Tracking the evolutionary loss of hemoglobin expression by the white-blooded Antarctic icefishes. Gene

ARTICLE *in* GENE · SEPTEMBER 2002

Impact Factor: 2.14 · DOI: 10.1016/S0378-1119(02)00691-1 · Source: PubMed

4 AUTHORS, INCLUDING:

Guido di Prisco
Italian National Research Council
233 PUBLICATIONS **3,676** CITATIONS
SEE PROFILE

Ennio Cocca
Italian National Research Council
32 PUBLICATIONS **415** CITATIONS
SEE PROFILE

Sandra Parker
Northeastern University
24 PUBLICATIONS **657** CITATIONS
SEE PROFILE

ELSEVIER

Gene 295 (2002) 185–191

GENE
AN INTERNATIONAL JOURNAL ON GENES AND GENOMES

www.elsevier.com/locate/gene

Review

Tracking the evolutionary loss of hemoglobin expression by the white-blooded Antarctic icefishes

Guido di Prisco[a,*], Ennio Cocca[a], Sandra K. Parker[b], H. William Detrich III[b]

[a]*Institute of Protein Biochemistry and Enzymology, Consiglio Nazionale delle Ricerche,Via Marconi 12, I-80125 Naples, Italy*
[b]*Department of Biology, Northeastern University, Boston, MA 02115, USA*

Received 22 April 2001; accepted 7 May 2002

Abstract

The blood of Antarctic icefishes (family Channichthyidae, suborder Notothenioidei) is completely devoid of hemoglobin. Icefishes have developed compensatory adaptations that reduce oxygen demand and enhance oxygen transport. Oxygen delivery to tissues occurs by carrying the gas physically dissolved in the plasma. To evaluate the evolutionary pathway leading to the icefish hemoglobinless phenotype, the adult and embryonic/juvenile gene complexes from a closely related, red-blooded notothenioid species were isolated and characterized. The hybridization pattern of notothenioid adult globin cDNAs showed that the genomes of three icefish species retain transcriptionally inactive α1-globin-related DNA sequences, which are identical truncated variants of the α1-globin gene of the red-blooded fish, containing part of intron 2, all of exon 3, and the 3′-untranslated region. The icefish genomes have no β-globin genes. Furthermore, Southern blots of genomic DNA from red- and white-blooded (two species) notothenioids, probed with fragments of the genes flanking the ends of the embryonic/juvenile complex, indicated that icefishes have also lost embryonic/juvenile globin genes. It is proposed that inability to express hemoglobin arose from a single, large-scale deletional event, which removed all icefish globin genes with the exception of the 3′ end of *α1*.

Keywords: Globin gene organization; Gene deletion; Mutational clock; Cold adaptation

1. Introduction

The oxygen transporter hemoglobin has generally been regarded as a *sine qua non* for adult vertebrate life. What a surprise it must have been to zoologists to read Ruud's seminal *Nature* article on icefishes (family Channichthyidae, suborder Notothenioidei), 'Vertebrates without erythrocytes and blood pigment' (Ruud, 1954). Indeed, Ruud himself harbored doubts about the existence of the *blodlaus-fisk* (bloodless fish) that Norwegian whalers reported to inhabit the shelf waters of South Georgia. He wrote:

> I first heard about these 'bloodless fish' on a visit to South Georgia in 1929; but no specimens were forthcoming, and I did not take them seriously. I was reminded about their existence, however, when Mr. D. Rustad, biologist in the *Norvegia* Expedition (1927–28), presented me with some photographs of a 'white crocodile fish' caught by him at Bouvet Island, mentioning the fact that its blood was colourless.

When in 1953 Ruud captured four specimens of the 'white crocodile fish' *Chaenocephalus aceratus* at South Georgia, he measured some of the hematological parameters of the species. The nearly transparent blood contained leukocytes at $< 1\%$ of total blood volume but few, if any, hemoglobinless erythrocytes. The oxygen capacity of the icefish blood was 10–12% that of the red-blooded South Georgian nototheniids *Notothenia rossii* and *N. coriiceps*, and the iron content of the blood was very low (<5% that of typical red-blooded fishes). Thus, Ruud concluded that "the blood of *Chaenocephalus aceratus* is to be considered a plasma with a moderate content of leucocytes". Indeed, most of the 15 known icefish species exhibit a similar blood profile, although small numbers of hemoglobinless erythrocyte-like cells may be present (Hureau et al., 1977).

How can these large fishes survive without an oxygen transporter in their blood? Ruud speculated that the metabolism of these fishes was "rather low", and he argued that the ecological context of these fish was critical:

Abbreviations: bp, base pair(s); Hb, hemoglobin; kb, kilobase(s); Ma, million years ago; NCP, *Notothenia coriiceps* putative

* Corresponding author. Tel.: +39-081-7257-242/234; fax: +39-081-593-6689.
E-mail address: diprisco@dafne.ibpe.na.cnr.it (G. di Prisco).

