Evolution, Organization, and Expression of α-Tubulin Genes in the Antarctic Fish Notothenia coriiceps

ADAPTIVE EXPANSION OF A GENE FAMILY BY RECENT GENE DUPLICATION, INVERSION, AND DIVERGENCE

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To assess the organization and expression of tubulin genes in ectothermic vertebrates, we have chosen the Antarctic yellowbelly rockcod, Notothenia coriiceps, as a model system. The genome of N. coriiceps contains ~15 distinct DNA fragments complementary to α-tubulin cDNA probes, which suggests that the α-tubulins of this cold-adapted fish are encoded by a substantial multigene family. From an N. coriiceps testicular DNA library, we isolated a 13.8-kilobase pair genomic clone that contains a tightly linked cluster of three α-tubulin genes, designated NcGTbαa, NcGTbβa, and NcGTbαc. Two of these genes, NcGTbαa and NcGTbβa, are linked in head-to-head (5’ to 5’) orientation with ~500 bp separating their start codons, whereas NcGTbαa and NcGTbαc are linked tail-to-tail (3’ to 3’) with ~2.5 kilobase pairs between their stop codons. The exons, introns, and untranslated regions of the three α-tubulin genes are strikingly similar in sequence, and the intergenic region between the genes are strikingly palindromic. Thus, this cluster probably evolved by duplication, inversion, and divergence of a common ancestral α-tubulin gene. Expression of the NcGTbα gene is cosmopolitan, with its mRNA most abundant in hematopoietic, neural, and testicular tissues, whereas NcGTbαa and NcGTbβa transcripts accumulate primarily in brain. The differential expression of the three genes is consistent with distinct suites of putative promoter and enhancer elements. We propose that cold adaptation of the microtubule system of Antarctic fishes is based in part on expansion of the α- and β-tubulin gene families to ensure efficient synthesis of tubulin polypeptides.

Subjected to an increasingly severe thermal environment as the Southern Ocean began to cool approximately 25–40 million years ago (mya)1 (1), the coastal fishes of the Antarctic diverged from temperate fishes (2) and evolved compensatory molecular, cellular, and physiological adaptations that maintain metabolic efficiency and preserve macromolecular function in their now chronically cold marine environment (~1.86 to approximately 1 °C). The translational machinery of these fishes, for example, shows clear evidence of cold adaptation (3, 4), with rates of polypeptide chain elongation more than 10-fold greater than those measured in temperate fishes cooled to comparable temperatures. Similarly, the polymerization energetics of the actins of Antarctic fishes (5) and the ATPase activities of their skeletal myosins (6) support efficient myofibrillar assembly and function at their low habitat temperatures. Our goal is to determine the molecular adaptations, both qualitative and quantitative, that maintain the efficient expression of the tubulin genes and the polymerization capacity of the tubulin polypeptides of these extreme psychrophiles.

We and others (7, 8) have shown previously that the critical concentration for microtubule formation by the brain tubulins of Antarctic fishes (~1 mg/ml) is comparable to those of temperate poikilotherms and homeotherms at their much higher body temperatures. Conservation of the critical concentration by Antarctic fishes probably results from structural changes, both in primary sequences and in posttranslational modifications, intrinsic to their α- and β-tubulin subunits (9–12). The primary sequence of class II β-tubulin from the yellowbelly rockcod Notothenia coriiceps, for example, contains unique residue substitutions that increase both the hydrophobicity and the flexibility of the polypeptide chain (12, 13), two factors that should favor microtubule formation in an energy-poor environment. Similar alterations have been observed in other α- and β-tubulin chains of this species.2 A second, related challenge confronting Antarctic fishes is the synthesis of sufficient quantities of the α- and β-tubulins to attain the critical concentration of tubulin dimers in their cells.

The abundant expression of tubulin in the brains of Antarctic fishes is likely to require compensatory adjustments in gene transcription to offset the rate-depressing effects of low temperature. Potential adaptations include increases in tubulin gene number, organization of tubulin genes into efficient transcription units, evolution of more efficient gene promoters, enhancers, transcription factors, and/or RNA polymerases, and enhancement of mRNA stabilization. To evaluate these possibilities, we have initiated analysis of the structure, genomic organization, and expression of the tubulin genes of N. coriiceps. Our results suggest that several of these modes of adaptation may be exploited by these cold-living vertebrates.

In higher vertebrates, the α- and β-tubulins are encoded by small gene families (~6–7 functional genes for α and a similar number for β), each member of which yields a structurally

1 The abbreviations used are: mya, million years ago; bp, base pairs; kb, kilobase pairs; PCR, polymerase chain reaction; UTR, untranslated region.

