

Paenungulata: A Comparison of the Hemoglobin Sequences from Elephant, Hyrax, and Manatee¹

Traute Kleinschmidt,* John Czelusniak,† Morris Goodman†‡
and Gerhard Braunitzer*

*Abteilung Proteinchemie, Max-Planck-Institut für Biochemie; †Department of Biology, Wayne State University; and ‡Department of Anatomy, Wayne State University School of Medicine

Inspection of the amino acid differences among hemoglobin sequences of a wide range of mammalian species suggested that at $\alpha 19$, $\alpha 110$, $\alpha 111$, $\beta 23$, $\beta 44$, and $\beta 56$, synapomorphies group manatee (*Trichechus inunguis*, Sirenia), Indian and African elephant (*Elephas maximus* and *Loxodonta africana*, Proboscidea), and rock hyrax (*Procavia habessinica*, Hyracoidea) into a monophyletic clade. Results obtained by parsimony analysis provide evidence for this grouping—and thus support for the genealogical validity of Simpson's superorder Paenungulata, which contains as the extant orders Proboscidea, Sirenia, and Hyracoidea. All of the 39 most, or nearly most, parsimonious of 10,395 trees constructed from a tandemly combined α - and β -hemoglobin sequence for 103 vertebrate species (of which 79 were mammals from 16 extant orders), depicted Paenungulata as one of the most anciently separated branches of Eutheria. It was found on examining thousands of alternative trees that to not group Proboscidea, Hyracoidea, and Sirenia in a monophyletic clade required at least four additional substitutions.

Introduction

In constructing higher taxa of the mammalian infraclass Eutheria, Simpson (1945) combined the extant orders Proboscidea, Sirenia, and Hyracoidea with several extinct fossil orders into the superorder Paenungulata but referred to the possibility that Hyracoidea might be closer to the ungulate order Perissodactyla. A more recent interpretation of morphological data supported such a hyracoid-perissodactyl group (McKenna 1975). Different phylogenetic schemes, based on gross anatomical characters, typically depict a single common origin for paenungulate and ungulate orders. Molecular results from tandemly combined amino acid sequences of α - and β -hemoglobin, myoglobin, lens α -crystallin A, and fibrinopeptides A and B support, with regard to extant eutherians, the validity of Simpson's superorder Paenungulata but do not suggest closer kinship with ungulates than with other eutherians (Goodman et al. 1985; Miyamoto and Goodman 1985). In earlier immunological work on serum proteins, Weitz (1953) observed significant cross-reactivity between hyrax and elephant, and Shoshani (1986; also Shoshani et al. 1981) as well as Sarich (1985; also Rainey et al. 1984) found smaller antigenic distances separating Proboscidea, Sirenia, Hyracoidea, and Tubulidentata (aardvark) from one another than from other orders of mammals. From maximum-parsimony trees constructed for lens α -crystallin A amino

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Address for correspondence and reprints: Dr. Traute Kleinschmidt, Max-Planck-Institut für Biochemie, Abteilung Proteinchemie, 8033 Martinsried bei München, West Germany.

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acid sequences, de Jong and Goodman (1982) provided cladistic evidence for the superorder Paenungulata expanded to include Tubulidentata as well as Proboscidea, Sirenia, and Hyracoidea; these maximum-parsimony results also displayed Edentata and Paenungulata as the earliest offshoots from the eutherian stem. Such ancient separation of Paenungulata is also depicted by the maximum-parsimony trees constructed for the tandemly combined amino acid sequences (Goodman et al. 1985; Miyamoto and Goodman 1985), the paenungulate branch most fully represented by amino acid sequence data being Proboscidea with all three globin chains, lens α -crystallin, and fibrinopeptides A and B tandemly combined for the African elephant.

Our current study focuses on the large body of α - and β -hemoglobin sequence data now available on eutherians and other vertebrates and uses these data to test the cladistic validity of Paenungulata. Included in these hemoglobin data are the two living species of Proboscidea, the Indian elephant *Elephas maximus* (Braunitzer et al. 1982) and the African elephant *Loxodonta africana* (Braunitzer et al. 1984), the rock hyrax *Procavia habessinica* as a representative of Hyracoidea (Kleinschmidt and Braunitzer 1983a), and the Brazilian manatee *Trichechus inunguis* representing Sirenia. The experimental details of determination of the manatee sequence will be published elsewhere.

