Analysis of fish *ZP1/ZPB* homologous genes – evidence for both genome duplication and species-specific amplification models of evolution

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The vertebrate egg envelope is composed of a family of related proteins, the zona pellucida (ZP) proteins, which are characterized by the presence of a conserved zona pellucida domain. Analysis of teleost fish *ZP* gene sequences has demonstrated that there are no direct orthologues of the mammalian *ZPB* and *ZP1* genes, but that teleost fish contain multiple copies of two classes of genes (*ZPXa* and *ZPXb*) that are equally related to *ZPB* and *ZP1*. The two classes of genes are further distinguished by expression in

liver or ovary, respectively, indicating there was probably an initial duplication event, followed by a switch to hepatic expression of one of the paralogues. This switch was followed in some species by additional amplification of one of the paralogues with the subsequent loss of the other. It is proposed that the expansion of the number of *ZPXa* and *ZPXb* genes and the acquisition of dual sites of synthesis are the result of an ancient polyploidization event, followed by additional species-specific gene amplifications.

Introduction

The generation of complexity by the process of gene duplication is widely accepted to occur (Ohno, 1970) and is believed to underlie the phenotypic diversity of vertebrates. Particular support for this hypothesis has been provided by analysis of the *hox* gene clusters. The presence of seven *hox* gene clusters in zebrafish has led to the suggestion that an additional genome duplication occurred in ray-finned fish (Actinopterygii) compared with other vertebrates (Amores *et al.*, 1998). Recently, doubts have been raised about this model and the additional gene copies in teleost fish have been ascribed to frequent independent (species-specific) duplications (Robinson-Rechavi *et al.*, 2001). Comparison of gene sequences across closely related species has demonstrated greater divergence in genes that are involved in sexual reproduction compared with those that are expressed in other tissues (Swanson and Vacquier, 2002), and vertebrate egg envelope genes in particular seem to be under positive Darwinian selection (Makalowski and Boguski, 1998; Swanson *et al.*, 2001; Swanson and Vacquier, 2002).

Egg envelope genes provide an opportunity to assess models for evolution, as they are conserved throughout

vertebrates. The zona pellucida proteins, named for the mammalian zona pellucida, share a common ZP domain, a large motif of approximately 260 residues with eight conserved cysteines. ZP domains are also found in a number of extracellular matrix proteins and are predicted to be involved in protein–protein interactions (Bork and Sander, 1992; Jovine *et al.*, 2002). The prevailing view is that the mammalian zona pellucida is composed of three glycoproteins encoded by three genes *ZP1*, *ZP2* and *ZP3* (McLeskey *et al.*, 1998), also known as *ZPB*, *ZPA* and *ZPC*, respectively (Harris *et al.*, 1994). However, *ZP1* and *ZPB* are not orthologous, and the human genome actually encodes four *ZP* genes: *ZPB*, *ZP1*, *ZP2* and *ZP3* (Hughes and Barratt, 1999). This contention is supported by the identification of distinct *ZPB* and *ZP1* orthologues in chickens (Bausek *et al.*, 2000). Additional *ZP* genes have also been identified in amphibians (Lindsay *et al.*, 2001, 2002). *ZPB/ZP1*-related genes have been isolated from various fish species, but the use of varying, and in some cases incorrect, nomenclature has prevented easy categorization of these genes. In the present study, a phylogenetic analysis was performed on the available teleost fish *ZPB/ZP1*-related sequences. This analysis has revealed that the evolution of the *ZP1/ZPB* genes is more complicated than was previously realized and raises interesting questions about the composition, synthesis and assembly of the egg envelope in fish.

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Table 1. Currently available fish ZPX protein sequences obtained from NCBI protein database