0378-1119/02/$ - see front matter © 2002 Published by Elsevier Science B.V.
PII: S0378-1119(02)00691-1

> Since these fish presumably descend from ancestors with hemoglobin in their blood, one imagines that only in the cold water of the polar regions could a fish survive which had lost its blood pigment. The *Chaenocephalus* specimens came from water rather less than 2 °C. In the winter farther south the water temperature descends to around −1.7 °C, and is always well aerated.

Implicit in Ruud's hypothesis is the concept that a phenotype, the absence of hemoglobin, that is deleterious for fishes living at high temperature may be selectively neutral (or perhaps deleterious but non-lethal) at low temperature. Consistent with this possibility are observations that the red-blooded Antarctic notothenid *Trematomus bernacchii* can survive under resting conditions when its hemoglobin is converted to the carbon monoxy form or when its hematocrit is progressively reduced to $< 1\%$ by withdrawal of blood aliquots (di Prisco et al., 1992). Others have suggested that the near elimination of erythrocytes may be advantageous because it should reduce the energetic cost of circulating a viscous, corpuscular blood fluid (di Prisco et al., 1991; Eastman, 1993; Macdonald et al., 1987). However, the development in icefishes of compensatory physiological and circulatory adaptations that reduce tissue oxygen demand and enhance oxygen delivery (e.g. modest suppression of metabolic rates, enhanced gas exchange by large, well-perfused gills and through a scaleless skin, and large increases in cardiac output and blood volume) argues that loss of hemoglobin and erythrocytes was probably maladaptive under conditions of physiological stress.

Channichthyids diverged from other Antarctic notothenioids ~7–15 Ma, but radiation of species within the icefish clade appears to have been confined to the last 1 million years (Bargelloni et al., 1994). Their inability to express hemoglobin probably arose in the ancestral channichthyid by mutation of globin genes to transcriptional inactivity or by large-scale deletion of globin genes. To evaluate the evolutionary pathway leading to the hemoglobinless phenotype of icefishes, we first investigated the organization and expression of adult and embryonic/juvenile globin genes in closely related red-blooded notothenioids.

2. Adult and embryonic/juvenile globin gene complexes of a red-blooded notothenioid fish

To establish a comparative framework for analysis of icefish globin genes, we have isolated and characterized the adult and embryonic/juvenile gene complexes from the closely related, but hemoglobin-expressing, notothenid *Notothenia coriiceps* (family Nototheniidae) (Cocca et al., 2000; Lau et al., 2001). Fig. 1A shows that the adult α1/β-globin complex is composed of the α1-globin gene linked in head-to-head (5′ to 5′) orientation with the gene for β-globin, with 4.3 kb of intergenic DNA separating the start codons of the two genes. (α1 denotes the α chain of the major adult hemoglobin Hb1; the β chain is shared by Hb1 and Hb2, the minor adult component.) The α1- and the β-globin genes are each composed of three exons separated by two introns, and the positions of their introns conform to the vertebrate norms for globin genes (Efstratiadis et al., 1980; Lawn et al., 1980; Liebhaber et al., 1980). That this complex constitutes the major functional globin locus of adult *N. coriiceps* is demonstrated by: (1) the exact match of the coding sequences and the 5′- and 3′-untranslated regions of the α1- and β-globin genes of *N. coriiceps* to the corresponding regions of the adult globin cDNAs (Cocca et al., 1995; Zhao et al., 1998) of this species; and (2) by the identity of the amino acid sequences of the encoded α1 and β globins to those obtained by automated Edman degradation of the polypeptide chains of Hb1 (D'Avino and di Prisco, 1989). Furthermore, the *N. coriiceps* globin genes lack the structural features characteristic of typical globin pseudogenes (Lacy and Maniatis, 1980; Proudfoot and Maniatis, 1980) or processed pseudogenes (Vanin, 1985).

The *non-adult* globin genes of *N. coriiceps* are organized as a compact complex of two consecutive sets of linked α- and β-globin genes, α_{NCPI} and β_{NCPI} forming set I and α_{NCPII} and β_{NCPII} forming set II (Fig. 1B). The sets share the same orientation and are separated by a spacer of ~1 kb. As for the adult globin genes, the α- and β-globin gene pairs of this complex are oriented 5′ to 5′, and each gene is composed of three exons and two introns. However, the intergenic regions that separate the α and β genes are quite short, ~0.8 kb. Several lines of evidence indicate that this gene cluster encodes embryonic and/or juvenile globin chains. First, the two identical α_{NCP} chains and the two similar β_{NCP} globins (β_{NCP1} and β_{NCP2}, 97% identical) differ significantly in amino acid sequence from their adult counterparts: α_{NCP} is 53% identical to adult α1-globin and 71% to adult α2 (the α chain of the minor adult Hb 2), whereas β_{NCP1} and β_{NCP2} are 61 and 63% identical, respectively, to the sole adult β chain. Second, molecular phylogenetic comparison reveals that the NCP globins cluster with embryonic globins from several non-notothenioid fish species. Third, the *NCP* genes are expressed at higher levels in the hematopoietic tissues of juvenile *N. coriiceps*.

