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A minor globin gene of the bivalve mollusc *Anadara trapezia*

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A minor haemoglobin gene was isolated from an *Anadara trapezia* genomic library using a synthetic oligonucleotide probe based on the identical amino acid sequence of the F-helical region of all the major *Anadara* globins previously sequenced. The amino acid sequence inferred from the coding region of the gene indicated that it is different from that of the three major chains α, β and γ, but most like the β-chain. This β-variant sequence shows 100% homology in the conserved F-helix region. The minor gene was found to contain two long intervening sequences, 1214 bp and 1435 bp, longer than those present in the genes for vertebrate globins or leghaemoglobins but shorter than those in myoglobin genes.

Introduction

Blood clams such as *Anadara trapezia* [1], *A. broughtonii* [2], *A. senilis* [3] and *Scapharca inaequivalvis* [4] contain two types of haemoglobins that bind oxygen co-operatively. These are formed from three distinct polypeptide chains. The major haemoglobin is formed from two of the polypeptide chains (designated α and β) which associate to form heterotetramers of the $\alpha_2\beta_2$ type. The minor form is made of a third type of chain that associates with itself to form a homodimer. This chain in *A. trapezia* is designated γ_2^A and γ_2^B since it exists in two forms the expression of which is temperature dependent, with expression following a latitudinal cline along the east coast of Australia [5]. Haemoglobin γ_2^A is dominant (called HbIIa by Nicol and O'Gower [1]) in warmer waters.

The amino acid sequences are known for the α [6], β [7], γ^B [8] and γ^A [9] chains of *A. trapezia*, and for the γ chain of *A. broughtonii* [10] and *S. inaequivalvis* [11]. Comparisons of the amino acid sequences show that each chain exhibits approx. 53% amino acid homology with each of the remaining chains. However, there is 100% sequence identity between each of the three chains in the F-helix, while there is 75–100% identity in the E-helix.

These clam haemoglobins have the normal myoglobin fold with helical regions A to H. However, each of the α, β and γ chains has additional amino acid residues as an amino terminal extension compared with mammalian globin chains. This is consistent with the presence of an additional pre-A helix identified as a feature of the tertiary structure of *S. inaequivalvis* [12] that is associated with a different quaternary structure for the tetramer compared with vertebrate haemoglobins. In this clam haemoglobin the monomers assemble to dimers by contacts between the E and F helices whereas in vertebrate haemoglobins contacts involve predominantly the G and H helices. This finding led Royer et al. [12] to describe the tetramer as being assembled 'back to front' relative to vertebrate haemoglobins.

The genes of vertebrate globins are characterized by three exons separated by two introns whose positions are strictly conserved [13]. Plant globin genes have an additional intron that splits the second exon [14–16]. The gene structure for invertebrate globins is not resolved and there is a possibility that some invertebrate globin genes may also have a third intron. Surprisingly, globin genes of the insect *Chironomus thummi thummi*

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contain no introns [17], while the genes for a globin from the earthworm *Lumbricus terrestris* possess the two intron three exon gene organization of vertebrates although they contain much longer introns [18]. cDNA from the clam *Barbatia reeveana* [19] has been cloned and sequenced but the gene structure has not been reported.

In the work reported here we present the sequence of 3775 bases of a gene that encodes a globin chain of *A. trapezia* that closely resembles the β-chain in amino acid sequence. It appears to be a variant β-chain that may be expressed as a minor haemoglobin.

Experimental procedures

Preparation of Anadara DNA

Packed red blood cells from *A. trapezia*, collected from the estuarine waters of Empire Bay on the central coast of New South Wales, were stored at −20°C until ready for use [20]. Packed red cells (3 ml) were lysed by the addition of 30 ml H_2O and centrifuged at 3000 × *g* for 15 min at 4°C. The supernatant was removed and the pellets washed twice with 40 ml 10 mM Tris-HCl/1 mM EDTA (TE) buffer (pH 8) containing 0.5 ml chloroform and once with 50 ml of lysis buffer (10 mM Tris-HCl/10 mM NaCl/10 mM EDTA (pH 8.0)). The cell pellets were resuspended in 10 ml lysis buffer and then 0.5 ml 10% sodium lauryl sulphate (SDS) and 0.5 mg proteinase K were added, mixed and incubated at 50°C for 2 h. The suspension was then extracted at 50°C with phenol saturated with TE buffer [21]. After centrifugation at room temperature the colour remained in the phenol layer. This extraction was repeated and followed by ethanol precipitation and rapid resuspension of the DNA in TE buffer. The DNA was dialysed against TE buffer.