2 Detailed analysis of the structural changes unique to Antarctic fish tubulins will be presented elsewhere (S. K. Parker, E. Nogales, K. H. Downing, and H. W. Detrich III, manuscript in preparation).
distinct polypeptide (14–16). These genes are generally thought to be unlinked and dispersed throughout the genome (17). In a study of the chicken α-tubulin gene family, for example, Pratt and Cleveland (18) found that four of five genomic clones contained single α-tubulin genes; the fifth contained two α-tubulin genes, one functional and the second a pseudogene. The genomes of lower, nonvertebrate eukaryotes, by contrast, frequently contain tightly linked tubulin genes. Protozoan parasites possess tubulin gene ensembles, either as separate tandem groupings of α- or β-tubulin genes (Leishmania spp. (19, 20)) or as linked α/β tandem repeats (Trypanosoma brucei (21, 22)). Similarly, some of the tubulin genes of the sea urchin Lytechinus pictus are organized in distinct α or β clusters (23).

The regulation of tubulin gene expression occurs at both transcriptional and translational levels. The tissue-specific and hormonally regulated expression of the β-tubulin genes of Dro sophila is controlled both by upstream promoter elements and by negative and positive regulatory elements (silencers and activators) generally located within the first introns (24–28).

Genomic Southern blotting was performed as described (12), and positive plaques were detected autoradiographically as described above. A total of 159 candidate α-tubulin clones were isolated from three screens (632,000 total recombinant phage) of the first library in 212 candidate plaques were detected autoradiographically as described above. One strongly hybridizing clone, designated Sa4, was isolated through use of a panel of plaque purification and screening, and single plaques were picked for clone stock preservation.

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**EXPERIMENTAL PROCEDURES**

**Collection of Fish Tissues**—Specimens of the Antarctic yellowbelly rockcod, N. coriiceps, were collected by bottom trawling from the R/V Hero or from the R/V Polar Duke near Low and Brabant Islands in the Palmer Archipelago. They were transported alive to Palmer Station, Rockford, 22° N (263, 000 total recombinant phage) of the second library that corresponded to the α-tubulin gene pair and the widespread expression of α-tubulin gene sequences by hybridization to an N. coriiceps α-tubulin cDNA, NcTb1, essentially as described for α-globin genes by Zhao et al. (43). Twelve candidate α-tubulin genomic isolates were obtained from a primary screen of 200,000 recombinant phage. Six of these isolates were carried through two additional rounds of plaque purification and screening, and single plaques were picked for clone stock preservation.

**cDNA Library Construction and Screening**—Total RNA was isolated from brain tissues of N. coriiceps (38, 44), and poly(A)+ RNA was selected by oligo(dT)-cellulose affinity chromatography (45). Two different libraries were made. Oligo(dT)-primed cDNA synthesis and construction of the first library in gt10 followed the procedures described by Huynh et al. (46). The second library was constructed in AZAP II(Stratagene); cDNA synthesis was primed with a mixture of random hexanucleotides (75%) and oligo(dT) (25%).

The libraries were screened for recombinant clones bearing α-tubulin coding sequences by hybridization of nick-translated cDNA (38, 49) or by random priming (47). In early screens cDNA was used as probe, whereas in later screens α-tubulin cDNAs from N. coriiceps were employed. Hybridization and washing of the membranes were performed as described (12), and positive plaques were detected autoradiographically. A total of 159 candidate α-tubulin cDNA isolates were obtained from three screens (632,000 total recombinant phage) of the second library that corresponded to the α-tubulin gene pair and the widespread expression of α-tubulin gene sequences by hybridization to an N. coriiceps α-tubulin cDNA, NcTb1, essentially as described for α-globin genes by Zhao et al. (43). Twelve candidate α-tubulin genomic isolates were obtained from a primary screen of 200,000 recombinant phage. Six of these isolates were carried through two additional rounds of plaque purification and screening, and single plaques were picked for clone stock preservation.
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GenBank Accession Numbers—The sequence of the N. coriiceps α-tubulin gene cluster reported in this paper has been deposited in the GenBank™ data base under the accession number AF082027. The sequence of the cluster has been scanned against the GenBank™ data base using the BLASTN program (National Center for Biotechnology Information) to identify sequences with significant relatedness. Related sequences, and their accession numbers, are presented under “Results.”

Northern Analysis of α-Tubulin Gene Expression—Total RNAs from testis, brain, gill, liver, spleen, blood, and muscle were isolated from tissues by a modification (44) of the acid guanidinium thiocyanate/phenol/chloroform method (51). RNAs (5 µgslot) were applied to nylon membranes (MagnaGraph, MSI) by vacuum aspiration using a Bio-Rad Bio-Safe slot apparatus. Sets of seven RNA samples were hybridized to PCR-generated, 32P-labeled probes specific for the 3′-UTRs of the cDNAs NcTb2 (α gene), NcTb3, NcTb7 (aa gene), or NcTb8 (ac gene). To estimate the total α-tubulin mRNA in each tissue, a control set of RNA samples was hybridized to a fragment of NcTb1 encoding amino acid residues 1–430. Prehybridization and hybridization of the membranes were performed in 5× SSPE (1× SSPE = 0.18 M NaCl, 0.01 M Na2HPO4·7H2O, 0.001 M EDTA), 5× Denhardt’s solution (41), 50% formamide, 0.2% SDS at 42 °C for 2 and 18–20 h, respectively, after which the membranes were washed sequentially with buffers of increasing stringency (final wash conditions = 0.1× SSPE, 42 °C, 15 min). The membranes were exposed to Fuji RX x-ray film at −70 °C with intensification.