3. Loss of globin genes by Antarctic icefishes

The 15 known species of icefishes all share the hemoglobinless condition (Barber et al., 1981; Eastman, 1993; Hureau et al., 1977). One may ask, 'What has befallen their globin genes?' To address this question, we compared initially the hybridization patterns of notothenioid adult globin cDNAs to the genomes of three icefish species (*Chaenocephalus aceratus*, *Champsocephalus gunnari*,

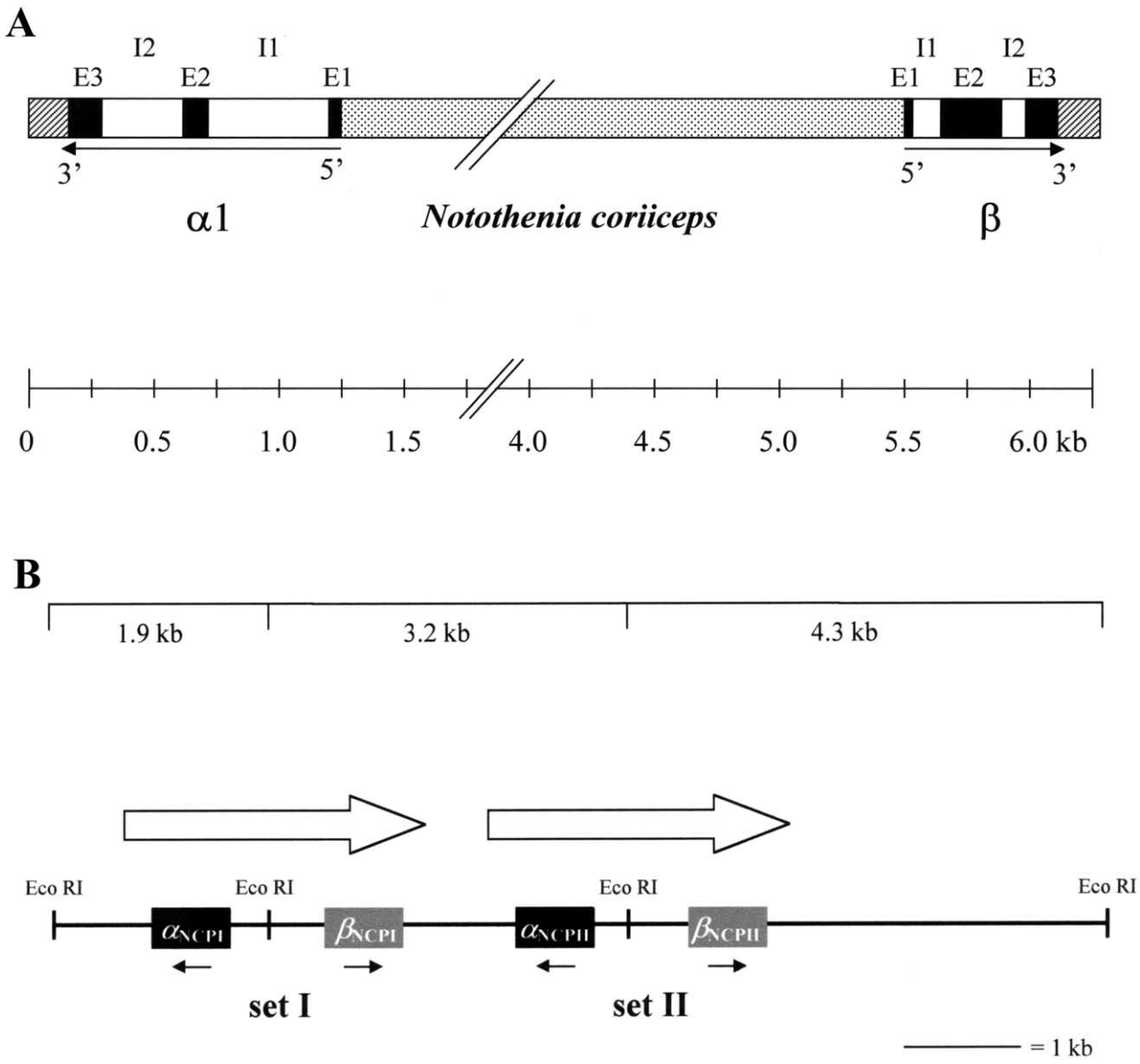

Fig. 1. Organization of the globin gene complexes of the Antarctic rockcod *N. coriiceps*. (A) The major adult α1- and β-globin genes are linked in 5′ to 5′ orientation. The exons (E1–E3) and introns (I1, I2) of the globin genes are represented by filled and open rectangles, respectively. The intergenic region, which is defined as the 4.3-kb nucleotide sequence that separates the initiator codons of the gene pair, is shown by a stippled rectangle, and 3′-downstream regions are indicated by diagonally hatched rectangles. The direction of transcription (5′ to 3′) is indicated for each gene. Lengths of sequence components can be estimated from the scale below the bar diagram. Reprinted with permission from Detrich (2000). Copyright 2000 by Springer-Verlag. (B) Organization of the *non-adult* globin genes. Each pair of the *NCP* globin genes is linked as a divergent transcription unit (direction of transcription indicated by black arrows). The two gene pairs and their intergenic regions share high sequence similarity (regions covered by the open arrows). Note the compactness of the four-gene complex relative to the adult complex. Reprinted with permission from Cocca et al. (2000). Copyright 2000 by The Fisheries Society of the British Isles.