Construction of a genomic library

Anadara DNA was subjected to partial *Sau*3A digestion and size fractionation on a 10–40% sucrose density gradient according to the method of Maniatis et al. [22]. Fractions containing DNA in the 10–20 kb range were dialysed against TE buffer and concentrated by butan-2-ol extraction before ethanol precipitation and resuspended to 0.5 μg/μl concentration. The DNA was treated with alkaline phosphatase to prevent self ligation.

EMBL 4 DNA (Promega) was digested with *Bam*HI and heated in 15 mM EDTA at 70°C for 10 min to inactivate the enzyme. The DNA was recovered by ethanol precipitation, digested with *Sal*I and, after complete digestion, EDTA again added to 15 mM concentration before phenol extraction and ethanol precipitation.

Ligation of the digested *Anadara* DNA to the prepared vector, packaging of the ligation product into bacteriophage, and transfection in Q359 host *Escherichia coli* cells followed standard procedures [22].

Probe construction

Oligonucleotide probes (Table I) used for library screening were derived from amino acid sequence data [6–8] using the methods of Lathe [23] to minimize the redundancy and synthesised using a Model 380A Automatic DNA synthesiser (Applied Biosystems). The base sequence of a degenerate probe expected to detect all globin genes was based on the conserved amino acid sequence of the F-helical region of all *Anadara* globin chains.

TABLE I

Oligonucleotide probe sequences

Peptide sequences were used to derive the nucleotide base sequence. Degeneracy was minimized by utilisation of codon useage tables and the known infrequency of C-G base sequences [23]. Degenerate codons are shown with the alternative nucleotide below the third position. Peptides are shown N-terminus to C-terminus and probes are shown as the 3′ to 5′ complement.

F helical exon 2	C V V E K F A V N H I	
Probe 1	3′ ACGCAGCAGCTCTTCAAGCGGCAGTTGGTGTAG	5′
	A A A A A A	
	based on known gene fragment sequence	
Probe 2	3′ ACACAACATCTTTTTAAACGGCAGTTGGTATAG	5′
	I I P E M K E T L K A	
α-Chain exon 3	3′ TAGTAGGGGCTCTACTTCCTCTGGGAATTCCG	5′
	D M E G T G L M L M A N	
β-Chain exon 1	3′ CTATACCTCCCATGACCAGAATACGAATACCGATTG	5′
	G T T G G G	
	M G S Y F D E D T V	
β-Chain exon 3	3′ TACCCGAGGATAAAACTACTTCTATGTCA	5′
	TC C G A	
	D K K G D G M A L M T T	
γ-Chain exon 1	3′ CTATTCTTCCCACTACCATACCGAGAATACTGGTG	5′
	G T G T G G	

Screening of the genomic library and isolated clones

The EMBL4 library was propagated in *E. coli* Y1090 cells and screened in aqueous solutions essentially as described by Maniatis et al. [22]. Plaque dot assays were performed according to the method of Powell et al. [24].

Clone sequencing

Insert DNA from individual clones was subcloned into M13 bacteriophage [25]. Insert DNA was excised after separation on low melting agarose gel or by Geneclean (Bio 101) treatment of normal agarose gel fragments. Fragments too long for complete sequencing using the standard universal primer were reduced in size by *Bal*31 nuclease digestion, Klenow fill-in and reclon-ing into M13 bacteriophage [22]. The dideoxy chain termination sequencing method [26] was used in con-junction with [α^{35}-S]dATPαS using a modified T7 DNA polymerase [27] (United States Biochemical). Nucleo-tide sequences were analysed using programmes within the package GENEUS [28].

