Expression of Major histocompatibility complex genes in carp (*Cyprinus carpio* L.)

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Expression of Major histocompatibility complex genes in carp (*Cyprinus carpio* L.)

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Preface

This thesis is the result of four years of research accomplished at the department of Experimental Animal Morphology and Cell Biology (EDC). Many people have contributed in different ways to the success of this thesis, both from inside and outside the lab.

I would like to start to give my thanks to everybody in EDC which in one way or another have manage to cope with me and my "southern european" way ... bedankt allemaal ! I specially want to thank René for his trust and the things he taught me, and I hope that our lively constructive discussions will continue in "an office" somewhere ! I also want to thank Trudi for working with me sometimes with some unusual schedules...!!! I will keep great memories of our work together. Of course thanks to Egbert, Saskia and Marie-José. I also would like to thank the students that have worked with me; Laura, Jeroen and Ank and also the others that have made the lab more lively. From the "buitenlanders" working at EDC during this period I can just say that they were sometimes my lifeline. Very special thanks to Brian with whom I had very fruitful conversations and the luck of working together and we had a lot of fun in the lab ... especially with loud music ! Nuno, Dolores and Maria that made EDC a more Iberian place to be... ! From outside EDC I would like to thank all my friends in Wageningen that made my live enjoyable and with a lot of fun. I want to thank Luis who put up with my for more than three years as a housemate... I am sure that we will cook together again ... but I hope this time in somebody else kitchen !!! Thanks to Anna for all the support and the wonderful times that we spend together during this years...tak, now lets look to the future! In the end I HAVE to finish this preface thanking my parents and family for helping me through my live and giving me all the support and understanding that I needed even when that meant me living away from home the most of my adult live, OBRIGADO.

Stellingen

1 Temperature-dependent regulation of ß2m transcription and its implication on MHC class I cell surface expression could explain the occurrence of "winter kills" in aquaculture. *Dit proefschrift.*

2 If ontogeny reflects phylogeny, MHC class II genes evolved before the class I genes. *Dit proefschrift.*

3 "PCR hell" can be heaven in science.

4 Some things get done better after office hours ...!

5 The wide distribution of a PhD thesis reduces the individual scientific work to a mass product.

6 When so many hours have been spent convincing myself that I am right, is there not some reason to fear I may be wrong ?
- Jane Austen -

7 There is an obvious discrepancy between scientific achievements of researchers and their present status within the university.

8 Nowadays we do not die anymore from dangerous things, we live so long that we die from things generally considered harmless ... (comment made in the Zodiac cantine about the soup).

Stellingen behorend bij het proefschrift
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General Introduction.

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1. The Organization of the Teleost MHC

The mammalian major histocompatibility complex is a set of linked genes spanning 4 Mb which encode among others products involved in antigen presentation, notably the MHC class I and class II genes (Campbell *et al.*, 1993). The complex is also unique in the sense that it contains an unusually high density of transcribed genes. While the β_2-microglobulin gene is not located in the MHC of mammals, its gene product is non-covalently associated with MHC class I. Since the β_2-microglobulin gene may also have a common evolutionary origin with the other MHC genes, a discussion of its teleost representatives has been included in this introductory chapter. In mammals the MHC also contains several other genes such as those encoding complement factors and the 70 kiloDalton (kD) heat shock protein (HSP70). These genes, sometimes referred to as the class III genes, are located between two discrete regions containing the MHC class I and class II genes. This organization is different in the MHC of mice and rats, where one class I gene is located beyond the class II genes, which are separated from the remaining class I genes by the class III region as usual (Klein, 1986).

1.1. The Teleost MHC

The organization of the genes within the teleost MHC is unresolved at present. While experiments using mixed lymphocyte reactions have indicated the existence of multiple MHC loci in both carp and trout, as reviewed by Stet and Egberts (1991), the recent isolation of several teleost MHC genes now makes it possible to effectively establish segregation patterns of these different MHC genes. While there has been some discussion regarding the fact that in zebrafish (*Brachydanio rerio*) class II α and β gene pairs were found clustered on the same genomic clones, but that they segregate away from the β_2-microglobulin gene, nothing has yet been published in this regard (Kronenberg *et al.*, 1994). The HSP70 gene may be contained in one MHC cluster, while some of the other known MHC genes are in another, similar to the situations found in chickens and *Xenopus*. A complicating factor in this kind of analysis is that some of the currently known teleost MHC genes may in fact be pseudogenes or non-classical (see discussion below) in which case they might not be located near the classical MHC genes at all. More information regarding the functional status and expression patterns of these genes by means of serological detection of the gene products will be required in addition to data about the linkage groups, before a comprehensive map of the teleost MHC can be drawn.

Given that the avian, and perhaps the amphibian, MHC regions show little of the organization into discrete regions found in the mammalian MHC's, it is difficult to predict what we will

find in the teleost MHC. However, the fact that the heavy and light chain immunoglobulin genes are arranged in different fashions in cod (*Gadus morhua*) and rainbow trout (*Oncorhynchus mykiss*) (Daggfelt *et al.*, 1993), and in various manners in many other species (reviewed in Pohajdak *et al.*, 1993), indicate that some surprises may be found in the organization of the fish MHC. In the next few years the organization of the fish MHC will certainly be elucidated, and hopefully it will bring information that will aid us in understanding the evolutionary origins of the MHC and the genes within it.

1.3. Teleost MHC Gene Structure

Very little is known about the intron-exon organization of the teleost MHC and $ß_2$-microglobulin genes, as only a few full-length genomic sequences have been obtained to date. The investigation of the teleost MHC has so far mostly involved the isolation of cDNA clones or PCR fragments containing only partial genomic sequences. The gene structure of the $ß_2$-microglobulin gene is, however, better documented. The intron-exon structure of a gene can reveal a lot about its evolutionary origin by showing evidence of exon-shuffling or intron-loss. The evolutionary processes which produced MHC genes, and other genes of the Ig super family, from an ancient gene encoding possibly an immunoglobulin-like domain is still unknown. Perhaps the intron-exon structure of teleost MHC genes will provide some clues regarding this question. What little is known about the intron-exon structure of teleost MHC genes now follows, but it merely illustrates that they contain many interesting features and deserve further study, before firm statements can be made about their origin.

1.3.1. $ß_2$-Microglobulin

Mammalian $ß_2$-microglobulin (*B2m*) genes contain four exons and three introns (Klein, 1986), as shown in figure 1A. Exon 1 contains the 5' untranslated sequences, and encodes the leader sequence and two residues of the mature protein. Exon 2 encodes the bulk of the mature protein (93 out of the 99 amino acids (aa)), while exon 3 contains a small part of the coding sequence (4 aa) and part of the 3' untranslated sequence. The rest of the 3' untranslated sequence is encoded by exon 4 (Klein, 1986). In most mammalian genes the first intron is the longest, for example the first intron of the human B2m gene is 4.5 kb long (Glüssow *et al.*, 1987). One of the most important features to note when discussing introns is phase. The phase of an intron is the position within the codon where it interrupts the coding sequence. A phase 1 intron divides the codon between the first and second nucleotide, while a phase 2 intron divides the codon between the second and third nucleotide and a phase 0 intron divides exactly between codons. The introns of mammalian MHC genes are all first phase introns.