PCR-based Gene Linkage Analysis—To determine the potential linkage of α-tubulin genes in the N. angustata genome, we employed a PCR-based strategy using as template phage DNAs purified from the six tertiary genomic clones (see “Genomic Library Construction and Screening”). Nondegenerate primers corresponding to highly conserved regions of the primary sequence of the N. coriiceps α-tubulins were synthesized as follows: 1) sense primer, 5′-CAGTTTGTGGACTGGTGC-3′; 2) antisense primer, 5′-AGCTCCAGTCTCACTGAAG-3′. PCR products not shared with sense alone and PCR products not shared with antisense alone reactions). Each PCR reaction contained 3–5 ng of template DNA, 1.6 µM primers (0.8 µM of each primer when different), and CLONTECH Advantage™KlenTaq polymerase mix (optimized for long distance PCR) (52). Touchdown PCR (53) was performed for 29 cycles using the following parameters: 1) denaturation steps, 94 °C, 30 s; 2) annealing steps, first 9 cycles ramping the temperature from 70 to 62 °C in 1° increments followed by 20 cycles at 62 °C; and 3) extension steps, 68 °C, 6 min. PCR products were analyzed on 1% agarose gels containing 1× TBE (0.089 M Tris borate, 2 mM EDTA, pH 8.0) and 0.0005% ethidium bromide. The ends of the PCR products were sequenced by the automated procedure to establish α-tubulin gene orientation.

Genomic Southern Analysis of a Repetitive DNA Element—During characterization of the N. coriiceps α-tubulin gene cluster, we discovered a 285-bp repetitive element. To determine the abundance, organization, and species distribution of this fragment, we hybridized it to Southern replicas of HindIII-digested genomic DNAs from Antarctic and temperate notothenioids, other temperate fishes, an amphibian, and a reptile. Restriction endonuclease digestion, electrophoresis, and transfer to nylon membranes were performed as described previously (12). Prehybridization of the membrane and subsequent hybridization to the 285-bp probe labeled with 32P by random priming (47) were performed as described by Detrich and Parker (12) with the following exceptions: 1) prehybridization was for 2 h; 2) the prehybridization/hybridization temperature was 63 °C; and 3) the membranes were washed to final stringencies of 0.1–1× SSC, 63 °C, for 15–40 min. The membranes were exposed to Fuji RX x-ray film as described above.

Genomic DNA from the zebrafish (Danio rerio) was prepared from total body tissues (36). Samples of genomic DNAs from the African lungfish (Protopterus aethiopicus), the clearance skate (Raja eglantaria), the goldfish (Carassius auratus), the horned shark (Heterodontus francisci), the sea lamprey (Petromyzon marinus), the spotted ratfish (Hydrolagus coliei), the sturgeon (Acipenser fulvescens), the clawed frog ( Xenopus mulleri), and the snapping turtle (Chelydra serpentina) were generously provided by Dr. Chris Amemiya (Boston University School of Medicine).

Fig. 1. Hybridization of α-tubulin cDNA probes to genomic DNA from N. coriiceps. Southern blots of restriction endonuclease-digested testis DNA were hybridized to 32P-labeled recombinant plasmids pT1 (A; chicken cα1 cDNA insert) or pcf10–2 (B; C. reinhardtii α10–2 cDNA insert). Lanes B, H, P, and E contain genomic DNA digested with BamHI, HindIII, PstI, and EcoRI, respectively. Lanes U contain undigested genomic DNA. The molecular weights of DNA standards (in kb) are indicated on the vertical axes.

RESULTS

Estimation of α-Tubulin Gene Number in N. coriiceps—To estimate the number of α-tubulin genes possessed by N. coriiceps, we probed its genome for sequences complementary to α-tubulin cDNAs from the chicken (cα1) and from Chlamydomonas (α10–2). Fig. 1 shows that the α-tubulin probes hybridized to 10–15 different fragments in each restriction digest of the fish DNA. Furthermore, the hybridization patterns generated by the two heterologous cDNAs were virtually identical. These results suggest that the α-tubulins of N. coriiceps, like its β-tubulins (12), are encoded by a multigene family that is larger than those of higher vertebrates (14, 16, 39). Of particular note, the strong hybridization signals observed for some of the fragments raised the possibility that they contain multiple, linked α-tubulin genes.