Results and Discussion

The genomic library originally containing $2 \cdot 10^5$ in-dependent clones was amplified once.

The base sequences of the probes used in this study are shown in Table I. Hybridisation with probe 1 (F helix exon 2, see Table I) detected 22 positive clones when screening was carried out at 43°C. Rescreening of these at 55°C detected only four clones. When these four clones were digested with the four base restriction enzyme *Alu*I, suitably small fragments were generated that were positive to the F-helix probe. These fragments were cloned into the *Hin*cII site of the vector M13 mp8 and sequenced. From the sequence data obtained, only one clone was found to represent a genuine globin gene. The other clones were false positives that had nucleo-tide sequences coding for five consecutive amino acids of the 11 residue F8 helix peptide sequence used to deduce the probe nucleotide sequence. Later, when the non-degenerate probe 2 (F-helix exon 2, Table I) was prepared it was found to hybridise at 55°C only with the globin clone.

The size of the clone containing the globin gene was calculated to be approx. 15.1 kb in length from the sizes of five *Eco*RI fragments. Restriction and hybridisation analysis using the probes shown in Table I were used to construct the restriction map shown in Fig. 1.

A series of fragments that bracketed the exon regions of the gene were sequenced as shown in Fig. 1. The sequence determined was 3775 bp in length and is shown in Fig. 2.

An initiation codon is present at nucleotide position 447 preceding exon 1 which codes for an amino acid sequence homologous to the N-terminal pre-A helix sequence of the β-globin chain [7]. There are the normal conserved dinucleotides GT and AG acting as splice site signals [29] commencing at nucleotide positions 572 and 1784; 2006 and 3439, giving exons 1, 2 and 3 with the splice sites at positions that have been retained in globin chains derived from a common ancestor. This results in exons coding for 41, 73 and 37 amino acids, respectively.

The intron sizes are 1214 and 1435 nucleotides bp, respectively.

There is a termination codon at position 3552 and a polyadenylation signal commencing at position 3731.

The amino acid sequence of the protein coded for by the three exons is shown in Fig. 2. There are 35 varia-tions in amino acid sequence between the β-chain previ-ously sequenced [7] and the predicted amino acid se-quence based on the nucleotide sequence presented here. The changed amino acids (underlined in Fig. 2) are spread throughout the chain and there is greater homology between the predicted amino acid sequence and β-chain (77%) than between α (52%) or γ-chain (54%).

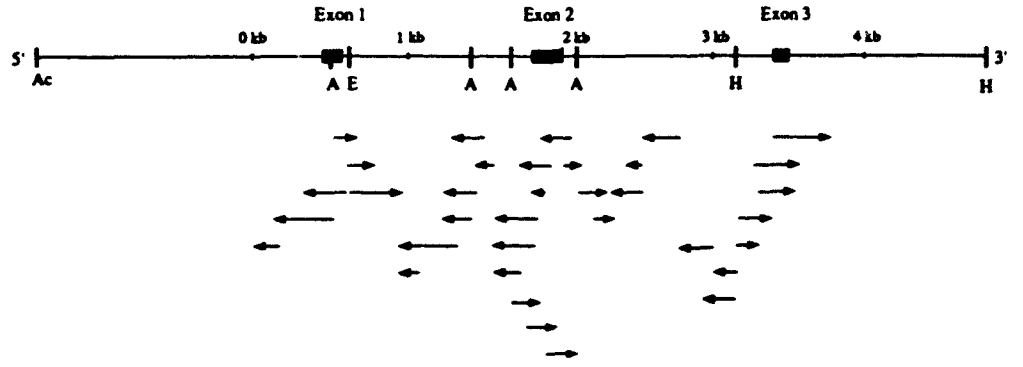

Fig. 1. Restriction map and sequencing strategy for a minor globin gene of *Anadara trapezia*. Arrows beneath the map indicate those regions that were sequenced and the direction in which they were sequenced. Sites for the following restriction enzymes are shown: A, *Alu*I; Ac, *Acc*I; E, *Eco*RI; H, *Hin*dIII.