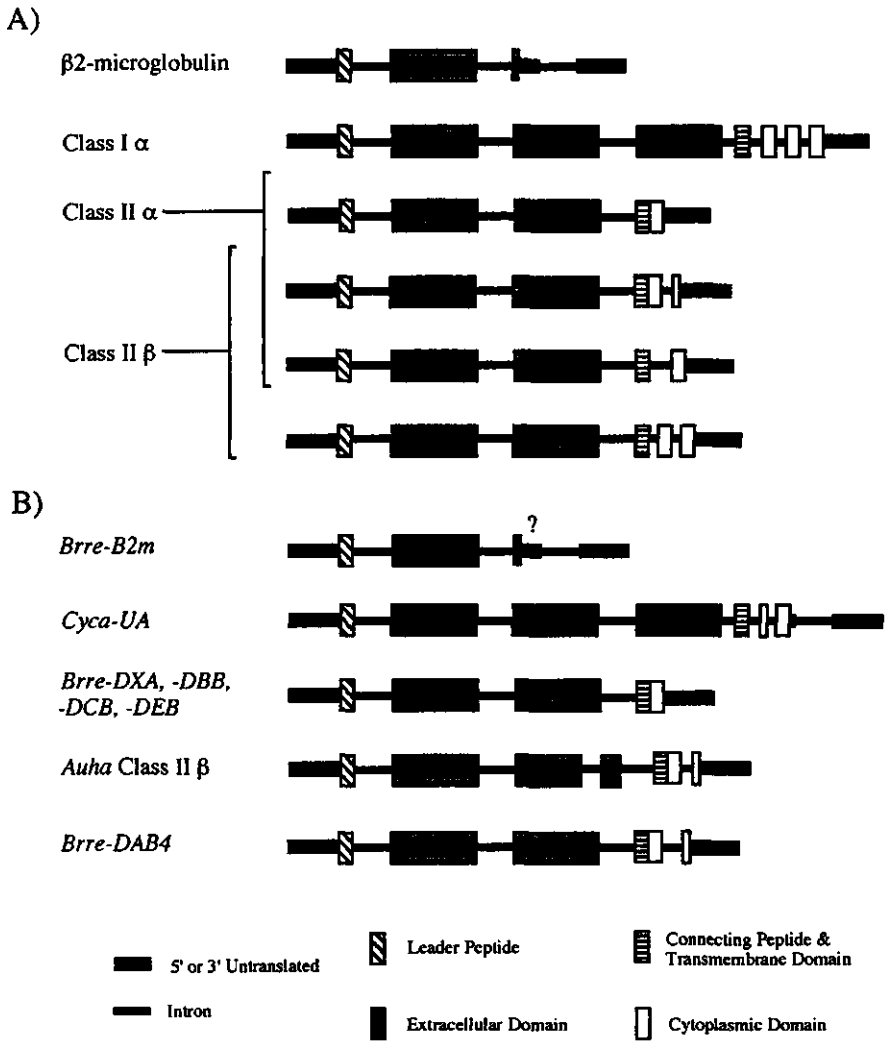

Figure 1. **(A)** The possible intron-exon structures of mammalian MHC genes. Class I α represents the structure of mammalian classical class I MHC genes (see text). **(B)** The structures of the full-length teleost MHC genes known to date. *Brre-B2m*, zebrafish $ß_2$-microglobulin (Ono et al., 1993*a*). *Cyca-UA*, carp class I (van Erp *et al.*, 1996*a*). Brre-DXA, zebrafish class II α (Sültmann *et al.*, 1993). *Brre-DBB*, *-DCB*, *-DEB*, zebrafish class II ß (Sültmann *et al.*, 1994). *Auha* Class II ß, a composite gene structure constructed from the sequences reported by Ono *et al.* (1993*c*). *Brre-DAB4*, zebrafish class II ß (Ono *et al.*, 1992;Sültmann *et al.*, 1994). The key below indicates which exon (or part of an exon) encodes each protein domain or untranslated region. Since intron/exon boundaries do not always correspond to protein domains there may be one or two codons from a domain in the previous or following exon. These have been omitted in this figure for clarity, however. Question mark indicates the position of the 9 bp gap in the *Brre*-ß2m gene (see text). Since intron size can vary greatly, even within a single species, this figure is not drawn to scale.

The only teleost *B2m* gene for which the intron-exon structure is known is from the zebrafish (*Brachydanio rerio*) (Ono *et al.*, 1993*a*). While it is much shorter than the corresponding mammalian genes (only 2.5 kb in total length), its structure closely resembles mammalian *B2m* genes, with one possible exception (see Figure 1B). The 5' untranslated sequence and the sequence encoding the leader are found in exon 1, which is followed by a 1 kb intron. Exon 2 encodes most of the mature protein, and exon 3 contains a sequence encoding the last four amino acids, the stop codon and ten nucleotides of the 3' untranslated sequence. Following this there is a 9 base pair insertion or deletion, which the authors suggest may be an intron (marked by a ? in Fig. 1B), followed by 13 bases of 3' untranslated sequence, then a 94 bp intron and an exon containing the rest of the 3' untranslated sequence (Ono *et al.*, 1993*a*). All of the teleost *B2m* introns are phase 1, consistent with that of mammalian *B2m* introns. The 94 bp intron corresponds with the third intron of mammalian *B2m* genes, but the alleged 9 bp intron is without precedent. It begins with a GT and ends with AG, and is absent in some cDNA sequences from different individuals of the same species (Ono *et al.*, 1993*a*) and also from the only other known teleost *B2m* sequence (*Cyca-B2m*; Dixon *et al.*, 1993). As the libraries used in these experiments were from poly A+ mRNA pooled from a number of individuals, it was not possible to determine whether this insertion or deletion represents allelic variation between individuals, or has a transcriptional basis. Further evidence for allelic variations in teleost *B2m* genes was shown by Southern blot analysis of carp and tilapia DNA, which indicated the presence of different alleles of the *B2m* gene. In fact there may be multiple copies of the gene in carp (Dixon *et al.*, 1993). The conclusion that the multiple bands represent alleles was based on the assumption that the *B2m* gene would only be present in a single copy per haploid genome as it is in mammals, but, given that there may be two MHCs in chickens and *Xenopus*, perhaps this observation indicates that there are in fact two copies of the *B2m* gene per haploid genome in fishes. In any case, there are still some interesting features waiting to be found in teleost *B2m* genes.

1.3.2. Class I MHC Genes

Class I MHC genes encode the large polypeptide chain which, in association with *B2m*, forms the functional class I antigen-presenting molecule. The class I a chain consists of three extracellular domains, as well as a connecting peptide, transmembrane domain and a cytoplasmic domain. The genes which encode these polypeptides are generally very conserved in structure. In mammals there are two types of class I MHC genes, classical and non-classical. Classical class I MHC genes encode polypeptides which form receptors to present intracellular peptides. Non-classical class I genes, however, can be distinguished from the classical genes by several criteria. Firstly, there is a high degree of sequence dissimilarity between the two groups,

secondly, non-classical genes are expressed in an erratic or tissue-specific manner, and thirdly, the polymorphism of non-classical genes is much lower than that of classical genes (Klein *et al.*, 1994). Many of the non-classical sequences reported are in fact pseudogenes, and those which are expressed do present antigenic peptides, but in a limited number of tissues (Bahram *et al.*, 1994), and/or peptides of a different nature than classical class I genes, resulting in different activation patterns as reviewed by Shawar *et al.* (1994).