Organization of an N. coriiceps α-Tubulin Gene Complex—To investigate the organization of the α-tubulin genes of N. coriiceps, we selected a strongly hybridizing clone, S2, that carried an insert of ~13.8 kb. Preliminary restriction mapping and Southern hybridization analysis suggested that the insert contained two or more α-tubulin genes in a segment of ~10 kb. Subsequent sequence analysis revealed that S2 contains two complete α-tubulin genes, designated NcGTbαa and NcGTbαc, and one partial gene, NcGTbβb, that abuts one end of the genomic fragment. Fig. 2 presents the organization and salient features of this gene complex. Two of the genes, αa and αb, are linked in head-to-head; or 5′ to 5′, orientation with ~500 bp separating their start codons. By contrast, the αc and αe genes are linked tail-to-tail (3′ to 3′) with ~2 kb between their poly(A) signal sequences. The approximately 4 kb of sequence to the left of the αc gene is devoid of α-tubulin coding sequences.

N. angustata Is a Temperate Congener of N. coriiceps—To determine whether this mesophilic species shares a similar organization of its α-tubulin genes, we probed its genome by

(50) as implemented by DNASTAR Align.
Southern blot hybridization to the *N. coriiceps* NcTbα1 cDNA. The α-tubulin fragment patterns observed for HindIII-digested *N. angustata* and *N. coriiceps* DNAs shared some similarities in the low molecular weight region, but the temperate species contained few of the strongly hybridizing fragments of high molecular weight (>5 kb) that are suggestive of gene clustering in the cold-living fish (data not shown). We also examined six genomic α-tubulin clones from *N. angustata* for gene clustering by use of PCR-based linkage analysis. Two of the six clones gave the same 3-kb amplification product, which corresponded to head-to-tail linkage of a pair of α-tubulin genes with approximately 2.5 kb separating their respective coding sequences (data not shown). The remaining four clones apparently contained single α-tubulin genes. Although these surveys were not exhaustive, they do suggest that the extent of α-tubulin gene clustering in *N. angustata* is smaller than that of the Antarctic fish.

Three α-Tubulin Genes and Their Encoded Polypeptides—Fig. 3, A—C, shows the nucleotide sequences and translations of the *NcGTbα*, *NcGTbβ*, and *NcGTbγ* genes, respectively. (For comparative purposes, the sequence of the αβ gene downstream of Glu<sup>168</sup> has been completed from its cDNA NcTbα2.) Table I gives estimates of the sequence similarities of these genes and subregions thereof. The αα, αβ, and αc genes are quite similar to each other (80–83%), which suggests that they may have arisen by duplication and divergence of a common ancestral gene. Each gene contains three introns that interrupt the nucleotide sequence after codon 1, within codon 76, and after codon 125, positions that are highly conserved in other vertebrate α-tubulin genes (54). In general, αα appears to be most closely related to αβ, except that its small introns 2 and 3 are quite similar to those of αc.

The three genes encode distinct, but closely related, α-tubulin polypeptides (Fig. 4). Compared pairwise, the αα- αβ-, and αc-tubulin chains are 98.4–98.9% identical to each other. With respect to other vertebrate α-tubulins, the three fish chains are very closely related to the α66-tubulin of the ray *Torpedo marmorata* (97.6–97.8% sequence similarity; GenBank<sup>TM</sup> accession number P36220) and to two mammalian α chains, α1-tubulin of Chinese hamster (97.3–97.6% sequence similarity; accession number P05209) and the Ms2 isoform of mouse (97.1–97.3% sequence similarity; accession number P05213). Somewhat surprisingly, the *N. coriiceps* α-tubulins are only ~94% similar to two α-tubulin polypeptides from salmonid teleosts, the rainbow trout (*Oncorhynchus mykiss*) testis-specific α chain, accession number P18288, and the chum salmon (*O. keta* α chain, accession number P30436). However, this apparent discrepancy most likely reflects the multiplicity of α-tubulin isoforms in vertebrates and the paucity of fish tubulin sequences available for comparison.

Like the Nenβ1 β-tubulin cDNA of *N. coriiceps* (12), the *NcGTbα*, *αb*, and *αc* genes show a strong preference for codons containing G or C in the third position. Although 57 (αα) to 58 (αβ, αc) codons are used, the frequency of codons ending in G or C is 2.05 (αα) to 2.07 (αβ, αc) times that of codons with third position A or T. The codon bias of the three α-tubulin genes stands in striking contrast to their A + T rich introns (see below) and to the G + C content (39–43%) of the genomes of closely related Antarctic nototheniid fishes (55). α-Tubulin genes from the chum salmon (accession number X66973) and the rainbow trout (accession number M36623) are similarly biased to third position G or C (G + (C/A) + T = 2.22 and 2.39, respectively), those of mammals are slightly less so (mouse αβ and α6 tubulins = 2.15 and 1.81, human = 1.72; accession numbers M13442, M13441, and K00558, respectively), but Xenopus (accession number X07046) and the electric ray show little codon bias (third position G + (C/A) + T = 0.96 and 1.13, respectively). Overall, the pattern of codon usage in the *N. coriiceps* α-tubulin genes is strongly reminiscent of that found for a set of 22 genes of the Atlantic salmon *Salmo salar* L. (56),...
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FIG. 3. Nucleotide sequences of the NcGTbαa, NcGTbαb, and NcGTbαc genes and primary sequences of the encoded α-tubulins. A, NcGTbαa, B, NcGTbαb, and C, NcGTbαc. Coding sequences are indicated by uppercase roman text, and the encoded amino acid residues are presented below in the three-letter code. Introns are shown in lowercase roman. 5′-UTR and -upstream sequences are presented in uppercase italics, and 3′-UTR and -downstream sequences are given in lowercase italics. Potential TATA boxes and maternal and neural enhancer motifs are indicated by reversed text, double underlining, and open boxes, respectively. The Kozak sequences for translation initiation (57, 58) are given in underlined boldface, and potential polyadenylation signal sequences in the 3′-UTRs are shown boldfaced, underlined, italic text. Gene-specific probes derived from the 3′-UTRs, used to assess the expression of these genes in major tissues (Fig. 5), are shown underlined.