Species	Annotation in database	Protein accession number	Site of expression	Predicted size (amino acids)	Reference
Carp (<i>Cyprinus carpio</i>)	ZP2	CAA96575	Ovary	632	Chang <i>et al.</i> , 1997
	ZP2	CAA96574	Ovary	269	Chang <i>et al.</i> , 1997
	ZP2	CAA96573	Ovary	555	Chang <i>et al.</i> , 1997
	ZP2	CAA96572	Ovary	544	Chang <i>et al.</i> , 1997
Goldfish (<i>Carassius auratus</i>)	ZP2	CAA96576	Ovary	580	Chang <i>et al.</i> , 1997
Japanese medaka (<i>Oryzias latipes</i>)	ZPB-domain containing protein	AAD38905	Ovary	401	Kanamori, 2000
	Choriogenin H	BAA13994	Liver	591	Murata <i>et al.</i> , 1997
	Choriogenin H minor	BAA76901	Liver	634	Sugiyama <i>et al.</i> , 1998
Flounder (<i>Pseudopleuronectes americanus</i>)	ZP protein	AC59642	Liver	509	Lyons <i>et al.</i> , 1993
Salmon (<i>Salmo salar</i>)	Eggshell protein	CAA04221	Liver	467	Oppen-Berntsen <i>et al.</i> , 1990
	Eggshell protein	CAA04220	Liver	439	Oppen-Berntsen <i>et al.</i> , 1990
Trout (<i>Oncorhynchus mykiss</i>)	Vitelline envelope protein α	AAF71258	Liver	563	Hyllner <i>et al.</i> , 2001
	Vitelline envelope protein β	AAF71259	Liver	524	Hyllner <i>et al.</i> , 2001
	Zona radiata structural protein	AAK97529	Liver	403	Arukwe <i>et al.</i> , 2001
Zebrafish (<i>Danio rerio</i>)	ZP2 (variant A)	AAK16578	Ovary	430	Mold <i>et al.</i> , 2001
	ZP2 (variant B)	AAK16577	Ovary	431	Mold <i>et al.</i> , 2001
	ZP2 (variant C)	AF331967	Ovary	432	Mold <i>et al.</i> , 2001
	ZP2	AAK16580	Ovary	430	Mold <i>et al.</i> , 2001
	ZP2	AAD49112	Ovary	428	Wang and Gong, 1999
Japanese eel (<i>Anguilla japonica</i>)	eSRS3	BAA36592	Ovary	446	Miura <i>et al.</i> , 1998

Only complete sequences are shown.
ZP: zona pellucida.

Materials and Methods

Database analysis

Nucleotide and protein sequences showing similarity to ZP1/ZPB were identified using the BLAST search programme (<http://www.ncbi.nlm.nih.gov/blast/>) to search the non-redundant database. Human ZP1 (Hughes and Barratt, 1999) and human ZPB (NM_021186) sequences were used as query sequences.

Representative sequences from each fish species were used to search the dbEST database.

Alignment and phylogenetic analysis

Complete nucleotide and protein sequences were aligned using the program ClustalW (<http://www.ebi.ac.uk/clustalw/>) with default settings.

Phylogenetic trees were constructed using ClustalW (Higgins *et al.*, 1994) and PIE (<http://www.hgmp.mrc.ac.uk>).

Bootstrap analysis was constructed with 1000 replicates. Trees were plotted using Treeview (Page, 1996).

Results

Searches of the non-redundant nucleotide and protein databases identified a large number of sequences from fish showing homology to ZPB and ZP1 (Table 1), which were restricted to teleost fish. With the exception of the Japanese eel (*Anguilla japonica*), the fish belong to the Euteleostei subdivision. Partial sequences were not included in subsequent analyses. Initial investigations demonstrated that teleost fish do not have distinct ZP1 and ZPB proteins, but instead have ZP proteins that show almost equal homology to both the ZP1 and ZPB proteins of higher vertebrates. For example, the carp (*Cyprinus carpio*) protein (accession number CAA96575) shows 36% identity to human ZP1 and 40% identity to human

ZPB. The fish *ZPB/ZP1*-related genes will be referred to as *ZPX*. It is worth noting that a number of these genes had been incorrectly identified as *ZP2*-related, and therefore annotated as *ZP2* (carp: Chang *et al.*, 1997; zebrafish: Wang and Gong, 1999; Mold *et al.*, 2001), even when the authors had noted greater similarity to the mammalian *ZP1/ZPB* genes (Mold *et al.*, 2001). No orthologues of the mammalian *ZP2* genes have been identified in fish to date.

In humans and other higher vertebrates, the *ZP* genes exist as single copy loci in the genome; in contrast, it is clear that there are multiple copies of the *ZPX* gene in a number of fish species (Table 1). BLAST searches of the dbEST database indicate that carp have at least six copies, the Japanese medaka (*Oryzias latipes*) has at least seven copies and the zebrafish (*Danio rerio*) has at least ten *ZPX* genes. As the data set is incomplete, it is not possible to predict how many *ZPX* genes any one fish species might have.

The multiple copies of carp, Japanese medaka (*Oryzias latipes*), salmon (*Salmo salmar*) and trout (*Oncorhynchus mykiss*) *ZPX* sequences show high levels of sequence identity at the DNA level except in exon 1. These differences can be attributed to the presence of a repeat domain of variable length. This motif comprises most of exon 1 and is rich in proline, glutamine and lysine residues. The number of repeats within the motif varies between the different *ZPX* genes even within a species. As a result, exon 1 varies considerably in length (354–967 bp). Repeats within a protein show greater similarity than between different proteins, and the repeat sequences differ more markedly between orders than between species. This sequence divergence is indicative of expansion of these repeats after gene duplication. The repeat domain is not present in all the *ZPX* genes identified. Of the three Japanese medaka sequences, AF128808 does not contain the repeat, and none of the zebrafish sequences do.