Mammalian classical MHC class I genes consist of eight exons and seven introns (ref. 7, see Fig. 1A). Again the 5' untranslated sequence and the sequence encoding the leader peptide are found in exon 1. Exon 2 encodes the α_1 domain, and exons 3 and 4 encode the α_2 and α_3 domains, respectively. The connecting peptide and transmembrane domain are encoded by exon 5, and exons 6 and 7 contain sequences which encode the cytoplasmic domain. The final part of the cytoplasmic domain and the 3' untranslated sequence are found in the last exon. Once again the introns are all phase 1. The third intron is the longest, and can be up to 2 kb long in mouse class I genes (Klein *et al.*, 1986).

While some non-classical class I genes in higher vertebrates can have the same intron/exon organization as the classical genes (Steinmetz *et al.*, 1981;Watts *et al.*, 1989), it has also been demonstrated that, in addition to the above-mentioned differences, some non-classical class I genes can also have an intron/exon organisation different from that of classical genes (Bahram *et al.*, 1994;Mashimo *et al.*, 1992;Nakayama *et al.*, 1991;Wang *et al.*, 1993). The exons encoding the 5' untranslated, leader peptide and extracellular domains are consistent, but the exons encoding the connecting peptide, transmembrane domain, cytoplasmic domain and 3' untranslated region vary considerably. For example, the cytoplasmic domain and 3' untranslated region can be encoded by a single exon (Bahram *et al.*, 1994;Mashimo *et al.*, 1992), or the region encoding the connecting peptide, transmembrane domain, cytoplasmic domain and 3' untranslated may be contained in a single exon (Nakayama *et al.*, 1991;Wang *et al.*, 1993). These situations are similar to those found in class II α and ß genes (see Fig 1A), perhaps indicating a common evolutionary origin for these classes of MHC genes, or simply the loss of introns, a common event in gene evolution (Marchionni and Gilbert, 1986). Some of these sequences contain introns which are not phase 1, or sequences in the 3' untranslated region which are reported as introns, but since these sequences all represent pseudogenes these features cannot be considered as *bona fide* variations in MHC gene organisation (Nakayama *et al.*, 1991; Wang *et al.*, 1993).

There has been only one chicken MHC class I gene isolated to date, the *B-FIV* gene (Kroemer *et al.*, 1990). It has a seven-exon, six-intron gene organisation; the only difference from the mammalian organisation is that the sequence encoding the cytoplasmic region is divided between two exons, not three. However the amino acid sequence derived from the cDNA clone used

to obtain this genomic clone revealed a shorter than usual cytoplasmic domain. In addition, this genomic clone contained a putative exon between the two exons encoding the cytoplasmic domain. This putative exon would produce an amino acid sequence found in the cytoplasmic domain of the derived amino acid sequence from a similar chicken MHC class I cDNA, *B-F19*, making the cytoplasmic domain consistent with the size of those found in mammalian MHC class I genes (Kroemer *et al.*, 1990). Alternative splicing events which exclude the corresponding exon from the resulting mRNA have been reported for these genes (Kaufman *et al.*, 1994). Therefore, this clone is in fact an eight-exon, seven-intron gene (the same gene organisation as found in mammals, see Fig. 1A) which gives rise to two different products by alternative splicing. While the introns in this gene are all phase 1, they are much shorter than the introns of mammalian MHC class I genes, varying from 73 to 346 base pairs. The largest reported intron is the one which contains the putative extra exon, and therefore may represent two much shorter introns.

Discussion regarding the gene structure of teleost class I genes is difficult since there have been no complete genomic sequences published to date. The only class I gene sequence published (*TLAI* $\alpha 1$, later designated *MhcCyca-ZA1*) is that of Hashimoto *et al.* (1990). This sequence seems to be present in a genomic clone containing two regions, approximately 5 kb apart, with identical sequences based on hybridization and restriction analyses. The sequence analysed contains only three exons and two complete introns. The sequences in these exons correspond to exons 2, 3 and 4 of mammalian MHC class I genes, but the authors could not find an exon encoding the transmembrane and cytoplasmic domains or the leader peptide, despite the fact that they sequenced approximately 500 bp downstream and 3.5 kb upstream, respectively. The sequence directly before the first exon does end with a CAG splice consensus sequence. Although it is possible that the leader sequence is present further upstream, similar introns in mammalian class I genes are usually less than 200 bp. The two introns which are present in this clone are both phase 1, and are shorter than the mammalian equivalents (180 compared to approximately 200 bp, and 440 bp compared to an intron which is between 570 and 2060 bp for mammalian introns 2 and 3, respectively). The fourth intron in mammalian class I genes is usually about 120 bp in size, but no exon was found 3' of the last exon. Unfortunately, the authors only sequenced some 500 bp downstream to try and identify the connecting peptide transmembrane, cytoplasmic, and 3' untranslated exons and corresponding introns, compared to the 3.5 kb that they investigated upstream. While the incomplete nature of these genes might indicate that they are pseudogenes, they may in fact represent non-classical class I MHC genes, since corresponding cDNAs have been obtained from kidney only. A limited expression pattern is one of the defining features of nonclassical MHC genes (Klein and O'hUigin, 1994).

Our laboratory has recently determined the intron/exon structure of a new carp class I gene,

Cyca-UA (see Fig. 1B, van Erp *et al.*, 1996*a*). It has a unique intron/exon structure which consists of 8 exons and 7 introns. The introns are all phase 1, and are equivalent in size to mammalian genes; intron 2 is at least 1 kb, while introns 3 and 4 are both approximately 1 kb long. The unique features in the organisation of this gene are in the exons encoding the cytoplasmic domain and the 3' untranslated region. Unlike mammalian class I genes the cytoplasmic domain is encoded in only two exons, the first encoding only 6 amino acids and the second encoding the rest. There is a unique intron 11 bp after the termination codon, following which is the exon encoding the remainder of the 3' untranslated sequence (Fig. 1B). Introns in the 3' untranslated sequence are usually found in *B2m* genes, but not in MHC genes. This intron is located in a different position in the 3' untranslated region than the intron of *B2m* genes, however. It is most equivalent in position to the 9 bp gap found in the *Brre-B2m* gene, suggesting that perhaps this "gap" region originated as an intron, but has become nonfunctional.

In conclusion, the intron-exon structure of teleost class I MHC genes appears to correspond well to that found in the exon 2 to exon 4 region of higher vertebrate class I genes, but the structure outside these regions seems variable. While the teleost class I MHC genes reported to date, like avian class I MHC genes, seem to contain smaller introns than their mammalian equivalents this may be a reflection of the function and phylogeny of the genes discovered to date, since genes with larger introns are now being discovered.

1.3.3. Class II Genes

Class II MHC genes are classified by their products; class II α genes produce the α polypeptide chain of the MHC class II protein and similarly class II ß genes encode the ß chain. Both of these polypeptides contain two extracellular domains, designated α_1 and α_2 or β_1 and β_2, and the gene organisation follows a one-extracellular-domain, one-exon format.