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which suggests that mutational bias is a major factor influencing choice of synonymous codons in both fishes.

**Expression of the α-Tubulin Gene Cluster**—To determine whether the α-tubulin genes of the *N. coriiceps* cluster are functional, we used gene-specific probes complementary to their 3'-UTRs (see Fig. 3) to measure steady-state mRNA levels in seven tissues of *N. coriiceps*. Fig. 5 shows that the αc gene is expressed most widely (all tissues except liver), whereas αa and αb expression is restricted primarily to brain. The mRNAs for all three genes accumulate significantly, and to comparable levels, in neural tissues. αc mRNAs are also prominent in red blood cells and testis. A fourth α-tubulin gene that is not part of this cluster (represented by the NcTb3 CDNA) also shows widespread expression. We conclude that each of the three α-tubulin genes of this cluster is functional and that regulation of the αaαb gene pair differs from the αc gene.

**Structural Features and Potential Regulatory Motifs of the Three α-Tubulin Genes**—The striking similarity of the αa, αb, and αc genes, together with their unusual organization and differential expression, prompted a detailed comparison of their coding and noncoding regions.

5'-Promoter and -Untranslated Regions—The organization of the αa and αb genes as divergent transcription units with potentially overlapping promoters, and their probable evolution by gene duplication, inversion, and divergence, suggest that the two genes may share structural features in their 5'-noncoding sequences (i.e. promoters and untranslated sequences). Indeed, Fig. 6A shows that the 479-bp DNA segment that separates the start codons of the αa and αb genes possesses an axis of 2-fold rotational symmetry. Thus, this intergenic region is substantially palindromic (overall similarity index for the two halves = 78%), and the 5'-promoter-untranslated regions of the two genes are strongly related. It is not surprising, then, that the two genes show an identical pattern of expression (Fig. 5). The initiator codons of the αaαb genes are strongly related. It is not surprising, then, that the two genes show an identical pattern of expression (Fig. 5).

| αa | αb | αc |
|----|----|----|
| A | B | A |

Despite the symmetry of the αalβ intergenic region, we have found it difficult to identify basal and tissue-specific promoter elements that would explain the neural expression of the two genes. A potential, but noncanonical, TATA box (consensus TATAAA) (59) found upstream of the αaαb gene pair differs from the αc gene.

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Fig. 6 shows the 5′-noncoding sequences of the αc gene. The sequence of this region differs substantially from those upstream of the αa and αb genes (Table I). Consistent with its pattern of expression, the αc gene contains promoter elements characteristic of hematopoietic, neural, and testicular genes (63, 64). A consensus TATA box begins 450 bp upstream of the start codon, and noncanonical TATA motifs are located at 2119 and 2470. Two bona fide CCAAT elements (60) begin at positions 2159 and 2210. Two GATA sites (consensus WGATAR) (64), the targets of GATA-binding transcriptional activators in subsets of blood, neural, and testicular cells (64–66), are found at position −411 and downstream of the proximal TATA box. One CACCC element (64) is located upstream of the distal CCAAT box, and a c-Myb (consensus ATTGAC) (63) site is present downstream of the proximal TATA box. Other sites that may contribute to expression of αc include single Sp1, Hox-1, and octamer motifs (63, 67, 68).

Introns—The introns of the three N. coriiceps α-tubulin genes are noteworthy for their generally small sizes (986–1149, 83–102, and 102–103 bp for introns 1, 2, and 3, respectively) and their uniformly high contents of A + T residues (62–71%)


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**Table I**

Sequence comparison of regions of the N. coriiceps αa-, αb-, and αc-tubulin genes

Percentage similarities were calculated as the similarity index of Dayhoff (50). For the alignments, the K-tuple was set at 3, the gap penalty at 1, and the range at 40.

| Nc gene | Complete gene | Promoter + 5′-UTR | Coding | 3′ UTR | Intron 1 | Intron 2 | Intron 3 |
|---------|---------------|-------------------|--------|--------|----------|----------|----------|
| NcGTb αa | 83 | 82 | 78 | 56 | 97 | 98 | 78 | 71 | 69 | 63 | 65 | 78 | 70 | 94 |
| NcGTb αb | 80 | 58 | 97 | 66 | 62 | 63 | 68 |

a Calculated for the coding sequence, introns, 100 nucleotides of sequence upstream of the translation start codon, and the 3′-UTR to and including the polyadenylation signal sequence. The αb sequence downstream of codon 168 was completed from its cognate cDNA, NcTbα2.

b Calculated jointly because transcription start sites were not mapped. The 479-bp region between the start codons of the αa and αb genes was divided into two equal fragments followed by alignment of the sense strands (cf. Fig. 5A). The αa and αb gene segments were also compared to a 240-bp segment upstream of the αc start codon.

c Calculated for sequence commencing after the stop codon and terminating after the probable polyadenylation signal.