and *Chionodraco rastrospinosus*) and of four red-blooded relatives (Cocca et al., 1995). Fig. 2 shows that the presence of α1-globin-related DNA sequences (Fig. 2A), and the absence of β-globin genes (Fig. 2B), are features common to icefish genomes that represent both primitive (*Champsocephalus*) and advanced genera (*Chaenocephalus*, *Chionodraco*). By contrast, three red-blooded Antarctic notothenioids [the rockcods *Gobionotothen gibberifrons* and *N. coriiceps* (family Nototheniidae), and the dragonfish *Parachaenichthys charcoti* (family Bathydraconidae)] and a temperate notothenid (the New Zealand black cod *Notothenia angustata*) give strong hybridization signals for both α- and β-globin probes. These results suggest that establishment of the hemoglobinless phenotype preceded the evolutionary radiation of the icefish genera and involved, at a minimum, the deletion of the adult β-globin gene.

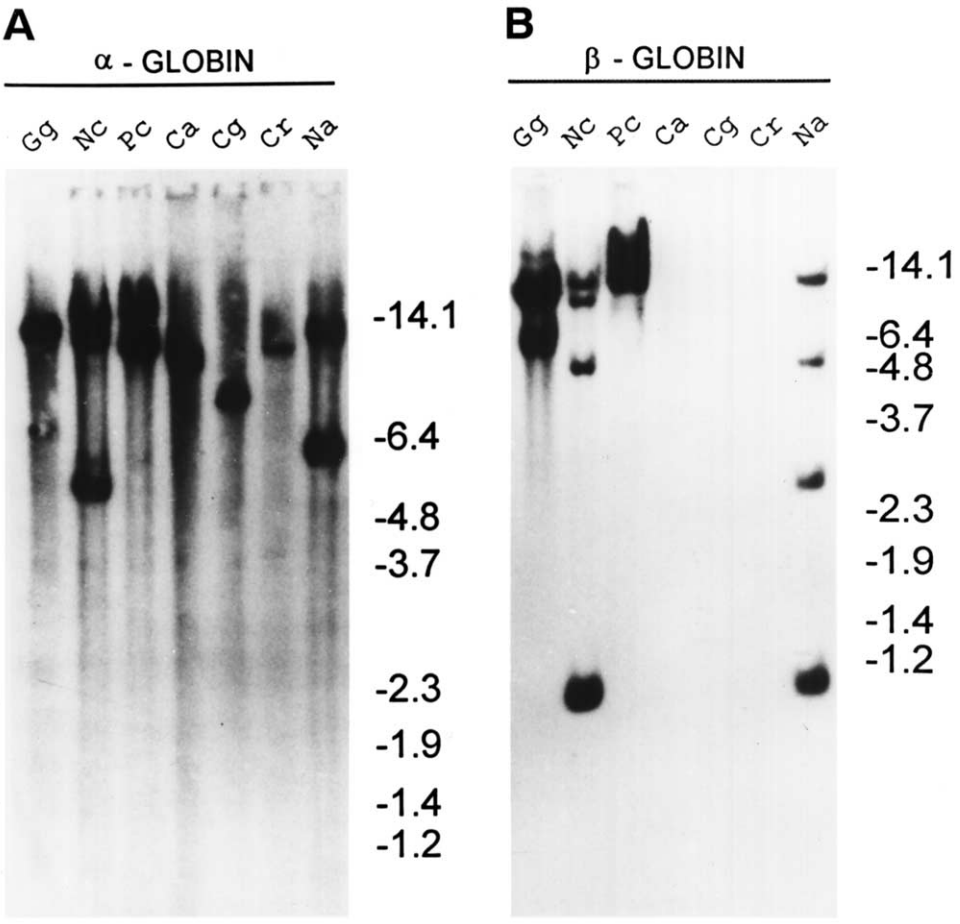

Fig. 2. Globin-related sequences in the genomes of red- and white-blooded Antarctic fishes. Southern blots of genomic DNAs from three nototheniids (Gg, *G. gibberifrons*; Na, *N. angustata*; Nc, *N. coriiceps*), a bathydraconid (Pc, *P. charcoti*), and three channichthyids (Ca, *C. aceratus*; Cg, *C. gunnari*; Cr, *C. rastrospinosus*) were probed with *N. coriiceps* cDNAs for α-globin (A) or for β-globin (B). DNAs were digested with *Bam*HI. The molecular weights of DNA markers are indicated on the vertical axes in kb. See Cocca et al. (1995) for hybridization and wash conditions. Reprinted with permission from Cocca et al. (1995). Copyright 1995 by The National Academy of Sciences of the USA.