Results and Discussion

The hemoglobins of manatee, rock hyrax, and Indian and African elephants each consist of only one component. Manatee and elephant hemoglobin do not show any remarkable properties. However, hyrax hemoglobin shows two characteristics not present in other mammalian hemoglobins: (1) 20% of the N-terminal valine residues in the β -chains are acetylated, and (2) the hyrax α -chains have an insertion of the amino acid glutamine in the GH-interhelical region between the glutamate positions 115 and 116. Gap events, i.e., deletions and insertions, have not been found so far in normal mammalian hemoglobins, with the exception of the terminal regions. They are also rare in abnormal human hemoglobins. Apart from a few deletions, only one case of an insertion is known: in Hb Grady Glu-Phe-Thr in positions 116–118 (GH4, GH5, and H1) is repeated as nine nucleotides encoding the three additional amino acid residues (Huisman et al. 1974). This event follows the common rule that insertions are usually tandem duplications of one or more codons. This rule is not valid for the insertion of glutamine in the hyrax α -chains, unless one assumes that there was an earlier duplication of a glutamic acid codon followed by a single nucleotide substitution that changed the coding to glutamine.

Indicative of the close relationship between *Elephas* and *Loxodonta*, the hemoglobins of these two elephants show only four amino acid differences, three in the α -chains— $\alpha 5(A3)Lys \rightarrow Asn$, $\alpha 49(CD7)Ser \rightarrow Gly$, and $\alpha 57(E6)Gly \rightarrow Ala$ —and one in the β -chains— $\beta 52(D3)Asp \rightarrow Glu$. Otherwise, judged by the numbers of amino acid differences in pairwise comparisons, each paenungulate hemoglobin diverges as much from each of the other paenungulate hemoglobins as it does from human hemoglobin. Closer inspection of the amino acid differences among hemoglobins of a wide range of mammals (α -chains in fig. 1 and β -chains in fig. 2) does indicate that there are synapomorphies grouping Proboscidea, Sirenia, and Hyracoidea into a monophyletic clade. In particular, figures 1 and 2 show amino acid replacements at positions $\alpha 19$, $\alpha 110$, $\alpha 111$, $\beta 23$, $\beta 44$, and $\beta 56$ that are likely candidates for synapomorphies grouping Sirenia, Hyracoidea, and Proboscidea into a monophyletic Paenungulata.

