb domains of invertebrates containing two-domain Hb chains. The reported identity between the amino acid sequences of the Hb domains of invertebrates belonging to different genera is generally low (less than 21% identity), while that between the amino acid sequences of two domains of the same Hb chain is much higher (60 to 80%; Fig. 4, underlined). A unique feature conserved in D. magna Hb chains is that the identity between the amino acid sequences of the first and second domains, either in the same chain or in different chains, is exceptionally low (21–24%; Fig. 4, shadowed), while the identity between the amino acid sequences of the corresponding domains in different chains (D1/D1 or D2/D2) is high (78–92%).

Genomic Sequences of Hb Chains—Chromosomal walking analysis was carried out by means of PCR, for which total DNA prepared from D. magna was used as a template and oligonucleotides synthesized according to the nucleotide sequences of the above described cDNAs were used as primers. Five genomic fragments, a to e, were amplified. The nucleotide sequences of the amplified fragments showed that there are four Hb genes (designated as dhb1 to dhb4) on the D. magna chromosome in the same direction and in the order of dhb4, dhb3, dhb1, and dhb2, with very short intergenic regions of a few kb (Fig. 5A).

FIG. 3. The amino acid sequences of D. magna Hb chains deduced from the nucleotide sequences of cDNAs. The amino acid position numbers are shown at the right. The gaps denoted by hyphens were introduced into the amino acid sequences to obtain maximum matching. Amino acid residues conserved in any two of the three sequences are shadowed. Those invariant in all or most Hb from other organisms are indicated by asterisks. The partial amino acid sequence of DH-b4 was predicted from the genomic sequence by assuming its homology with those of other Hb chains.

FIG. 4. Percentage identity between the amino acid sequences of various invertebrate Hb domains. The amino acid sequences corresponding to that from Ser256 to Lys175 of DH-b1 shown in Fig. 3 are compared. Abbreviations: D1, first domain; D2, second domain; B.l., clam B. lima two-domain chain (6); A.s., nematode A. suum two-domain chain (8).

FIG. 5. Organization of cluster and structures of D. magna Hb genes. A, organization of the D. magna Hb gene cluster. Open arrows indicate the positions and directions of the Hb genes. Bars at the top, a–e, denote the positions and lengths of the genomic DNA fragments amplified by PCR. At the bottom in parentheses are the lengths of the genes (transcribed regions) and intergenic regions shown. Vertical bars in the intergenic regions represent HIF-1 binding motifs (marked + or −, depending on the strand they are located on) and the putative TATA box (denoted by asterisks). B, the exon, intron structure common to D. magna Hb genes. The shadowed bars, E1–E7, and open bars, I1–I6, indicate exons and introns, respectively. The numbers at the top in parentheses show the lengths of the exons and introns in the dhb2 gene (base pairs).

FIG. 6. A phylogenetic tree of the amino acid sequences of various invertebrate Hb domains. The amino acid sequences of the Hb domains of clam B. reeveana (5) and nematode P. decipiens (7), and domain E1 of Artemia Hb (29), in addition to those compared in Fig. 3, were analyzed. The tree was constructed by using the PAUP (Phylogenetic Analysis Using Parsimony) program developed by Swofford (30). The amino acid sequence of Paramecium Hb (31) was used as an outer group sequence. The numbers on the bars indicate the levels of confidence (%).
the 3’ region of the dhb4 gene was fortuitously amplified by using a 5’ primer whose sequence was conserved in the three cDNAs. Amplification of DNA fragments containing the 5’ region of the dhb4 gene was unsuccessful. The sequences of the dhb1, dhb2, and dhb3 genes, compared with their cDNAs, showed that they have a seven-exon, six-intron structure (Fig. 5B). A similar structure was reported for the gene of a two-domain Hb chain in the clam, B. reeveana (5). An intron bridging the two large repeated regions is conserved in both organisms. The organizations of the two large repeated regions encoding the two heme-binding domains are similar in both organisms, having the three-exon, two-intron structure characteristic of animal Hb genes (26). However, in contrast to the first intron located in the 5’-untranslated region of the B. reeveana gene, the first intron separates an exon encoding a secretory signal sequence in D. magna Hb genes. The lengths of introns were much shorter in D. magna genes (less than 100 bp on average), which, together with the short lengths of the intergenic regions, makes the gene cluster very compact. The positions of introns were perfectly conserved in the three Hb genes. Several sequences homologous to the octamer binding motif (TAGTGGCT, underlined nucleotides were forced to be conserved) for mammalian hypoxia inducible factor-1, HIF-1 (27), were found in each of the intergenic regions (Fig. 5A).