In the absence of its β-globin gene partner, what has become of the adult α1-globin gene of icefishes? The most plausible fate of the icefish α1-globin gene is mutation to transcriptional inactivity without extinction of the entire sequence from the channichthyid genome. To answer this question, we cloned the α1-globin genes of the icefishes *C. aceratus* and *C. rastrospinosus* (Zhao et al., 1998). Fig. 3 shows that the icefish α1-globin genes are truncated variants of the *N. coriiceps* α1-globin gene; each contains part of intron 2, the entirety of exon 3, and the 3′-untranslated region. The apparent 5′ chromosomal breakpoint within intron 2 is identical in the two icefish genes, and the 5′-flanking sequences preceding the breakpoint are unrelated to any portion of the *N. coriiceps* α1-globin gene. Beyond the 3′ polyadenylation signal, the icefish and *N. coriiceps* genes share sequence similarity for at least 180 bp. In the absence of selective pressure for expression, the two icefish α-globin gene remnants have diverged at a rate of ~0.25% per My. Together, these observations are consistent with deletional loss of 5′-upstream α1-globin sequences, including the 5′-untranslated region, exons 1 and 2, intron 1 and part of intron 2, prior to divergence of these two relatively advanced icefish species. Determination of the status of the α1-globin gene in the ancestral channichthyid will require analysis of more primitive icefish species (e.g. *Champsocephalus* spp.).

Like adults, embryonic and juvenile icefishes also fail to express hemoglobin (data not shown). Are embryonic and juvenile globin genes, corresponding to those described here, present in channichthyid genomes? Southern blots of genomic DNA from red- and white-blooded notothenioids were probed with fragments of α_{NCPI} and β_{NCPII}, the genes that flank the ends of the embryonic/juvenile complex (Fig. 1B). Both probes hybridized strongly with one or two DNA fragments in the genomes of the red-blooded fishes *N. coriiceps*, *Trematomus bernacchii*, *T. newnesi*, and *Gymnodraco acuticeps* (Fig. 4). In striking contrast, no hybridization signals were observed for two icefishes, *Chionodraco hamatus* and *C. aceratus*. Although sampling of the icefish family is incomplete, our results suggest that the species of this group have lost both adult, embryonic, and juvenile globin genes.