One surprising feature in this investigation was the detection of a globin gene that had not been detected as an expressed protein chain in the haemoglobins of *A. trapezia*. The non-detection of the α, β and γ chain genes could be due to inadequate representation in the gene library or to the inadequacy of degenerate nucleo-

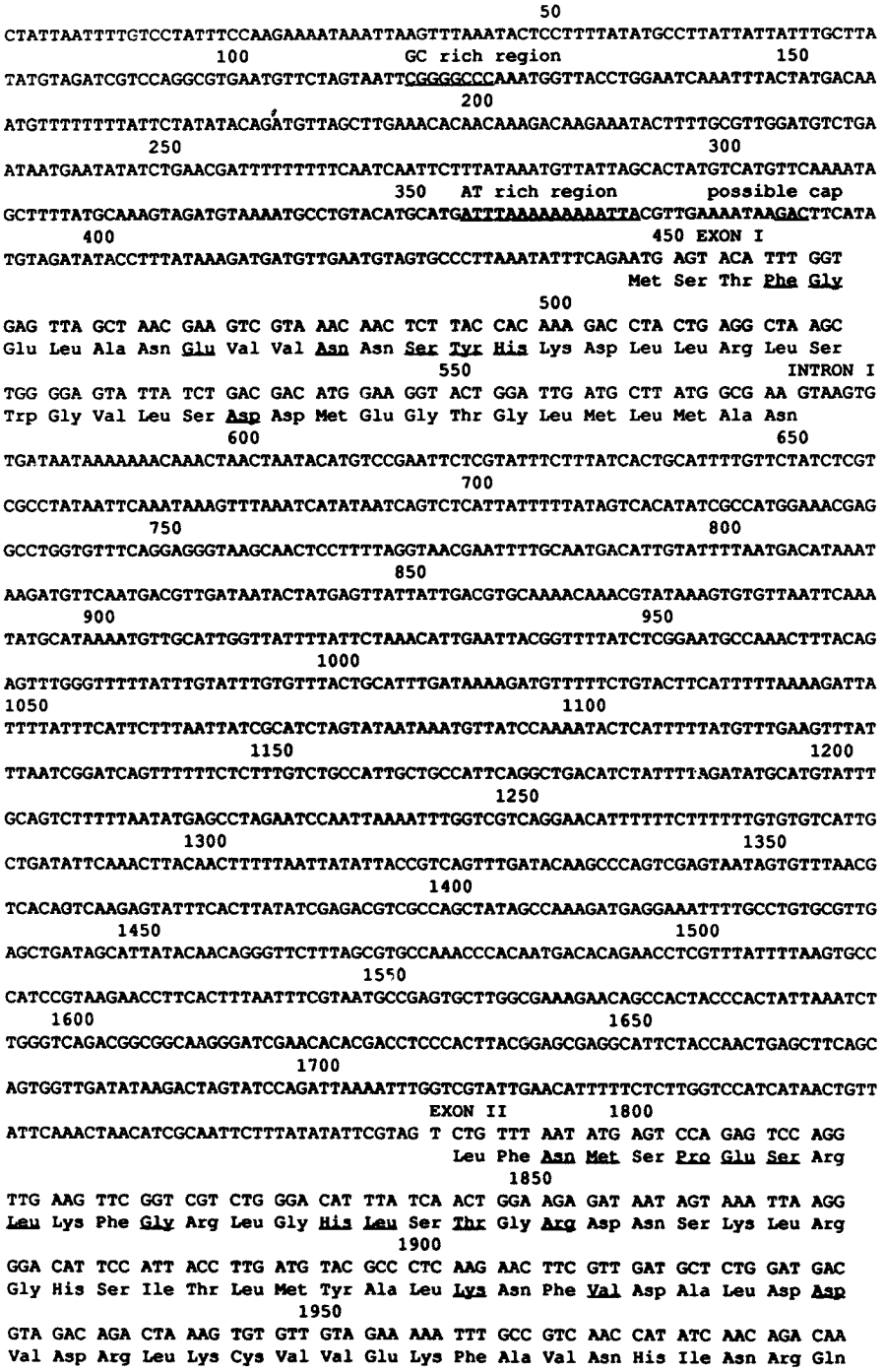

Fig. 2. Nucleotide and deduced amino acid sequence of *Anadara trapezia* minor globin gene. The two intron, three exon structure is shown and the underlined amino acids indicate variations with the known β-chain sequence [7]. The polyadenylation signal sequence as well as the possible upstream sites for capping, AT- and GC-rich regions are indicated by underlining.

```
                    2000        INTRON II
B ATC TCT GCT GAA GAA TTT GGG GTAAGTGATATTGCAACTACATTAAAATATAATAAAAACTACTTTAATTA
  Ile Ser Ala Glu Glu Phe Gly
                                                2100
TATCCTCTTTTATTTCAATTTCACACGTGTCAAGCGTAGTTAGCTAGGCTTAATTGCCTACTCTTCTTAAGCACCGAC
               2150                                                        2200
GTCTTTTGCCGTTTTGTAGATTGTGAATTGAATATTGACCTGTATGGTAGTGTTTAATTTCATTGAATATTGACCTGT
                                          2250
ATGGTAGTGTTTAATTTCATTGAATATTGACCTGTATGGTAGTGTTTAATTTCATTGAATATCAAAATAACAAACGAT
       2300                                                        2350
GGCTGGTTTAGGGCATGAACAGAAGTGATTTAGCTCGTTTAATGACGTAGTTTATCGTTAAAATGAGACACTTTTCGT
                                    2400
TATTATTAGAAACTATCTTCTTATAACGACCTAGTATTACGAAATTAATTCTTGTTGTAACGAGATAGTTTCTTGTTA
 2450                                                        2500
TAATGAAAAAGGGTCTAGTTTTTACGGCGTATGATTTCGTTATAACGATATAAATAAAAACATTCAAGTCCTTTACCA
                              2550                                                  2600
GCCACCAAACAAACCATATTTATTGGGTTTTTTTTTACTTTCTGATGAATGACTTCCATAAAAACTCTGTTTATATTA
                                                2650
ATTACTCTACCGAGTTCTAAGCACAAATAGTTTATACATGTATATTCGAAGGAAGTACAACTTATATTCCATTTTATT
                    2700                                                        2750
AGCTCAACTGGCTATAGCCAGCTTAGCTTATGGCATAGTGAAGCGTGCGTTCGTGCATCTGTCTGTGCGTTATCTTTG
                                          2800
ATCTTCTTCTCAGAAACCACTGAGCCAATTGAAATCAAATTTGGCGTGAAAAGTCCTTAGGTGGAGTAGATCAAAGTT
          2850                                                        2900