Class II gene structure varies in mammals (Klein, 1986). The class II gene can contain four, five or six exons, separated by either three, four or five introns, respectively (see Fig. 1A). There are however some similarities consistent with MHC class I gene structure. The 5' untranslated sequence and those sequences which code for the leader peptide are found in exon 1, as usual, with the addition of 4 aa of the β_1 domain in the case the ß gene. The second exon contains those sequences which code for either the α_1 or β_1 domain, and the third exon contains sequences for the α_2 or β_2 domain, depending upon which gene is examined. Class I α genes have a consistent intron-exon structure for the sequences encoding the connecting peptide, transmembrane domain, cytoplasmic domain and the 3' untranslated sequences, however class II genes show some variation in this region. In class II α genes these four features are encoded by a single exon, or by two exons, in which case the 3' untranslated is either encoded

by a separate exon or in an exon including a short stretch of the cytoplasmic domain (see Fig. 1A). This previous organisation is also found in class II ß genes, which can also have the connecting peptide plus transmembrane region, the cytoplasmic domain and the 3' untranslated regions encoded by separate exons. In class II α genes the first intron is the longest, usually greater than 1 kb, while the other introns are much smaller. In class II ß genes, both the first and the second introns are usually greater than 1 kb and can be up to 5 kb in size. The remaining introns are generally smaller than 1 kb. Consistent with other MHC genes, both in class II α and ß genes the introns are phase 1.

No avian MHC class II α genes have been isolated to date, but five MHC class II ß genes have been cloned (reviewed by Zoorob *et al.*, 1990). These genes all have the same intron-exon structure, which is one of the 5-exon/4-intron structures found in both mammalian MHC class II α and ß genes. In the case of chicken MHC class II ß genes the last exon contains sequences encoding both part of the cytoplasmic domain and the 3'-untranslated sequences (see Fig. 1A). The introns are once again much shorter than those found in mammalian genes, and are all phase 1. Intron 1 is the largest, but is usually only around 200 bp long.

The first teleost MHC class II genes discovered are still incomplete (Hashimoto et *al.*, 1990). The *TLAIIß-1*,(renamed *Cyca-YB*, which should be designated *Cyca-DYB*), sequence shows exons encoding $ß_1$ and $ß_2$ domains separated by a 190 bp, phase 1 intron. While the introns contain the correct splice sequences, extensive sequencing in both directions of this clone have once again, similar to the class I gene, shown no exons encoding leader sequences or connecting peptide/transmembrane/cytoplasmic domains. In this case, this clone may actually represent a pseudogene, since cDNAs produced from this gene have not been reported to date.

Teleost MHC class II gene structures do appear to be consistent with those of mammalian class II genes, however, Sültmann *et al.* (1993) have reported a full-length class II α gene from zebrafish (*MhcBrre-DXA*) which shows a four-exon/three-intron structure, similar to mammalian class II α genes (see Fig. 1 A and B). The first exon encodes the 5' untranslated region and the leader peptide, the second and third the α_1 and α_2 domains respectively, and finally the last exon encodes the connecting peptide, transmembrane domain, cytoplasmic domain and the 3' untranslated region. The three introns are again relatively short, being 126, 129 and 174 bp long, respectively. This gene is organized in a manner completely consistent with mammalian genes.

Ono *et al.* (1993*b*) have also described four genomic sequences which imply a six-exon/five intron class II ß gene in the cichlid fish *Aulonocara hansbaenschi* (*MhcAuha*-Class II ß; Fig. 1B). It is not the typical six-exon, five-intron structure shown in Fig. 1A, however. The 5' untranslated region, leader peptide and 3 aa of the $ß_1$ domain are apparently encoded by a single exon, as is the remainder of the $ß_1$ domain. The connecting peptide, transmembrane

region, cytoplasmic domain and 3' untranslated region seem to be split between two exons, with the intron dividing the sequences encoding the cytoplasmic domain in two. The genomic clones only include 11 bp of the 3' untranslated region, however, so there is a possibility that another intron could be located further downstream. The unusual thing about this gene is the fact that the β_2 domain encoding region is split into two exons. The introns which correspond to those of mammalian genes are all phase 1, but the intron which divides the sequences encoding the *Auha* class II β_2 domain in this unique way is phase 2. Since it is in an odd position, it does not correspond to an intron in any other MHC gene, and, most importantly, it differs from them in phase. Therefore, it almost certainly represents a recent intron gain, and most probably was not present in the ancestral class II ß gene as alternatively suggested by Ono *et al.* (1993*b*). While this structure appears to be consistent for this species (the authors describe at least two different loci), partial genomic sequences from the two cyprinid fish, carp and zebrafish, show the more classical one-extracellular-domain, one-exon structure. Like mammalian class II ß genes, the second intron is just over 1 kb, however, like the avian class II ß genes, the first intron is 200 bp or less.

The structure of the zebrafish class II b gene has recently been elucidated. Sültmann and co-workers, have recently submitted the sequences of six genomic clones called *Brre-DAB4, Brre-DBB, Brre-DCB, Brre-DDB, Brre-DEB*, and *Brre-DFB* to the Genbank and EMBL databases (Sültmann *et al.*, 1994). Only four of these sequences represent complete genes, and one of these, *Brre-DBB*, is presented as probably being a pseudogene. The *Brre-DCB* and *Brre-DEB* genes have four exons and three introns. The connecting peptide, transmembrane domain and cytoplasmic domain are all encoded by a single exon. The introns are relatively short, being between approximately 100 to 400 bases long, consistent with the reported length of intron 1 of this gene obtained from PCR experiments (Ono *et al.*, 1993*b*). This gene structure is found in class II α genes in higher mammals, but not class II ß genes (see Fig. 1). The fourth full length clone, *Brre-DAB4*, which corresponds to both of the previously reported alleles, *Brre-DAB4*01* and *Brre-DAB4*03* (Ono *et al.*, 1993*b*), has a five-exon, four-intron organisation, in which the cytoplasmic domain is encoded by two exons. Again the introns are shorter than 400 base pairs. This gene structure is consistent with those found in higher vertebrates, as shown in figure 1. All of the introns in these *Brre* class II ß genes are phase 1. The variation in intron number present in the zebrafish class II ß genes probably reflects the ongoing process of intron loss in some loci (Marchionni and Gilbert, 1986).

A new carp class II ß gene (*Cyca-DAB*) has been described as cDNA (Ono *et al.*, 1992). One of the cDNA clones (*Cyca-DAB*02*) has retained intron 3, which was only 173 bp long, separating exon 3, which encodes the β_2 domain, from the remaining transcribed sequence.

Table 1. A list of the teleost MHC cDNA full length sequences known until 1995, including the GenBank accession number, EMBL code, reference and the scientific common names used to derive the gene designations are included.