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**Fig. 4.** Primary sequences of the αa-, αb-, and αc-tubulins. Amino acid residues that differ between the three polypeptides are shown by shaded rectangles. Residues of the αb- and αc-tubulins that are identical to αa are indicated by periods. The sequence of αb-tubulin beyond Glu168 was deduced from the cognate cDNA, NcTbα2, of the αb gene.

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2 Numbering of the upstream sequences of αc is relative to its start codon because the transcription initiation site of this gene has not yet been mapped.
length-weighted mean = 65.5%). Corresponding introns in human and frog \(\alpha\)-tubulin genes (accession numbers X01703 and X07045, respectively) are considerably larger (intron 1 = 1527–3499 bp, intron 2 = 147–1024 bp, intron 3 = 183–303 bp), and their \(A + T\) contents range from 36 to 73% (weighted mean = 59.5). In contrast to their introns, the coding sequences of the \(\alpha\)-tubulin genes in tissues of \(N.\ coriiceps\) are considerably larger (intron 1 = 147–430 bp, intron 2 = 239 bp from its 5' end, or 721 bp upstream of the start codon for the \(\beta\)-tubulin gene of \(Drosophila\)).

Fig. 7 shows the exon-intron boundaries of the three \(\alpha\)-tubulin genes. The donor exon triplets located immediately to the 5' sides of the splice sites are unusual: ATG for the first junction, T/C/T/G for the second, and CTG for the third versus the vertebrate consensus (C/A)AG (69). Similarly, the 5' nucleotide of the downstream acceptor exon rarely matches the vertebrate consensus residue, G. By contrast, intron sequences adjacent to the donor and acceptor junctions conform well to the vertebrate consensus. In particular, each intron contains the triplet GT(A/G) at its 5' end and a pyrimidine-rich tract immediately upstream of the CAG triplet at its 3' end (see also Fig. 3, A–C).

The absence of definitive basal promoter elements in the compact \(aa/ab\) intergenic region linking the start codons of the \(aa\) and \(ab\) genes. The 2-fold rotational axis is indicated by the vertical line and attached arrows, and palindromic sequences are shown by light and dark shading. 5'-UTRs, deduced from the \(NcGTb\) and \(NcGTboe\) cDNAs that correspond to the \(aa\) and \(ab\) genes, are indicated by underlining. (Due to the high degree of similarity of the palindromic 5' sequences, it has been proven impossible to design gene-specific oligonucleotides for precise mapping of the transcription start (capping) sites by primer extension (85) or by S1-nuclease protection (86).) Because transcriptional start sites have not been mapped, sequences are shown as a darkened portion (85) or by S1-nuclease protection (86). Because transcriptional start sites have not been mapped, sequences are shown as a darkened portion (85) or by S1-nuclease protection (86).
Clustered α-Tubulin Genes in Antarctic Fish

## INTRON - EXON BOUNDARIES

| Vertebrate consensus | Donor exon / intron | ACceptor intron / exon |
|----------------------|---------------------|------------------------|
| NcTub | C TAA GTGAGT | T CAGT |
| Tubulin | ATG GCTGAGT | |

### Introns 1 and 2

| Introns | Donor exons | Acceptor introns |
|---------|-------------|------------------|
| 1       | A | ATG GAA | CCAACGAGCTGA |
|         | B | ATG GAGGC | CCAACGAGCTGA |
|         | C | ATG GAAGA | CCAACGAGCTGA |
| 2       | A | TCG GTAAT | CTCCACAGATGA |
|         | B | TTG GAGT | CTAATCAGATGA |
|         | C | TCG GTAAT | CTTGTCAGATGA |

### Introns 3

| Introns | Donor exons | Acceptor introns |
|---------|-------------|------------------|
| 3       | A | CTG GGGTC | ATCCGAGGCTGA |
|         | B | CTG GGGGC | TGTACAGAGT |
|         | C | CTG GGGGT | ATCCGAGGCTGA |

Fig. 7. Exon-intron boundaries of the three α-tubulin genes. Donor and acceptor splice junctions for the three introns present in each of the NcGTbaa, NcGTbabb, and NcGTbac genes are compared with the vetebrate (69) and tubulin (54) consensus sequences. Residues of the aa, ab, and ac junctions that match the tubulin and/or vertebrate donor and the vertebrate acceptor consensus sequences are shown in boldface.

respectively. These observations support our hypothesis that cis-acting sequences within the first introns of the clustered α-tubulin genes (particularly in the aa and ab genes) may contribute to regulation of their expression.