CGTGTATGGCAACCTTGTATGAAATCCAAAATGACTGCCGTAACTATAAATAGCAATATCACTAAATGGACATTATGC
                                    2950
ATTAAATTCGTCACCCATTCTCTTCATTTTTAAAACAGAGAAAGTATATAGCTAGCTATTTTTGATATGTAACGGGTT
   3000                                                        3050
TTTCCGACAATTTTTTCATTGACTAATTTTTGCTCATTTATGCACATGAGGTCTGATTTTTGACCAAATCTTTAAAAA
                              3100
TTTCTTCTCAGAAACCACAATGCCAACTGCAATCAAATTTGGTGTGGAAGGTCCCTAGGTGGAGAAAATCAAACAAGC
                                                3200
TTGTGCAAGGTTATCATGTATGAAATCAAAGATGACCGCTGTTACTATAAATAGCAAAATCACTAAAATGGCCCTTGT
                        3250                                                    3300
GTATTTATTTTTTCACCGATTTTCTCCATATTTGAAACTTATAAAGTAAAAATCCTAGCCATTTAATATATGCGAGTT
                                          3350
TTCCGAGAAATTTTTCTTTGACCTTATTTTAGCAAATGAGTTCAAATTATGCCAGTTGAGCATTACAGGCCTTTTGTT
                    3400                                                EXON III
TATATTTTAGCTGGATTCAGTGAGCGTATAGTAAATTCAAGAATCTTTATATTTTAG   AAA ATA GTT GGA
                                                            Lys Ile Val Gly
                                                          3500
CCA TTT AGA GCG GTA CTA AGG ATT AGA ATG GGA GAC TAT TTC GAT GAA GAA ATT GTT
Pro Phe Arg Ala Val Leu Arg Ile Arg Met Gly Asp Tyr Phe Asp Glu Glu Ile Val
                                                3550
GCC GCA TGG GCT GCA CTC ATT GCT GTT GTT CAA GCT GCA TTA TGA CTTGCTAGATTATATAAG
Ala Ala Trp Ala Ala Leu Ile Ala Val Val Gln Ala Ala Leu Stop
                              3600                                                  3650
TAAAAATTGAAGTATGACTGAACTAGCACTCAGTGTTTTATGCATTAAACATTTTGGTATAATAAATGATATCCTGAT
                                                3700
GTTTAATATTGATAGTAGTTCATTATGTAATGATCTGTTAAAACTTCTTGATTAACTTGTCTATATTCTTGCAAAATC
polyadenylation   3750
AAAATAAAACCAGTTTGTAATTAGCTATGCAGTGTTGTTTGTTTCAA
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Fig. 2. (continued).

tide probes of limited size. More recent work using longer probes prepared from mRNA by the polymerase chain reaction has resulted in detection of α and β-globin clones in a similar library, so it may be that the limited size and mismatches are mainly responsible for their inadequacy as probes.