Sequence	Accession Number	EMBL Code	Reference (see footnote)		Scientific and common name
β_2-microglobulin					
Brre-B2m	L05383	BRB2MICA	1	Brre-	Brachydanio rerio (zebrafish)
Cyca-B2m	L05536	CCB2MGLB	2	Cyca-	Cyprinus carpio (common carp)
Class I					
Brre-UA			3		
Cyca-UA			4		
Sasa-p23	L07605	SSMHIA	5	Sasa-	Salmo salar (Atlantic salmon)
Sasa-p30	L07606	SSMHIB			
Class II α					
*Brre-DXA1**2.1.4	L19445	BRMHCAA	6		
*Brre-DXA1**1.4.3	L19446	BRMHCAB			
*Brre-DXA1**1.3.4	L19450	BRMHCF			
Mosa-A-S5	L35062	MOZMHCIIAA	7	Mosa-	Morone saxatilis (striped bass)
Mosa-A-S8	L35063	MOZMHCIIAB			
Mosa-A-S9	L35064	MOZMHCIIAC			
Mosa-A-R2	L35065	MOZMHCIIAD			
Mosa-A-R5	L35066	MOZMHCIIAE			
Mosa-A-R7	L35067	MOZMHCIIAF			
Mosa-A-S7	L35072	MOZMHCIIAG			
Class II β					
Auha-M-231a	L13222	AHMCH2BA	8	Auha-	Auloncara hansbaenschi (African cichlid)
Auha-M-231b	L13223	AHMCH2BB			
*Brre-DAB1**01	L04805	BRMHDABAA9			
*Brre-DAB2**01	L04808	BRMHDABAD			
*Brre-DAB4**01	LO4819	BRMHDABA0			
*Cyca-DAB**01			10		
*Cyca-DAB**02					
Mosa-C-1	L33962	MOZMCIP	4		
Mosa-C-2	L33963	MOZMCIPA			
Mosa-C-22	L33964	MOZMCIPB			
Mosa-R-41	L33965	MOZMCIPC			
Mosa-S-1	L33966	MOZMCIPD			
Mosa-S-2	L33967	MOZMCIPE			
Sasa-DC144	X70165	SSMHC144	11		
Sasa-DC157	X70166	SSMHC157			
Sasa-DC22	X70167	SSMHC22			

(1) Ono *et al.*, 1993*a*; (2) Dixon *et al.*, 1993; (3) Klein *et al.*, 1996; (4) van Erp *et al.*, 1996*a*; (5) Grimholt *et al.*, 1993; (6) Sültmann *et al.* 1993; (7) Walker and McConnell, 1994; (8) Ono *et al.*, 1993*c*; (9) Ono *et al.*, 1992; (10) Ono *et al.*, 1993*b*; (11) Hordvik *et al.*, 1993

Intron 1 has been obtained by PCR, and like in the chicken and cichlid genes, it is shorter than the mammalian equivalent, being only 208 bp. A study currently underway in our laboratory using PCR to amplify the peptide binding region of *Cyca-DAB* alleles, has also revealed that introns may not necessarily be short in teleosts. The first intron in one of our alleles, *Cyca-DAB*04*, is 665 bp long. In the *Cyca* genes both intron 1 and 3 are phase 1, as expected.

2. Teleost MHC protein sequences

Although from several teleost species a number of MHC sequences have been reported, the majority of those sequences registered on the database (EMBL) are PCR fragments. However, the number of complete genomic or cDNA sequences to date are still relatively small. A list of teleost full-length cDNAs is shown in Table 1. We have deliberately restricted ourselves to the listing of these sequences only, because the first clue to functionality of the teleost MHC genes is that these genes are actively transcribed, and are spliced into proper mRNAs.

All of the teleostean MHC protein data that can be discussed are deduced from cDNA sequences. However, this still gives the opportunity to compare some of the their main features with established protein characteristics of human Mhc molecules, such as HLA-A2, which includes the β2-microglobulin molecule, and HLA-DR1 composed of at least one α and one β chain.

2.1. β_2-microglobulin

The first β_2-microglobulin cDNA reported was that of the carp (Dixon *et al.*, 1993), which was followed by its identification in two other Cyprinid species; zebrafish (Ono *et al.*, 1993*a*) and large African Barbel (Dixon *et al.*, 1995*b*). Alignment (Fig. 2) of the deduced protein sequences revealed that the cyprinid β_2-microglobulin molecule is only 97 residues long, compared to the 99 residues in avians and mammals. Based on overall sequence similarity to mammalian β_2-microglobulin, the predicted three dimensional structure of the fish β_2-microglobulin molecule is that of the barrel-shaped immunoglobulin domain, consisting of seven anti-parallel beta-strands in two pleated sheets. The beta-strands are connected by loops of variable length. There is a high degree of conservation between the different cyprinid species, especially in those regions which form the seven beta-strands. Most differences are

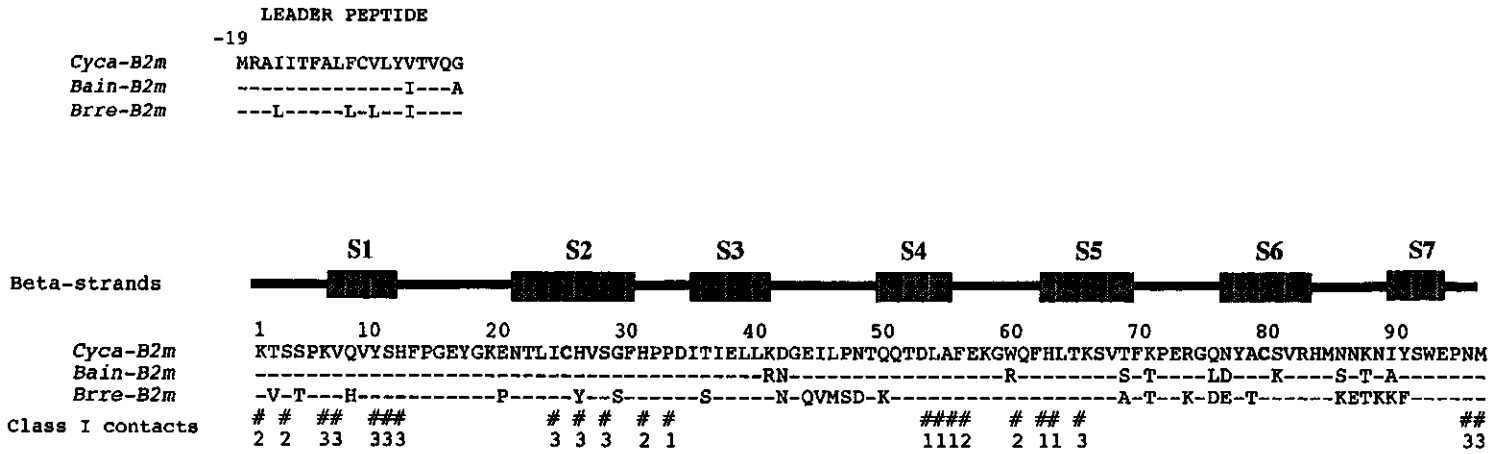

Figure 2. Alignment of beta2-microglobulin molecules deduced from cDNA sequences of *Cyprinus carpio* (*Cyca-B2m*; Dixon *et al.*, 1993), *Barbus intermedius* (*Bain-B2m*; Dixon *et al.*, 1995*b*) and *Brachydanio rerio* (*Brre-B2m*; Ono *et al.*, 1993*a*). Positions of beta-strands and contact points with class I are base on HLA-A2 (Bjorkman *et al.*, 1987).

found in the loops connecting the betastrands. More important, however, is the observation that the majority of the residues which form the contact points between β_2-microglobulin and the class I molecule, as deduced from HLA-A2 (Bjorkman *et al.*, 1987; Saper *et al.*, 1991), are identical between the three species. This suggests a co-evolution of the class I and β_2-microglobulin molecules in cyprinid fish.

Thus, although the teleostean fish possess β_2-microglobulin encoding mRNAs, it is not established whether these molecules do associate themselves with class I molecules in a fashion that, in conjuction with a peptide bound in the groove, is thought to be necessary for the assembly and stabilisation of the class I molecule (Townsend *et al.*, 1990). It remains to be investigated whether the poikilotherm nature of fish affects the biochemistry of this assembly.