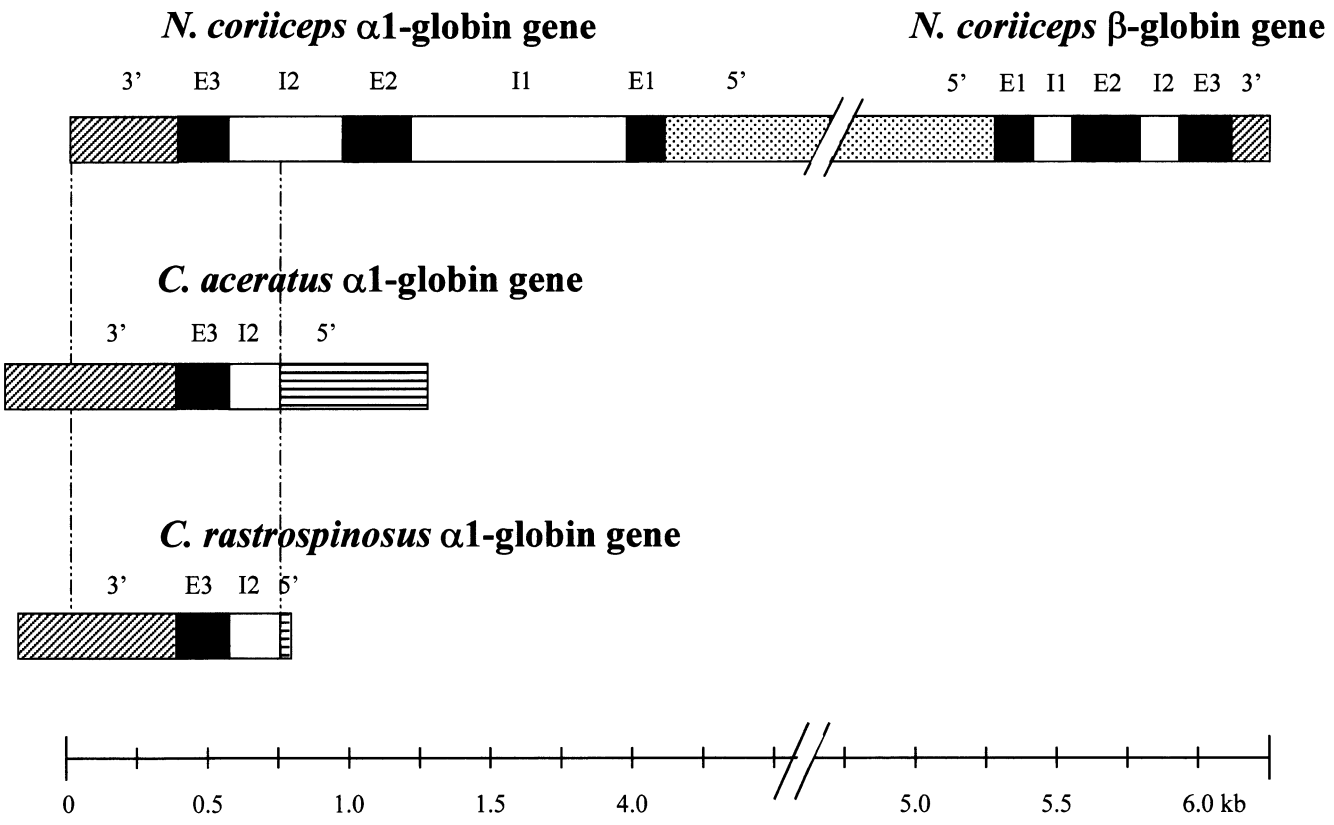

Fig. 3. Comparison of the α1-globin gene remnants of *C. aceratus* and *C. rastrospinosus* to the α1/β-globin gene complex of *N. coriiceps*. The exons (E1–E3), introns (I1, I2), intergenic region, and 3′-downstream regions of the wild-type *N. coriiceps* globin complex are represented by filled, open, stippled, and hatched rectangles, respectively. Sequences adjacent to the 5′ limits of the icefish α1-globin gene remnants are shown by horizontally striped boxes. Box dimensions encompass the extent of sequencing of each gene. Adapted with permission from Zhao et al. (1998). Copyright 1998 by the American Society for Biochemistry and Molecular Biology, Inc.

4. Evolutionary mechanism of globin gene loss

The evolution of icefishes to the hemoglobinless phenotype may have occurred by at least two mechanisms: (1) direct gene deletion or (2) a multi-step process involving transcriptional inactivation of the adult and the embryonic/juvenile globin complexes, followed by elimination of the non-functional genes themselves (Cocca et al., 1995; Zhao et al., 1998). Fig. 5 shows that a single deletional event (schema X) in the ancestral channichthyid, with breakpoints located within intron 2 of the α1-globin gene and downstream of the 3′-untranslated region of the β-globin gene, would eliminate the adult genes. Could such a deletional event have removed the *NCP* globin gene cluster as well? Although we have not established linkage between the adult and *NCP* complexes, Chan et al. (1997) have shown that the major embryonic and adult globin genes of the zebrafish are present on a single chromosomal fragment of ~550 kb. Thus, we propose that a single, large-scale deletional event (Fig. 5, schema Z) may have removed all icefish globin genes with the exception of the 3′ end of *α1*.

Based on the results reported here, we have formulated two mechanistic hypotheses to explain the evolution of the hemoglobinless condition of icefishes. We are currently testing these hypotheses by assessing the status of globin genes in other icefish species. Although the state of adult globin genes in *C. aceratus* and *C. rastrospinosus* has been characterized in molecular detail, the 13 remaining icefish species may yet reveal surprises that would have delighted Ruud.

Acknowledgements

We gratefully acknowledge the excellent logistic support provided to our Antarctic field research programs, performed at the U.S. Antarctic Program's Palmer Station and at the Italian Terra Nova Bay Station, by the staffs of the Office of Polar Programs of the National Science Foundation and of the Italian National Program for Antarctic Research, by the personnel of Antarctic Support Associates, and by the captains and crews of the *R/V Polar Duke* and the *ARSV Laurence M. Gould*. This work was supported by National Science Foundation Grants OPP-9120311, OPP-9420712, and OPP-9815381 (H.W.D.), and is in the framework of the Italian National Program for Antarctic Research (G. di P.).