The false positive clones that were detected using the F helix exon 2 probes were of two types. The false positives detected with probe 1 had a binding region of 24 nucleotides with two to four mismatches, compared with four mismatches, as subsequently became apparent, between the probe and the variant globin gene detected.

When a non-degenerate F helix exon 2 probe (probe 2) was used to screen the library, five false positive clones with repeating sequences of several different types showed a positive response. Short sequences of 7 to 13 consecutive bases (with none, one or two mis-

matches) that repeated 5 to 8-times in about 300 bases of sequence were apparently responsible for the probe binding.

These false positive clones hybridising to the non-degenerate exon 2 probe at 55°C did not hydridize when the temperature was raised to 70°C, only the variant β-globin remained bound.

These false positive clones did not hybridise to the α, β and γ degenerate probes that were based on amino acid sequences in other regions of those chains separate from the F-helical sequence.

It is possible that the gene coding for the variant β-chain is expressed as a minor haemoglobin, which would, by analogy, be expected to be of the $\alpha_2\beta_2$ type. There have been reports by various workers of minor haemoglobins [1,3,30,31], but they appeared more likely to be the result of changes after a short period of ageing of haemolysates. None have been characterised by amino acid sequence.

The nucleotide sequence obtained for the variant β-globin gene of *A. trapezia* does not have obvious barriers to expression. There are no termination codons or deletions present within the coding sequences, initiation and termination codons are present, the polyadenylation signal is present where expected and a possible cap site for the mRNA is present at position 383 which is 64 nucleotides from the initation codon. The consensus sequences for intron/exon boundaries are present and the sizes of the exons conform to those previously reported for globins. In the 5′ promoter region the putative control upstream sequences can be suggested although they do not exactly conform to the consensus sequences. However, there is enough variation in these sequences between different species to suggest that the sequence in this region is not critical for expression.