2.2. Class I alpha chain

The first full-length class I cDNA reported was that from the Atlantic salmon (*Sasa*-p30; Grimholt *et al.*, 1993). In cyprinids the only full-length cDNAs characterized are those in the common carp (*Cyca-UA* ;Van Erp *et al.*, 1995*a*) and zebrafish (*Brre-UB* ;Takeuchi *et al.*, 1995), which belong to a different lineage than the previously isolated partial cDNAs from *Cyca-ZA*, *-ZB* and *ZC* (Okamura *et al.*, 1993). The deduced protein sequences of these cDNAs have been aligned, including an almost full-length class I sequence from the coelacanth *Lach-UB* (Betz *et al.*, 1994), to illustrate some of the conserved structural and functional characteristics (Fig. 3).

Binding of peptides in the groove formed by the α_1 and α_2 domains of class I molecules involves six binding pockets designated A-F, of which A and F contain as conserved residues mainly tyrosines interacting with the amino- and carboxyl-terminus of the peptide (Saper *et al.*, 1991). Most of these are conserved in the teleostean molecules, with the most prominent exception being the tyrosine (Y) at position 82, which is replaced by arginine (R). However, the coelacanth class I protein sequence clearly differs from the consensus in the peptide-binding residues. Known contact pairs between β_2-microglobulin and class I domains involving residues Q91, D118, and Q236 are very well conserved compared to human and mouse contact pairs (Fremont *et al.*, 1992).

During antigen presentation, the interaction between the MHC class I molecule and the T-cell receptor (TCR) is stabilized by the interaction of the CD8 co-receptor, which binds to an exposed loop, containing many acidic residues (Salter *et al.*, 1990). A similar homologous stretch of mainly acidic residues is found at position 217-223, containing mainly aspartic acid (D) and glutamic acid (E) residues. However, in the coelacanth this conserved feature is not so prominent, indicating that, based on the low similarity between the respective class I molecules, the CD8-binding region in this species might be located in a different position.

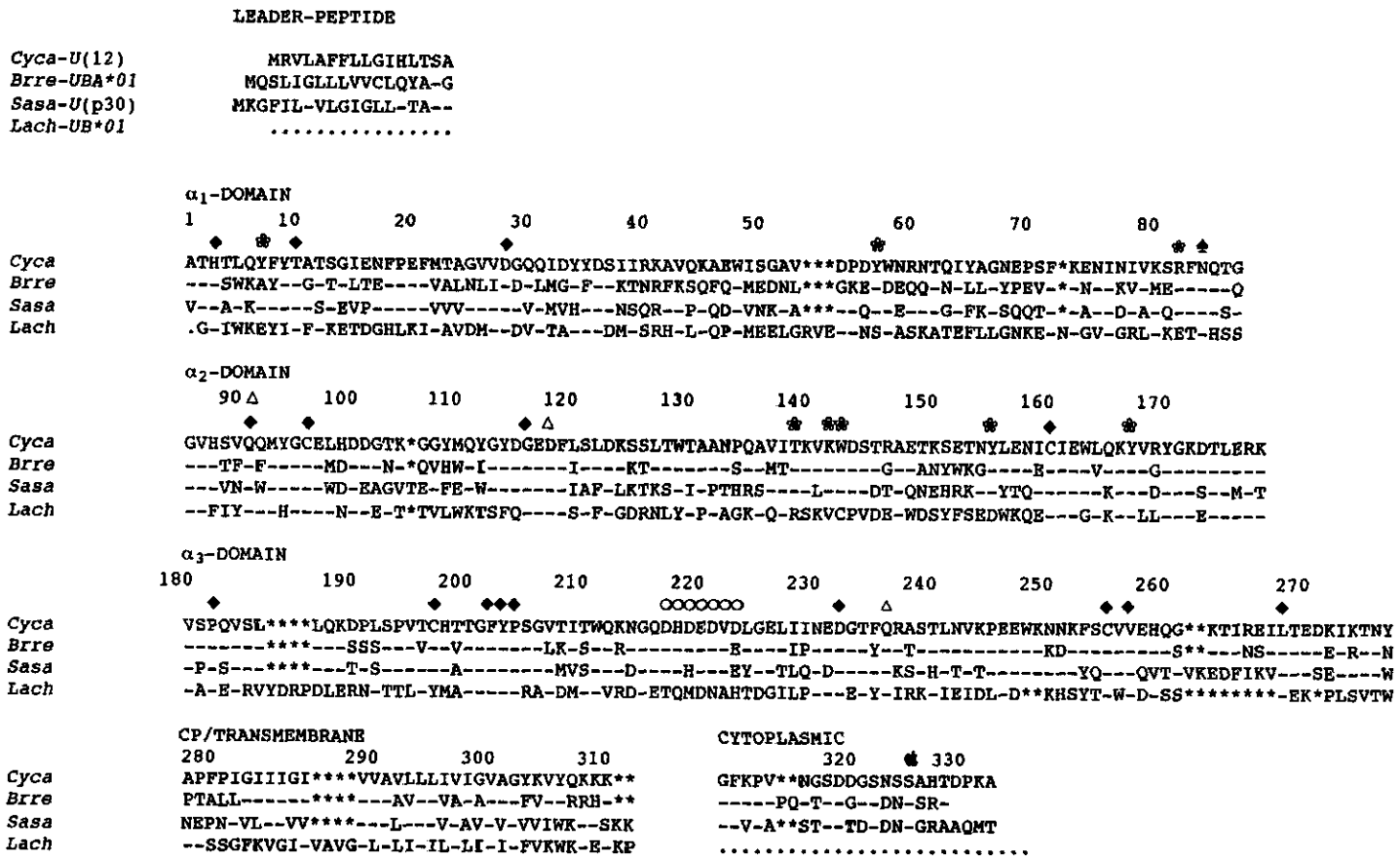

Figure 3. Alignment of MHC class I alpha-chain molecules deduced from cDNA of *Cyprinus carpio* (*Cyca-U*(12); Van Erp *et al.*, 1995a), *Brachydanio rerio* (*Brre-UBA*01*; Takeuchi *et al.*, 1995), *Salmo salar* (*Sasa-U*(p30); Grimholt *et al.*, 1993), and *Latimeria chalumnae* (*LachUB*01*; Betz *et al.*, 1994). Features indicated are residues involved in peptide binding (flower), N-linked glycosylation site (♠), beta2-microglobulin contacts (△), CD8 binding (O), a kinase site (apple), and conserved residues (♦), some with unknown function.

The connecting peptide and transmembrane region are, apart from containing many hydrophobic residues, not as conserved as that of the class II α and ß chain transmembrane regions. Clearly, the reason for this is that only the α chain of the class I heterodimer has to span the cell membrane, instead of both α and ß chain in the case of class II molecules (*vide infra*). There are many other conserved residues, such as the cysteines (C96, C160, C197 and C255) forming the intra-chain disulfide bridges, residues involved in the formation of salt-bridges (D28/H3, H88/D115), the N-linked glycosylation site (N(Q/H)(T/S); 84-86), and a phosphorylation site (S325) in the cytoplasmic region, all of which are characteristic of functional class I molecules (Kaufman *et al.*, 1994). In addition, many other conserved residues with unknown function can be identified.