### 3'-Coding and -Untranslated Sequences—Comparison of the carboxyl-terminal coding sequences and the 3'-UTRs of the aa, ab, and ac genes reveals a remarkable degree of similarity (Table I and Fig. 8). The aa and ab UTRs are the most closely related, with 88% identity in the first 90 bp, and an overall similarity of 78%. However, the aa 3'-UTR is considerably shorter (127 bp) than those of ab and ac (270 and 309 bp, respectively).

A Repetitive Sequence Element in the Notothenioid Genome—During characterization of the N. coriceps α-tubulin gene complex, we also scanned the ~2-kb intergenic region located between the aa and ac genes against the GenBank™ data base to determine whether it shared significant sequence features with other genes. We found that a 285-bp fragment (Figs. 1 and 9) of the aa/ac intergenic region is ~90% similar to a bipartite element from intron 4 of the trypsinogen gene (accession number U58835) (74) of the Antarctic toothfish, Dissotichus mawsoni. No other significant matches were detected. The lone match to the Dissotichus intronic fragment is striking and raises the possibility that this shared sequence might constitute a repetitive element of notothenioid fishes. To determine the abundance and species distribution of this fragment, we hybridized it to Southern replicates of a panel of HindIII-digested genomic DNAs from Antarctic and temperate notothenioids, other temperate fishes, an amphibian, and a reptile (Table II). Among the notothenioid fishes, ~40~50 discrete bands were detected against a smeared background of positive DNA fragments, consistent with the partially structured dispersal of many copies of this element throughout their genomes (data not shown). This pattern is reminiscent of the distribution of two short interspersed nuclear elements in a subgroup of salmonid fishes (75). By contrast, the 285-bp fragment did not hybridize at all to the genomic DNAs of non-notothenioid fishes and more distantly related vertebrates. Given the apparent restriction of this repetitive element to the notothenioid suborder, we provisionally designate it Nto1. We are currently investigating the possibility that Nto1 is a mobile genetic element.

### Evolutionary Divergence Times for the α-Tubulin Genes—Using as a metric the nuclear gene divergence rate (0.12–0.33%/million years) recently determined for the nonfunctional globin gene remnants of Antarctic icefishes (43), we can estimate the time of α-tubulin gene duplication. We considered substitutions at positions of 4-fold degeneracy in the coding sequences, which minimizes the influence of selection on molecular differences (76). Furthermore, transversions were analyzed because they accumulate linearly with respect to time (77). Taken pairwise, the 2.3–3.7% transversion frequency observed for the NcGTbaa, NcGTbabb, and NcGTbac genes at 4-fold degenerate codons yields an estimated divergence time of ~7–31 million years. Thus, the cluster apparently evolved as the Southern Ocean cooled (1, 2).

### DISCUSSION

In this report we describe the first example of a vertebrate tubulin gene cluster, a complex of three tightly linked α-tubulin genes from the Antarctic yellowbelly rockcod, N. coriceps. The aa, ab, and ac genes probably evolved by duplication, inversion, and divergence of an ancestral gene during the period when the Southern Ocean was cooling. We propose that cold adaptation of microtubule assembly in Antarctic fishes entails both the expression of numerically large α- and β-tubulin gene families and the unique sequence features of the encoded tubulin polypeptides.

Evolution of a Vertebrate α-Tubulin Gene Cluster by Gene Duplication—The striking similarity of the three α-tubulin genes that comprise the N. coriceps cluster (97–98% coding sequence similarity, 80–83% overall similarity), and the clearly palindromic structure of the aa and ab genes, suggests that they evolved relatively recently from a common ancestral gene. Given the apparently large number of α-tubulin genes possessed by this fish, the identity of the ancestral gene is unclear. Nevertheless, we consider it likely that aa is the direct ancestor of ab (or vice versa) and gave rise to the latter gene through a recent duplication/inversion event that preserved neural-specific expression. Subsequent conversion (78) of the segment of the aa gene containing introns 2 and 3 to that of ac (or of the corresponding region of the ab gene to that of a fourth α-tubulin gene) would explain the regional similarities and dissimilarities within the cluster. Determination of the most plausible evolutionary scenario that explains the origin of the entire cluster will depend on analysis of other members of the α-tubulin gene family of N. coriceps.

It is intriguing to speculate that other α-tubulin genes may be linked to the aa-ac cluster, upstream of ac and/or downstream of ab, in orientations that create additional divergent transcription units. We plan to evaluate these possibilities by analysis of genomic clones that overlap S2 and by PCR-based linkage studies.