Position	1	1	1	1	1	2	2	2	2	2	3	4	5	5	5	6	6	6	6	7	7	7	7	7	7	8	9	1	1	1	1	1	2	3	3					
Human	P	A	T	A	A	G	V	A	A	G	E	G	A	L	S	A	V	G	K	A	D	N	A	V	D	M	N	A	A	K	A	A	L	P	A	E	A	A	S	
PAENUNGULATA																																								
Elephant	D	N			T	S		D		S	D	V		F	G	G		A		G	E	Q	G	L		L	S		S	S	Q		T			E	S	N		
Manatee	D	E			T	F		I	T	T			G		G	I	A				R	G	L	E	L	G	T	E	R	S	S		R	E		D	S	S		
Hyrax	A		N	G		E		T						T			A	Q	G	A	K	G	L		L		D		S	R		E	Q			S	N			
PERISSODACTYLA																																								
Horse	A				S	G										A	G		L	G	L		L	G	N		V		N		D	S								
Wild ass	A				S	G		D								A	G		L	G	L		L	G	N		V		N		S	T								
Zebra	A				S	G										A	G		L	G	L		L	G	N		V		N		S	T								
Rhinoceros		T		T												A	G		Q	G	L		L	G			L	H	Q		S	N								
Tapir		T			S	S										A	G		Q	G	L		L	G			L	H	D		D	S	N							
ARTIODACTYLA																																								
Pig	A		A			G		A					D	A	Q		K	G	L		L	G				H	D		D	S	A	N								
Hippopotamus	A		N	S			N	P				S	A		A		K	G	L		L	G		D		H	S		D		N									
Llama	S	K	A	T			I	G	A	D				A	G		K	D	L		L	S				H	G		D		N									
Elk	A		T	S			G	P	A					A	E		N	K	G	L		L	G	T	D			S		D		N								
Bovine	A		G			G		A							A		A	K	E	L		L	G		E		S		S		D		N							
CETACEA																																								
Dolphin					G	T	S	I	N	S	A		I	G		I			K	G	I		L	D	E		L				D	S								
Whale		T	S		T	A	I	N	G	A			M	G					K	G	M		L	D	D		L				S									
OTHER MAMMALS																																								
Echidna	D		K	S	L		A	G		E					A			T	G	F	N		S			R	H			S	S	R								
Kangaroo	A		G		I		G				A	H		I	A			Q	E	I		L	G	T	K		C	H	G	D	A	E	A							
Mole	G		T	S			D					T		A				G	L		L	G				C	H	N	D		T									
Bat	S					D		G									G		G	L		L	G				N		S	D										
Armadillo	A				F		G		A				A		A			L	G	L		L	G	T		C		D	D		G									
Raccoon				A		T	D	I	G			G	A		A			L	G	L		L	G			C	H				T									
Rabbit				T		E	I	S	G			G	A		T	E	I	A		S	E	K	G	L	L	G	T	N	H	S										
Mouse	G		E	S			I	G	G	A			A		I	G			S	G	L		L	G			S	H		D										
Tree shrew		G	S			I	G		P	Q				I	A			T	G	L		L	T			C	H	G		D	E	N								

Loxodonta africana (1)
Trichechus inermis
Procavia habessinica (2)
Equus przewalskii caballus (3)
Equus hemionus kulan (4)
Equus zebra (4)
Ceratotherium simum (5)
Tapirus terrestris (6)
Sus scrofa domesticus (3)
Hippopotamus amphibius (7)
Lama guanicoe glama (8)
Alces alces alces (9)
Bos primigenius taurus (3)
Tursiops truncatus (10)
Balenoptera acutorostrata (11)
Tachyglossus aculeatus (3)
Macropus giganteus (3)
Talpa europaea (12)
Roussettus aegyptiacus (13)
Dasyurus novemcinctus (14)
Procyon lotor (15)
Oryctolagus cuniculus (3)
Mus musculus (3)
Tupaia glis (3)

FIG. 1.—Replacements among α -chains of Paenungulata and other mammalian hemoglobins. Only those positions are shown at which replacements in the Paenungulata compared to human α -chains have been found. Positions with asterisks have amino acid replacements that are likely candidates for synapomorphies of Paenungulata. Sources: (1) Braunitzer et al. 1984; (2) Kleinschmidt and Braunitzer 1983a; (3) Dayhoff 1972b; (4) Mazur and Braunitzer 1982; (5) Mazur et al. 1982; (6) Mazur and Braunitzer 1984; (7) Braunitzer et al. 1983; (8) Braunitzer et al. 1978; (9) Aschauer et al. 1984; (10) Kleinschmidt and Braunitzer 1983b; (11) Abbasi et al. 1984; (12) Kleinschmidt et al. 1981; (13) Kleinschmidt and Braunitzer 1982; (14) Kleinschmidt et al. 1982; (15) Brimhall et al. 1978.

An analysis of hemoglobin sequences (α - and β -chains tandemly combined) from 103 vertebrate species by the maximum-parsimony method supports the monophyly of Paenungulata but tends to reject a close connection to either a broad ungulate cohort or to individual ungulate orders such as Perissodactyla. Previously, we reported (Shoshani et al. 1985) results on the hemoglobin sequences of 83 vertebrate species of which the mammals (64 species) did not include any representatives of Sirenia. In our present analysis, 15 of the additional 20 species are eutherians and, of course, now include a sirenian. Using our branch-swapping algorithm (Goodman et al. 1979) and initiating dendrograms based on branching arrangements suggested in previous searches, we examined 5,800 alternative trees and found that each minimum-length tree has 2,690 nucleotide substitutions estimated via amino acid replacements, or NRs. There are 26 trees with this score, and all 26 depict a monophyletic Paenungulata grouping Hyracoidea, Proboscidea, and Sirenia. Most of these 26 alternatives involve changes within orders—such as, in Primates, joining Lorisidae to Haplorhini rather than to *Lemur*.