Thus, the teleostean class I proteins as shown are potentially capable of functioning as classical class I peptide-presenting heterodimers. Studies on one hallmark of MHC class I molecules, *i.e.* the high degree of polymorphism as seen in humans (Parham *et al.*, 1995), is still in its infancy in fish. So far, only in Atlantic salmon (Grimholt *et al.*, 1994) and pink salmon (Katagiri *et al.*, 1995) is there evidence for variability of class I genes. In both instances similar findings, revealing surprisingly low variablity at the polymorphic sites in either α_1 or α_2 domains, were obtained from limited analyses of stocks with different geographical origins. The fact that in the majority of polymorphic sites only two different residues were found might indicate that both species have gone through a recent genetic bottle-neck. Clearly, there is a need for systematic studies on class I polymorphism in commercially interesting species, as this polymorphic marker can be used in conjuction with microsatellite markers to detect correlations with polygenic immunological traits.

2.3. Class II alpha chain

In only three species class II α chain full-length cDNA sequences have been reported: zebrafish (*Brre* ;Sültmann *et al.*, 1993), striped bass (*Mosa* ; Hardee *et al.*, 1995) and common carp (*Cyca* ;Van Erp *et al.*, 1995*b*). The deduced protein is structurally organized in a fashion similar to that of the mammalian class II α chains (Brown *et al.*, 1993), with the exception that the α_1 domain, similar to the α_2 domain, now also has two cysteines (C13 and C67) which are able to form an intra-chain disulphide bridge (Fig. 4). These cysteine residues are not found in shark (Kasahara *et al.*, 1993), which suggests that the teleostean α_1 domain originates from an ancestral class II β_1 domain. In addition, it implies that on two separate occasions, once in the elasmobranchian fish and once in the tetrapods, the two cysteines have been lost. The exception to this is the HLA-DMα molecule, which has retained the two cysteines. Non-polymorphic residues involved in peptide binding are, with some conserved substitutions, present. The putative N-linked glycosylation site located only in the α_2-domain differs between

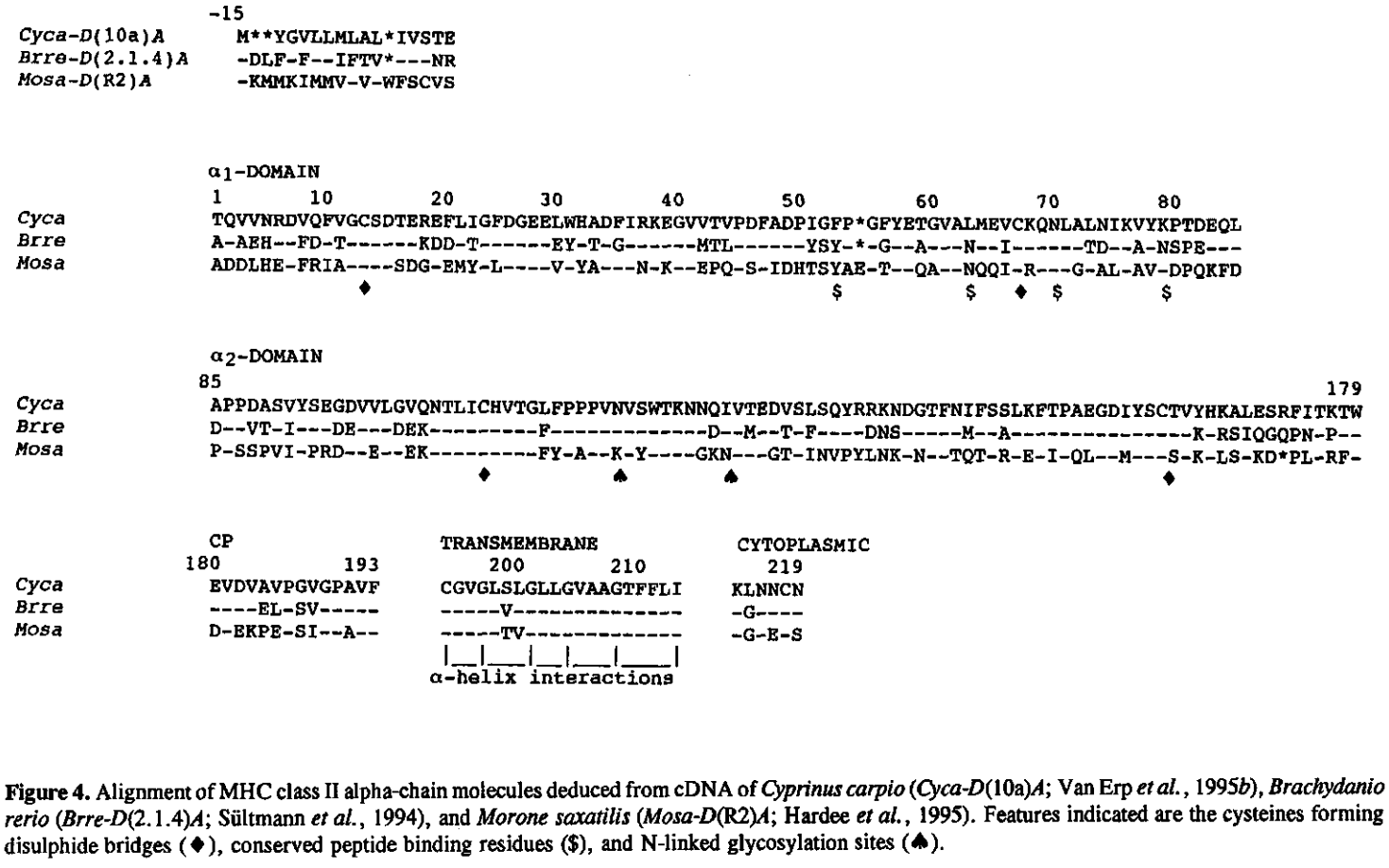

Figure 4. Alignment of MHC class II alpha-chain molecules deduced from cDNA of *Cyprinus carpio* (*Cyca-D*(10a)*A*; Van Erp *et al.*, 1995*b*), *Brachydanio rerio* (*Brre-D*(2.1.4)*A*; Sültmann *et al.*, 1994), and *Morone saxatilis* (*Mosa-D*(R2)*A*; Hardee *et al.*, 1995). Features indicated are the cysteines forming disulphide bridges (♦), conserved peptide binding residues ($), and N-linked glycosylation sites (♠).

cyprinids (NVS; 119-121) and striped bass (NVT; 128-130).

The transmembrane region is extremely well conserved between the teleostean species. Moreover, this conservation of residues forming the α-helix interaction is seen throughout almost all known class II α chain sequences (Kaufman *et al.*, 1994), and is thought to be essential for the formation of $\alpha\beta$ dimers (Cosson and Bonifacino, 1992).

Polymorphism of the α_1 domain, when observed, could be used to infer functional peptide binding characteristics. Although at this point it is difficult to distinquish between loci and alleles, which may account for some of the variability observed, it is clear from studies in both zebrafish and striped bass that allelic polymorphism is present at positions shown to be involved in peptide binding. It should, however, be noted that in mammals class II α chain genes can be mono- or oligomorphic, and still be functional. Thus, further proof awaits detection of peptide-loaded $\alpha\beta$ heterodimers capable of stimulating T cells.