Adaptive Expansion of Tubulin Gene Templates—Based on the divergence rate (0.12–0.33%/million years) recently determined for the nonfunctional nuclear globin gene remnants of Antarctic icefishes (43), we estimate that the N. coriceps α-tubulin gene cluster arose ~7–31 mya. Thus, duplication and divergence of members of the α-tubulin gene family apparently occurred in concert with, and probably was an adaptive change selected by, cooling of the Southern Ocean, which began ~38 mya and reached freezing temperatures during the mid-late...
Miocene (5–14 mya) (79). This conclusion must be qualified by recognition that gene conversion events within the α-tubulin cluster may have reduced the sequence heterogeneity of the individual genes (80), which would lead to underestimation of the true divergence time. However, it is noteworthy that the antifreeze glycoprotein genes of notothenioid fishes apparently evolved from a pre-existing pancreatic trypsinogen gene in a time frame similar to that which we have estimated for α-tubulin gene divergence (74).

We interpret the duplication of the α-tubulin genes as a molecular adaptation that amplifies the genomic templates for transcription of tubulin mRNAs, thus ensuring that RNA polymerase II, whose activity is likely depressed at the low body temperatures of Antarctic fish, can generate transcripts in quantities sufficient to support synthesis of α-tubulin polypeptides. To test this hypothesis rigorously, it will be necessary to examine the status of α-tubulin genes in a temperate notothenioid fish, such as the congeneric New Zealand black cod N. angustata. The black cod appears to have diverged from cold-adapted notothenioids and re-evolved a temperate phenotype as the waters around New Zealand warmed with the retreat of the Antarctic convergence; 5 mya (81, 82). With the relaxation of positive selection pressure for maintenance of a large α-tubulin gene family, it is likely that N. angustata has lost some of these genes (80), a prediction consistent with our preliminary observation that the black cod lacks an α-tubulin gene cluster comparable to that of N. coriceps. Further comparisons of α-tubulin gene numbers and organization in cold-adapted and...
temperate notothenioid is clearly warranted.

Gene Regulatory Elements—The widespread expression of the ac gene and the neurally restricted expression of the aa/ab pair indicate that the two groups have evolved distinct cis-acting regulatory elements. However, our inability to delineate clear examples of upstream basal (e.g. TATA boxes) and tissue-specific promoter elements, particularly for the a/ab gene pair, is perplexing. One possible interpretation is that the gene regulatory elements of Antarctic fishes may differ from those of higher vertebrates. We regard this possibility as unlikely because other notothenioid genes, such as the adult higher vertebrates. We regard this possibility as unlikely because other notothenioid genes, such as the adult a-globin and myoglobin genes of N. coriceps (43, 83), possess suites of consensal basal and tissue-specific promoter and enhancer motifs. Rather, we suggest that some promoter and enhancer elements will be found in the first introns of the three a-tubulin genes. For example, the first intron of aa contains two inverse TATA boxes that may control transcriptional initiation from the ab gene, and intron 1 of ab contains a near-perfect inverse TATA motif that may regulate aa expression. We have also tentatively identified neural- and maternal-specific enhancers (CAAAAT and CAAAAAT, respectively) in the first introns. Originally defined for the Drosophila b-tubulin gene (70), these sequences may function as specific regulators of tubulin expression throughout the metazoa. To test this proposal, we plan to determine the regulatory elements of the aa/ab intergenic region and its associated introns 1 by deletion analysis using a luciferase-expressing reporter vector and PC-12 cells to provide a neural microenvironment.

Biased Nucleotide Compositions of Noncoding Regions—We have reported previously that the introns and UTRs of a-globin genes from N. coriceps and other cold-living and temperate vertebrate ectotherms are markedly rich in A+T-rich residues, whereas the corresponding regions of a-globin genes from warm-bodied vertebrates are A+T poor (43). Similar results have been reported for the myoglobin genes of Antarctic icefishes (83). The introns and UTRs of the three N. coriceps a-tubulin genes described here share the A+T-rich intron/UTR profile of the cold-living ectotherms. The preference for AT base pairing may promote DNA replication and transcription at cold temperatures by facilitating strand separation, thereby enhancing access of DNA and RNA polymerases to their templates (43, 83). This advantage may be particularly important at the extreme and chronically low temperatures experienced by Antarctic fishes.

Repetitive Elements in the Notothenioid Genome—We have identified within the a-tubulin gene cluster a 285-bp DNA sequence, Noto1, that is strikingly similar to part of intron 4 of the notothenioid trypsinogen gene (74). Southern analysis indicates that Noto1 is a repetitive element unique to notothenioid fishes and characterized by partially structured genomic dispersal. Although its nature remains undefined, this repetitive element shares no sequence similarity with the pericentric/telomeric satellite DNA element p1F (84) of notothenioid fishes. Thus, Noto1 constitutes a second DNA repeat that may prove useful in establishing the phylogenetic relationships of the notothenioid suborder.

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Evolution, Organization, and Expression of α-Tubulin Genes in the Antarctic Fish Notothenia coriiceps: ADAPTIVE EXPANSION OF A GENE FAMILY BY RECENT GENE DUPLICATION, INVERSION, AND DIVERGENCE

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