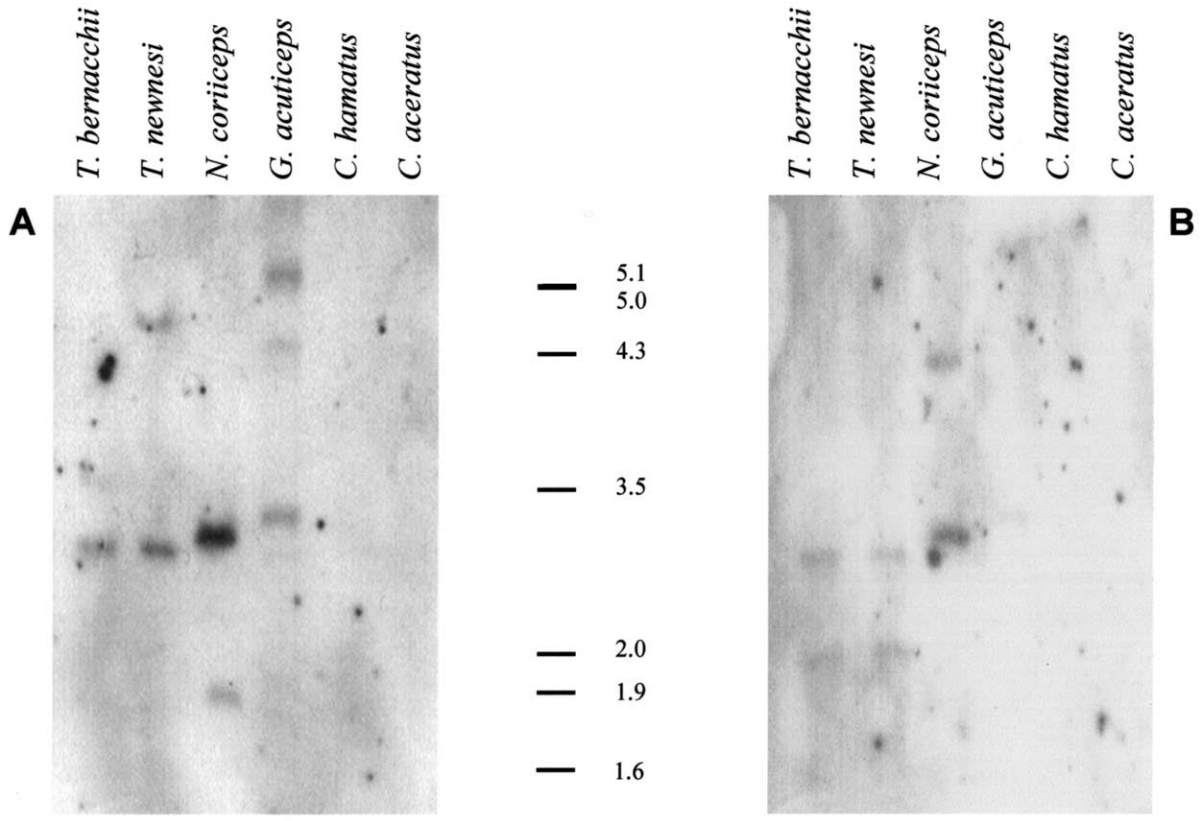

Fig. 4. *NCP*-related gene sequences in the genomes of Antarctic fishes. Southern blots of *Eco*RI-digested genomic DNAs from three notothenioids (*T. bernacchii*, *T. newnesi*, and *N. coriiceps*), one bathydraconid (*G. acuticeps*), and two channichthyids (*C. hamatus* and *C. aceratus*) were probed with α_{NCPI} (A) and β_{NCPII} (B) globin gene fragments. The sizes (kb) of DNA standards are indicated on the vertical axis. Reprinted with permission from Cocca et al. (2000). Copyright 2000 by The Fisheries Society of the British Isles.

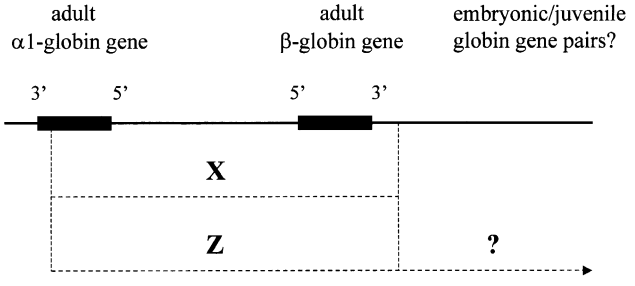

Fig. 5. Potential mechanisms of globin gene deletion by the Antarctic icefishes. Mechanism 1: Simultaneous deletion of the adult β-globin gene, the intergenic region, and the 5′ portion of the linked α1-globin gene (X), leaving the α1-globin 3′ gene remnant. Mechanism 2: If embryonic and juvenile α/β-globin gene pairs are linked in the notothenioid genome to the adult globin gene pair, then a single deletion (Z) could easily explain the loss of most of the globin genes in icefish genomes.

References

Barber, D.L., Mills Westermann, J.E., White, M.G., 1981. The blood cells of the Antarctic icefish *Chaenocephalus aceratus* Lönnberg: light and electron microscopic observations. J. Fish Biol. 19, 11–28.