The five zebrafish (*Danio rerio*) *ZPX* sequences show an unusual pattern of sequence homology. Identity levels are extremely high (approximately 99% at nucleotide level) in exons 1–6, but there is significant sequence variation in exons 7 and 8. The lack of neutral sequence divergence in the first six exons of the zebrafish *ZPX* genes is curious and may represent homogenization as a result of gene conversion (Li, 1997).

A dendrogram produced from alignments of *ZPX*, *ZP1* and *ZPB* protein sequences using a neighbour-joining algorithm is shown (Fig. 1). Trees produced using maximum likelihood analysis showed the same grouping of the sequences into distinct families (data not shown). Alignment of the corresponding DNA sequences gave analogous results (data not shown). The tree clearly shows the duplication and divergence of the *ZP1* and *ZPB* genes from a common ancestor. The segregation of the teleost *ZPX* genes is reinforced by the observation that one group (*ZPXa*) is expressed in the ovary and the other

(*ZPXb*) is expressed in the liver. Although representatives of both classes have been isolated from medaka, in many species only *ZPXa* (for example, zebrafish) or *ZPXb* (for example, salmon) homologues have been identified. A search of dbEST supported these findings, indicating that there is unlikely to be an ascertainment bias. It is worth noting that the only non-euteleost *ZPX* sequence available, from the Japanese eel, did not fall into either of the two groups and shows equal homology to both.

Discussion

Teleost fish sequences belonging to the *ZPB/ZP1* class of genes were analysed and several important observations were made. It is apparent that there are multiple copies of these genes in the fish species that have been analysed, thereby confirming previous observations (Mold *et al.*, 2001). These genes can be segregated into two groups (*ZPXa* and *ZPXb*) on the basis of sequence similarity, coincident with their site of expression (Table 1). The outlying position of the eel *ZPX* sequence is consistent with an ancient duplication after the divergence of the lineage leading to eels (Robinson-Rechavi *et al.*, 2001). Analyses of other gene families such as Na⁺/K⁺-ATPase subunits support this model (Cutler and Cramb, 2001; Serluca *et al.*, 2001). Teleost fish must have diverged from the vertebrate genealogy before the duplication that gave rise to separate *ZPB* and *ZP1* proteins. The divergence of *ZPB* and *ZP1* happened at some time before the separation of the Aves 150 million years ago.

After the ancient duplication in euteleosts, one of the duplicate copies appears to have substituted the ovary-specific expression common to most vertebrate egg envelope genes (McLeskey *et al.*, 1998; Hughes and Barratt, 1999) with expression in the liver, under the control of oestrogen. The E box which directs expression to the ovary (Liang *et al.*, 1997) was replaced with oestrogen response elements (Lyons *et al.*, 1993). Independently, *ZP3* is also expressed in the liver of a number of teleost fish species (Murata *et al.*, 1995; Del Giacco *et al.*, 1998; Hyllner *et al.*, 2001). Subsequently available sequence data indicate that different mechanisms may be operating in distinct euteleost species. Maintenance of copies of both genes ensured expression of *ZPX* in both liver and ovary (medaka), whereas loss of one of the genes led to a restriction of *ZPX* expression to either the liver (trout, salmon, winter flounder) or the oocyte (zebrafish, carp). There have also been recent gene amplifications within common ancestors of the fish species analysed, at least in part by tandem duplication (Mold *et al.*, 2001).

Early models of gene duplication assumed that duplicated alleles are either quickly lost or that one of the copies rapidly acquires new, divergent function. These models have been superseded by the duplication–degeneration–complementation (DDC) model, which predicts that after gene duplication, each copy acquires

Fig. 1. Dendrogram based on ClustalW multiple sequence alignment of ZPX, ZPB and ZP1 protein sequences. Accession numbers in the NCBI database are given for each sequence. Bootstrap values expressed as percentages of 1000 replicates are given at each node. Families of zona pellucida (ZP) genes are labelled (ZPXa, ZPXb, ZP1 and ZPB). ZPXa proteins are expressed in the ovary, whereas ZPXb proteins are expressed in the liver.