To explore variations in branching arrangement that test the genealogical validity of grouping Proboscidea, Sirenia, and Hyracoidea into a monophyletic clade, i.e., into superorder Paenungulata, we took the alternative among the 2,690-NR-length trees that was most consistent with other phylogenetic evidence (e.g., Lorisidae joined to *Lemur*) and subdivided this tree for the 103 species into eight subtrees or branches. The eight subtrees consisted of (1) all noneutherians serving as the outgroup to root Eutheria, (2) Edentata, (3) Perissodactyla, (4) artiodactyls plus Cetacea, (5) Hyracoidea, (6) Sirenia, (7) Proboscidea, and (8) a clade of the seven remaining eutherian orders, Rodentia, Carnivora, Lipotyphyla, Scandentia, Chiroptera, Lagomorpha, and Primates. With one exception, the branching arrangement followed within the eutherian subtrees always depicted monophyletic orders, i.e., no nonprimates occurred among Primates, no noncarnivores occurred among Carnivora, etc. The one exception was Artiodactyla. In the subtree containing artiodactyls, Pecora (bovids and deer) first joins Cetacea (dolphin and minke whale); then, as successively earlier offshoots, the subtree grows by addition of hippopotamus, camelids, and, finally, pig. The search for the 2,690-NR-length trees and earlier work indicated that to have a monophyletic Artiodactyla by placing Cetacea outside the artiodactyl-containing subtree increases NR length by at least 4 NRs.

We examined each of the 10,395 unrooted trees for eight branches, recording the branching arrangement and NR length of each tree. Then, using an approach developed by one of us (J.C.), we judged the strength of the groupings found in the most parsimonious trees by determining in a stepwise manner the minimum number of NRs that had to be added to the lowest NR length to break up the monophyletic groups depicted at this lowest length, until at the last step only a bush remained. We found a lower minimum NR length than in our previous search. There are two trees at 2,689 NR length, and they yield the consensus shown in figure 3. Inasmuch as the NR values within the eight invariant subtrees summed to 2,430 NR on constructing prelimsets by the method described in appendix A-1 of Goodman et al. (1979), the relevant NR score for the most parsimonious length is 259. Each of the two trees at this most parsimonious score depicts a monophyletic Paenungulata in which Sirenia (manatee) first joins Proboscidea (Indian and African elephants) and then Hyracoidea (rock hyrax) joins the clade. Edentata (armadillo) joins Perissodactyla (tapir, rhino, and equines), and these two joined subtrees then join the artiodactyl-Cetacea subtree. As the two alternative trees either join this Cetacean-ungulate-edentate clade to the