Thus, there is an 'AT' rich region 14–29 bp upstream from the possible cap site and this region is preceded by CAAT box regions a further 50–80 nucleotides further upstream. There is also a GC rich region CGGGCCC, normally found 80 to 100 nucleotides from the cap site, which in this gene is approx. 250 bp upstream. Whether

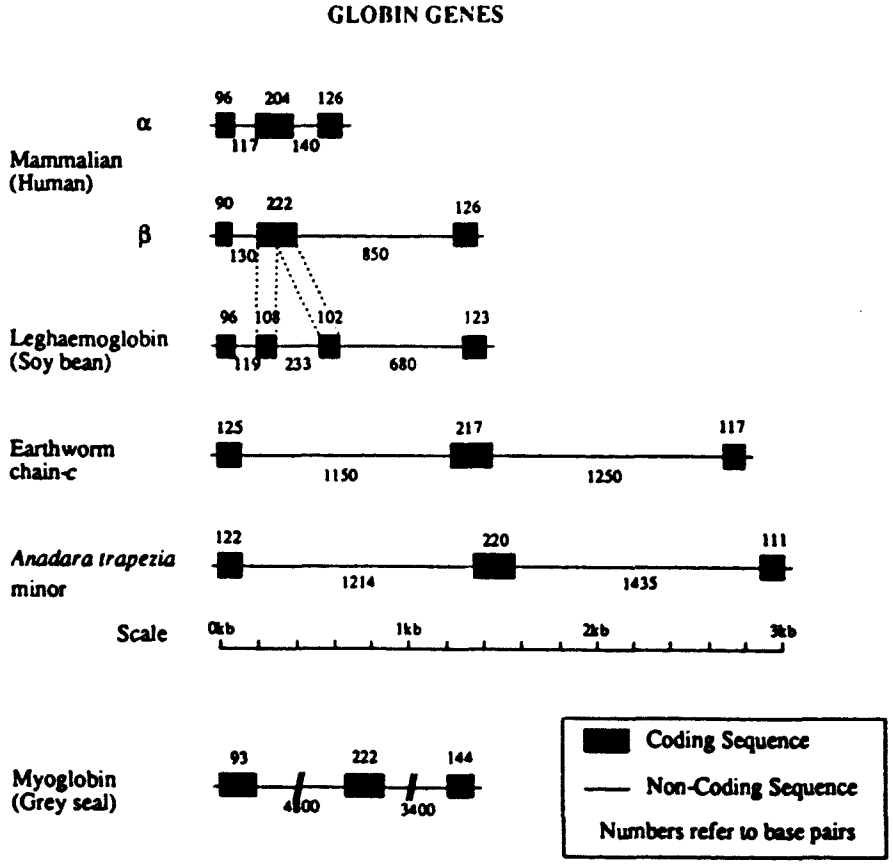

Fig. 3. The structure of *Anadara trapezia* minor globin gene compared with the globin genes of other species. The diagram indicates the relative sizes of the coding and non-coding sequences in the globin genes from a number of different species, including vertebrate [13,34], invertebrate [18] and plant [14] species.

the displacement of this GC rich region by approx. 150 bases is significant and could result in non-expression as a pseudogene is not supported by the variation in the type and placement of a CCAAT and ATA box in human θ globin gene [32] where there is displacement by insertion of a 200 bp GC rich sequence, yet the gene is transcribed in human fetal erythroid tissue.

In the sequence 22 bp 3′ to the polyadenylation signal there is a CA polyadenylation addition site followed by a run of TGT and T residues that is characteristic of many genes whose RNA's are polyadenylated [33].

Few genes for invertebrate globins have been characterised in terms of their intron sizes. For the insect *Chironomus thummi thummi* there are no introns [17]; whereas the gene for the globin chain C of the earthworm *Lumbricus terrestris* [18] shows the two intron, three exon globin gene organisation characteristic of vertebrate haemoglobins and myoglobins. The intron sizes for *L. terrestris* were 1150 and 1250 for introns 1 and 2, respectively. Thus, the variant β-chain gene for *A. trapezia* conforms to this pattern with introns of the same order, 1214 and 1435 bp, respectively; which are considerably larger than the introns of vertebrate haemoglobin chain genes or leghaemoglobin genes (Fig. 3), though not as large as for myoglobin genes [34,35].

It does not follow that the genes for the major α, β and γ chains of *A. trapezia* will closely follow the sizes of introns found in the variant β gene, although it seems likely that they will conform to the two intron, three exon pattern.

Whether any of the lower invertebrates represented from phyla such as nematodes, flatworms and protozoa have globin genes with a different exon-intron pattern remains to be investigated.

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