Bargelloni, L., Ritchie, P.A., Patarnello, T., Battaglia, B., Lambert, D.M., Meyer, A., 1994. Molecular evolution at subzero temperatures: mitochondrial and nuclear phylogenies of fishes from Antarctica (suborder Notothenioidei), and the evolution of antifreeze glycopeptides. Mol. Biol. Evol. 11, 854–863.

Chan, F.Y., Robinson, J., Brownlie, A., Shivdasani, R.A., Donovan, A., Brugnara, C., Kim, J., Lau, B.C., Witkowska, H.E., Zon, L.I., 1997. Characterization of adult alpha- and beta-globin genes in the zebrafish. Blood 89, 688–700.

Cocca, E., Ratnayake-Lecamwasam, M., Parker, S.K., Camardella, L., Ciaramella, M., di Prisco, G., Detrich III, H.W., 1995. Genomic remnants of α-globin genes in the hemoglobinless Antarctic icefishes. Proc. Natl. Acad. Sci. USA 92, 1817–1821.

Cocca, E., Detrich III, H.W., Parker, S.K., di Prisco, G., 2000. A cluster of four globin genes from the Antarctic fish *Notothenia coriiceps*. J. Fish Biol. 57, 33–50.

D'Avino, R., di Prisco, G., 1989. Hemoglobin from the Antarctic fish *Notothenia coriiceps neglecta*. 1. Purification and characterization. Eur. J. Biochem. 179, 699–705.

Detrich III, H.W., 2000. Recent evolution of the hemoglobinless condition of the Antarctic icefishes. In: di Prisco, G., Giardina, B., Weber, R.E. (Eds.). Hemoglobin Function in Vertebrates, Springer, Milan, pp. 39–49.

di Prisco, G., D'Avino, R., Caruso, C., Tamburini, M., Camardella, L., Rutigliano, B., Carratore, V., Romano, M., 1991. The biochemistry of oxygen transport in red-blooded Antarctic fish. In: di Prisco, G., Maresca, B., Tota, B. (Eds.). Biology of Antarctic Fish, Springer, Berlin, pp. 263–281.

di Prisco, G., Macdonald, J.A., Brunori, M., 1992. Antarctic fishes survive exposure to carbon monoxide. Experientia 48, 473–475.

Eastman, J.T., 1993. Antarctic Fish Biology: Evolution in a Unique Environment, Academic Press, San Diego, CA.

Efstratiadis, A., Posakony, J.W., Maniatis, T., Lawn, R.M., O'Connell, C., Spritz, R.A., DeRiel, J.K., Forget, B.G., Weissman, S.M., Slightom, J.L., Blechl, A.E., Smithies, O., Baralle, F.E., Shoulders, C.C., Proudfoot, N.J., 1980. The structure and evolution of the human β-globin gene family. Cell 21, 653–668.

Hureau, J.-C., Petit, D., Fine, J.M., Marneux, M., 1977. New cytological, biochemical, and physiological data on the colorless blood of the Channichthyidae (Pisces, Teleosteans, Perciformes). In: Llano, G.A. (Ed.). Adaptations Within Antarctic Ecosystems, Smithsonian Institution, Washington, DC, pp. 459–477.

Lacy, E., Maniatis, T., 1980. The nucleotide sequence of a rabbit β-globin pseudogene. Cell 21, 545–553.

Lau, D.T., Saeed, A., Parker, S.K., Detrich III, H.W., 2001. Adaptive evolution of gene expression in Antarctic fishes: Divergent transcription of the 5′-to-5′ linked adult α1- and β-globin genes of the Antarctic teleost *Notothenia coriiceps* is controlled by dual promoters and intergenic enhancers. Am. Zool. 41, 113–132.

Lawn, R.M., Efstratiadis, A., O'Connell, C., Maniatis, T., 1980. The nucleotide sequence of the human β-globin gene. Cell 21, 647–651.

Liebhaber, S.A., Gootsens, M.J., Kan, Y.W., 1980. Cloning and complete nucleotide sequence of human 5′ alpha-globin gene. Proc. Natl. Acad. Sci. USA 77, 7054–7058.

Macdonald, J.A., Montgomery, J.C., Wells, R.M.G., 1987. Comparative physiology of Antarctic fishes. Adv. Mar. Biol. 24, 321–388.

Proudfoot, N.J., Maniatis, T., 1980. The structure of a human α-globin pseudogene and its relationship to α-globin gene duplication. Cell 21, 537–544.

Ruud, J.T., 1954. Vertebrates without erythrocytes and blood pigment. Nature 173, 848–850.

Vanin, E.F., 1985. Processed pseudogenes: characteristics and evolution. Annu. Rev. Gen. 19, 253–272.

Zhao, Y., Ratnayake-Lecamwasam, M., Parker, S.K., Cocca, E., Camardella, L., di Prisco, G., Detrich III, H.W., 1998. The major adult α-globin gene of Antarctic teleosts and its remnants in the hemoglobinless icefishes: calibration of the mutational clock for nuclear genes. J. Biol. Chem. 273, 14745–14752.