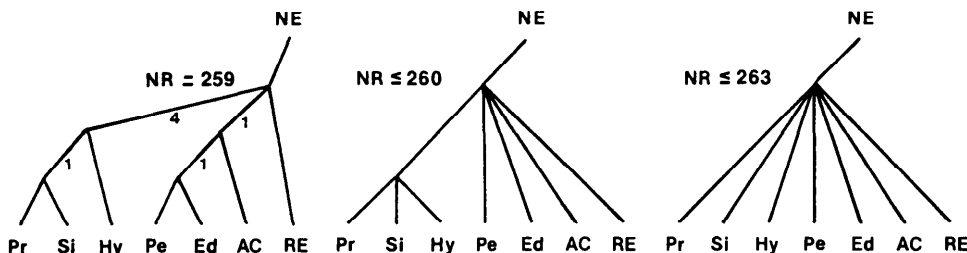


FIG. 3.—Testing hemoglobin amino acid sequence data to determine the genealogical validity of grouping Proboscidea, Sirenia, and Hyracoidea into the superorder Paenungulata. The 103 species represented by tandemly combined α - and β -hemoglobin amino acid sequences were divided into eight subtrees, and all possible alternative trees (10,395) were examined by the maximum-parsimony method. The eight subtrees are designated as follows: Pr, Proboscidea; Si, Sirenia; Hy, Hyracoidea; Pe, Perissodactyla; Ed, Edentata; AC, Artiodactyl-Cetacea; RE, remaining eutherian orders; NE, noncatherians. (See text for further explanation of these eight subtrees). Although the total lengths of these 10,395 trees range from 2,689 NR to 2,720 NR, the NR values of the invariant subtrees (Pr = 4; Si = 0; Hy = 0; Pe = 53; Ed = 0; AC = 218; RE = 797; NE = 1,358) sum to 2,430 NR. Thus the relevant scores for these 10,395 trees range from 259 to 290 NR. *Left*, strict consensus tree for the two lowest length trees (at score 259 NR). The numbers below the links do not represent NRs between ancestral and descendant sequences but are a summary of NR score information obtained from the consensus trees in the middle and on the right. *Center*, strict consensus tree for the eight trees at score 260 NR; this consensus also describes the nine trees at score 261 NR and the 20 trees at score 262 NR. *Right*, strict consensus tree for the 34 trees at score 263 NR. The numbers below the links in the tree on the left represent the number of additional NRs that would be required to break up the taxonomic group below. For 1 additional NR, there are trees that break up the specific grouping of Sirenia and Proboscidea, of Edentata and Perissodactyla, and of the latter branch and the artiodactyl-Cetacea branch. Not until 4 additional NR are there trees that break up Paenungulata. Thus the strongest grouping in these data places Proboscidea, Sirenia, and Hyracoidea in a monophyletic clade. For the tree regions that were free to vary, the NR scores for the 10,395 trees have the following distributional properties: range, 259–290; mean, 277; median, 278; and mode, 279.

Paenungulate clade or to the clade of the seven remaining eutherian orders, the consensus tree depicts a trichotomy for the three major eutherian clades. Among the eight trees at 260 NR length, four separate Edentata form the stem to the remaining eutherians and one of the four also joins the artiodactyl-cetacean subtree to the large subtree containing the remaining eutherian orders rather than to the Perissodactyla clade. Six of the eight trees at 260 NR length join Hyracoidea rather than Sirenia to Proboscidea, and one of the eight trees joins Hyracoidea first to Sirenia. The first 39 trees at the lower range of NR-length distribution all depict a monophyletic Paenungulata. Not until an NR score of 263 is reached do we find some trees (4 of the 35 at this length) that break up this Proboscidea-Sirenia-Hyracoidea clade. Thus the strongest grouping of subtrees among the eight free to vary is that (supported by at least 4 NRs) producing an anciently separated monophyletic Paenungulata. Moreover, these results plus those obtained in the earlier survey by our branch-swapping algorithm do not support McKenna's (1975) grouping of Hyracoidea with Perissodactyla.

Another overlapping series of 10,395 trees for eight subtrees corroborates the strength of the grouping of Proboscidea, Sirenia, and Hyracoidea into Paenungulata and tests the hypothesis (Storr 1780) that hyrax is a rodent. The eight subtrees for this series varied from those shown in figure 3 by treating Rodentia as a separate subtree—thus leaving six orders (Carnivora, Lipotyphyla, Scandentia, Chiroptera, Lagomorpha,

and Primates) rather than seven in the largest eutherian subtree—and by grouping Perissodactyla with the artiodactyl-cetacean branch into a single subtree. The most parsimonious trees in this series are at length 2,690 NR, and not until length 2,694 NR is reached do some trees fail to depict a monophyletic origin for the three paenungulate orders.

In conclusion, the parsimony evidence from hemoglobin sequences agrees with that from lens α -crystallin A sequences (de Jong and Goodman 1982) in depicting an anciently separated monophyletic Paenungulata clade grouping Proboscidea, Sirenia, and Hyracoidea. In this connection, aardvark (*Orycteropus afer*, Tubulidentata) needs to be included in the analysis of hemoglobin sequences in order to clarify the conflicting results obtained from the eye lens α -crystallins and myoglobins, the former grouping the aardvark with the Paenungulata (de Jong et al. 1981) and the latter grouping it with Chiroptera and Erinacidae (Dene et al. 1983).

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