**DISCUSSION**

Two-dimensional gel electrophoresis of Hb purified from D. magna clearly demonstrated that the animal contains six chemically different Hb chains, while analyses of the NH₂-terminal amino acid sequences of Hb chains and the genomic DNA sequences only confirmed the presence of four Hb genes. Analysis is in progress to determine whether each Hb chain constitutes a homomultimeric Hb or Hb chains are mixed and constitute heteromultimeric Hb. It is not clear at present whether DHbA and DHbB are the products of the same genes as those for DHbD and DHbE, respectively, showing different migration on two-dimensional gel electrophoresis due to post-translational modification, or they are the products of different genes having the same NH₂-terminal amino acid sequences. D. magna may contain more than four Hb genes, or it may contain only four Hb genes giving rise to six Hb chain species through some post-translational modification. Assuming that the latter is the case, one of the possible sites of modification is the NH₂-terminal Cys of DHbB, DHbE, and DHbF. Detection of these NH₂-terminal Cys after pyridylethylation of their sulfhydryl groups (22) was unsuccessful, while Cys in the insulin B chain was clearly detected in a control experiment. The sulfhydryl groups of these Cys might have undergone some modification that blocks pyridylethylation.

In our previous work (14), red D. magna was shown to contain a more than 12 times higher amount of Hb mRNA as compared with pale animals. The differential up-regulation by hypoxia of Hb chains demonstrated here is possibly a result of the transcriptional control of Hb genes. Post-translational modification of Hb chains, if any, may also be affected by hypoxia. Oxygen-responsive genes are found in a broad range of organisms, from bacteria to humans, and investigation of the molecular mechanisms underlying their regulation is one of the important and rapid-growing fields in current molecular physiology (27). The analysis of the cis-elements and trans-acting factors involved in the transcriptional regulation of Hb genes in D. magna is of particular interest. HIF-1 is a trans-acting factor playing a critical role in the hypoxic induction of several physiologically important genes in mammalian cells (27). Functional analysis of HIF-1 binding motifs found in the intergenic regions of the D. magna Hb gene cluster is in progress.

The amino acid sequences deduced from the nucleotide sequences of the cloned cDNAs show unique features of D. magna Hb chains. The exceptionally low identity between the amino acid sequences of the first and second domains suggests that the duplication of a single-domain Hb gene in D. magna occurred a very long time ago and then the resulting two single-domain Hb genes fused to form a two-domain Hb gene. The multiplication of the two-domain Hb gene seems to have occurred relatively recently, because the identity of the amino acid sequences as a whole between different chains is high. Supporting this notion, phylogenetic analysis showed that the first domains and second domains of D. magna Hb chains constitute different clusters (Fig. 6). The three-exon, two-intron structure encoding the two domains is conserved in all D. magna Hb genes, further indicating the dominance of this structure among animal globin genes (26). Another unique feature conserved in D. magna Hb chains is the presence of an unusual NH₂-terminal extension. This extension is reminiscent of the COOH-terminal extension found in the two-domain Hb chain of a parasitic nematode, A. suum, containing a repeat of the sequence, Glu-Glu-His-Lys (8, 28). This extension, named the polar zipper sequence, was proposed to be joined in an eight-stranded β barrel at the center of the molecule. The repeated Thr clusters and a Val residue in between them in the NH₂-terminal extension of D. magna Hb chains may also play a role in the multimerization of 16 subunit chains to yield a functional Hb molecule through hydrophilic and hydrophobic interactions of the side chains of Thr and Val, respectively.

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