mutations in its regulatory region resulting in expression of different members of the gene family in different tissues or at different stages of development. It is not clear what the advantage of expression of envelope genes in the liver might be, given that there is no evidence for any other functions of these proteins. There do not appear to be any obvious structural differences in the egg envelopes of different species of teleost fish (Dumont and Brummett, 1985). Multiple copies of a gene enable the production of larger amounts of protein, which is commonly seen in tumour progression or pesticide resistance in individual species (Guillemaud *et al.*, 1996). There is certainly a requirement for large amounts of egg envelope protein. Fish oocytes are relatively large and many species spawn frequently during the reproductive season, each time producing large numbers of eggs; for example, a mature female medaka can lay up to 20 eggs every day for up

to 2 months with the inner layer of each egg constituting approximately 25 μ g protein (Yamagami *et al.*, 1992). Gene amplification offers a simple way of responding to these physiological demands. An alternative possibility is that the availability of multiple copies of the ZPX genes allows for the generation of additional variation, which may increase the structural complexity and diversity of the egg coat. This is proposed to underlie the duplication of the carp fibroin-like substance and spider silk-forming protein genes (Guerette *et al.*, 1996; Chang and Huang, 2002). Like the ZPX genes, these genes also show variation in the number of copies of a repeat domain. In addition, the truncation of at least one of the carp ZPX sequences with a premature stop codon (Chang *et al.*, 1997) indicates that ZPX genes are subject to a number of evolutionary processes. The acquisition of stop codons could provide additional functionality or may

be repaired by gene conversion events; a Flounder ZP3 cDNA sequence in GenBank also contains a premature stop codon (AJ310719).

Evidence for interactions between the egg envelope and spermatozoa in fish is sparse and it seems likely that in fish the role of the zona pellucida proteins is purely structural. Teleost spermatozoa lack the acrosomal cap present in higher vertebrates, whereas the egg envelopes have an additional structure, the micropyle, which shows species-specific variation. As spermatozoa can enter the egg only through the micropyle, any sperm-binding molecules in the egg envelope would be expected to be found clustered around this opening. There is no suggestion of clustering of the zona pellucida proteins in fish egg envelopes around the micropyle. In the Japanese medaka, ZPX and ZP3 proteins are evenly distributed throughout the outer mucosal layer (Iwamatsu *et al.*, 1997).

There is an additional important consequence of our observations about the teleost fish ZPX genes. Fish egg envelope proteins have been assessed as potential biomarkers for exposure to environmental oestrogens, as an alternative to vitellogenin. In several studies to date, expression of the egg envelope proteins has been detected at lower concentrations of oestradiol than vitellogenin, and expression has been induced more rapidly than vitellogenin in a number of fish species including salmon (Celius and Walther, 1998a), trout (Celius *et al.*, 2000) and arctic char (Westerlund *et al.*, 2001). Similar results have been obtained with the xeno-oestrogen α -zearalenol (Celius and Walther, 1998b). The pesticide *o,p'*-DDT (1,1,1-trichloro-2-[2-chlorophenyl]-2-[4-chlorophenyl]ethane) is also capable of inducing expression of egg envelope protein but not vitellogenin (Arukwe, 2001). It is worth noting that the effects of these agents are observed in not only female but also male fish, and that there are pathological consequences of exposure to oestrogen. Male fish, and female fish outside the reproductive cycle, accumulate fluid in the peritoneum containing significant amounts of the envelope proteins (Yamagami *et al.*, 1992), which may be as deleterious as the feminizing effects of exposure to xeno-oestrogens (Arukwe, 2001). We propose that the increased sensitivity of egg envelope genes as xeno-oestrogen biomarkers is not a result of increased activity of the regulatory regions, but instead reflects the increased copy number of these genes. In addition, it is likely that fish species that lack the liver-expressed paralogous group will not display this sensitivity to the effects of xeno-oestrogens.

Many of the difficulties in addressing the function of the vertebrate egg envelope genes have arisen from the use of confusing and inappropriate nomenclature (Hughes and Barratt, 1999), a situation which is magnified in the case of the fish egg envelope proteins. We suggest that any nomenclature should be dependent on the relationship between related genes and should

always be supported by multiple alignment analysis. A recent study by Spargo and Hope (2003) analysed a cross-section of zona pellucida proteins. Although these workers did not focus on the fish genes and, thus, did not examine all the fish ZPX gene sequences, their phylogenetic tree clearly supports our findings despite using different analyses. It should be noted that Spargo and Hope (2003) used the term 'ZPX' in a different context to that used in the present study. As more fish gene sequences accumulate, clarification of the processes leading to increased complexity will be elucidated. Our analysis of the teleost fish ZPX gene sequences indicates that after the divergence of the lineage leading to eels, genome duplication in a euteleost ancestor probably created two paralogous classes of ZPX genes expressed in the liver and ovary, respectively. Subsequently, in different fish lineages there was either retention of both classes, or loss of one of the two classes, which has been followed by significant expansion of the copy number of the remaining gene or genes in recent ancestors of the species described herein. Therefore, these data support both the ancient genome duplication and the recent gene duplication models (Amores *et al.*, 1998; Robinson-Rechavi *et al.*, 2001). Confirmation of this model awaits the identification of these genes from more fish species. Elucidation of the functional significance of the expansion and varying sites of expression of the ZPX genes will prove an interesting challenge.

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