2.4. Class II beta chain

Undoubtedly, genes encoding the MHC class II ß chain are the best studied MHC genes in fish judging from the total number of sequences reported (Dixon *et al.*, 1995). However, only for common carp (Ono *et al*, 1993*b*; Van Erp *et al.*, 1996*b*), zebrafish (Ono *et al.*, 1992), Atlantic salmon (Hordvik *et al.*, 1993), rainbow trout (Glaman, 1995), an African great lake cichlid (Ono *et al.*, 1993*c*), and striped bass (Walker and McConnell, 1994) are the protein sequences derived from full-length cDNAs available for analysis (Fig 5). The molecule displays the usual structure seen in other class II ß chain molecules, namely two extracellular domains, and connecting peptide, transmembrane and cytoplasmic regions. Both extracellular domains have two cysteines forming intra-chain disulphide bridges. Initially, the regions surrounding these residues have been used to deduce PCR primers to obtain MHC sequences (Hashimoto *et al.*, 1990). However, in retrospect it is clear from the alignment of the fish sequences that other conserved regions in the β_2 domain would have been more suitable. These include the residues 136-140, which are part of the region shown to affect CD4 binding to class II in humans (König *et al.*, 1992). Other conserved features include two invariant peptide-binding residues, which in HLA-DR are W β61 and N β82, but in fish in most instances the tryptophan (W) is replaced by an asparagine (N). Residues involved in salt bridges (R/K67, E71) and turns (G44 and G49), important in the structural integrity of the domain, are also conserved. The N-linked glycosylation sites seem to vary in position with the fish species. In the salmonids and cyprinids the site is probably located at residues NST (36-38), while in the Atherinomorpha residues NST (12-14) are most likely used for this purpose. However, similar to the situation in the class II α-chain genes, the most consistent conserved feature is the transmembrane region, where residues involved in the alpha-helix are extremely well conserved throughout evolution.

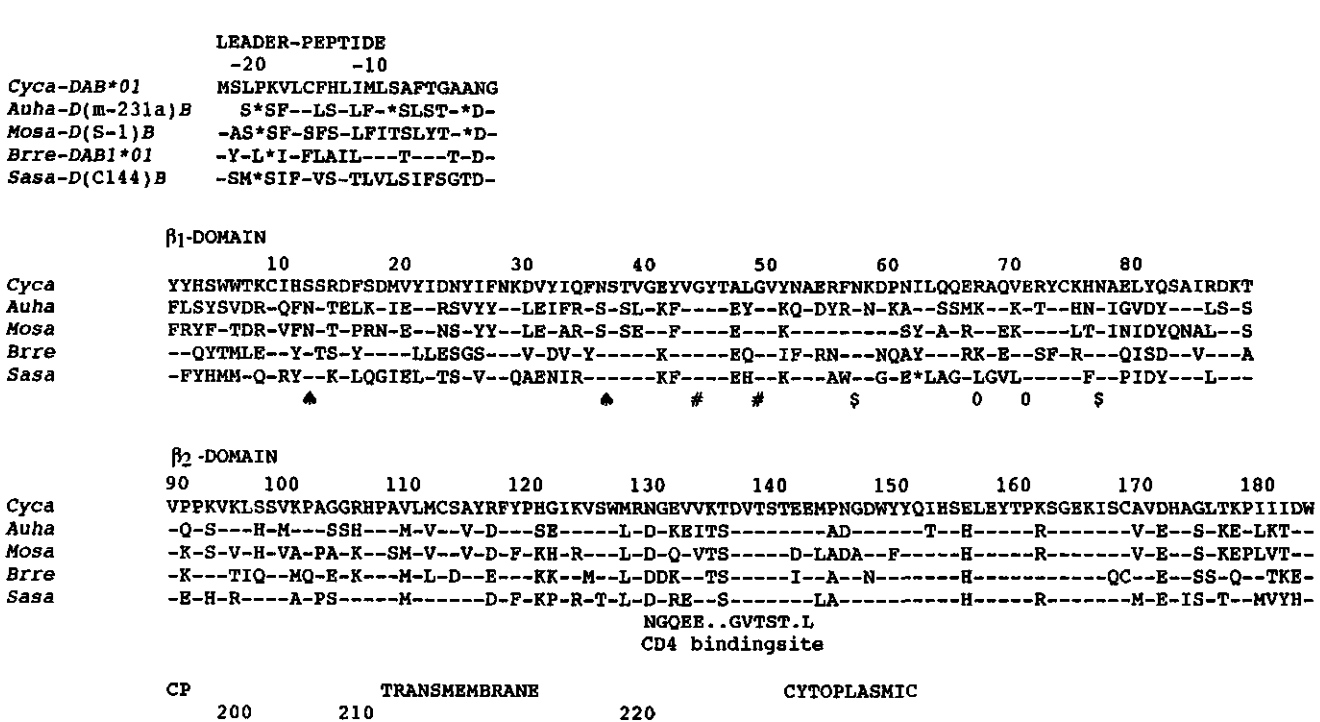

Figure 5. Alignment of MHC class II beta-chain molecules deduced from cDNA of *Cyprinus carpio* (*Cyca-DAB*01*, Ono *et al.*, 1993), *Auloncara hansbaenschi* (*Auha-D*(M-231a)*B*; Ono *et al.*, 1993), *Morone saxatilis* (*Mosa-D*(S-1)*B*; Walker & McConnel, 1993), *Brachydanio rerio* (*Brre-DAB1*01*, Ono *et al.*, 1992), and *Salmo salar* (*Sasa-D*(c144)*B*; Hordvik *et al.*, 1993). Features indicated are conserved peptide binding residues ($), N-linked glycosylation sites (♠), turns (#) and salt bonds (0).

The key characteristic to functionality of class II ß chain sequences is their polymorphism. The polymorphic residues are mainly located in the PBR at more or less fixed positions, whereas other positions are monomorphic and are important for maintaining the structural integrity of the molecule. Assignment of polymorphic residues is only possible when comparison of a number of alleles of any given gene is available. In order to ensure comparison of only alleles of the same gene and not alleles of different genes, it is essential to include introns in the analyses in those cases in which the relationship of the different genes is unclear. As a result of not including an identification of true alleles, it has been concluded that in studies on class II polymorphism in fish only isotypic variability has been identified (Kaufman *et al.*, 1994).

We are currently analysing class II ß-chain genes from members of a large African barbel (*Barbus intermedius*) species flock (Dixon *et al.*, 1995*b*). This species flock inhabits Lake Tana in Ethiopia, which is the origin of the Blue Nile. Based on eco-morphological characteristics 13 different morphotypes can be distinguished (Nagelkerke *et al.*, 1994). From selected morphotypes, representing fish under different pathogen loads, we are analysing intron 1 and exon 2 sequences of the class II ß-chain genes by PCR using primers based on *Cyca-DAB* sequences. A number of unique sequences have been characterized, and based on intron comparisons these sequences clearly belong to the same locus. The availability of a substantial number of sequences allows the determination of the polymorphic residues involved in peptide binding (Fig. 6). This analysis clearly shows that the highest variability is concentrated in those positions that are thought to be involved in peptide binding. Similar findings have been reported for the zebrafish and cichlids, although in these studies it is unclear whether alleles of the same locus are compared (Ono *et al.*, 1992; Ono *et al.*, 1993*c*). Studies in Atlantic salmon indicated that, although a large number of different sequences are obtained from *Sasa-DB*, at each polymorphic position the variability was low (Grimholt *et al.*, 1994). Again, as in the *Sasa* class I sequences, in most instances only two different amino acids were present.

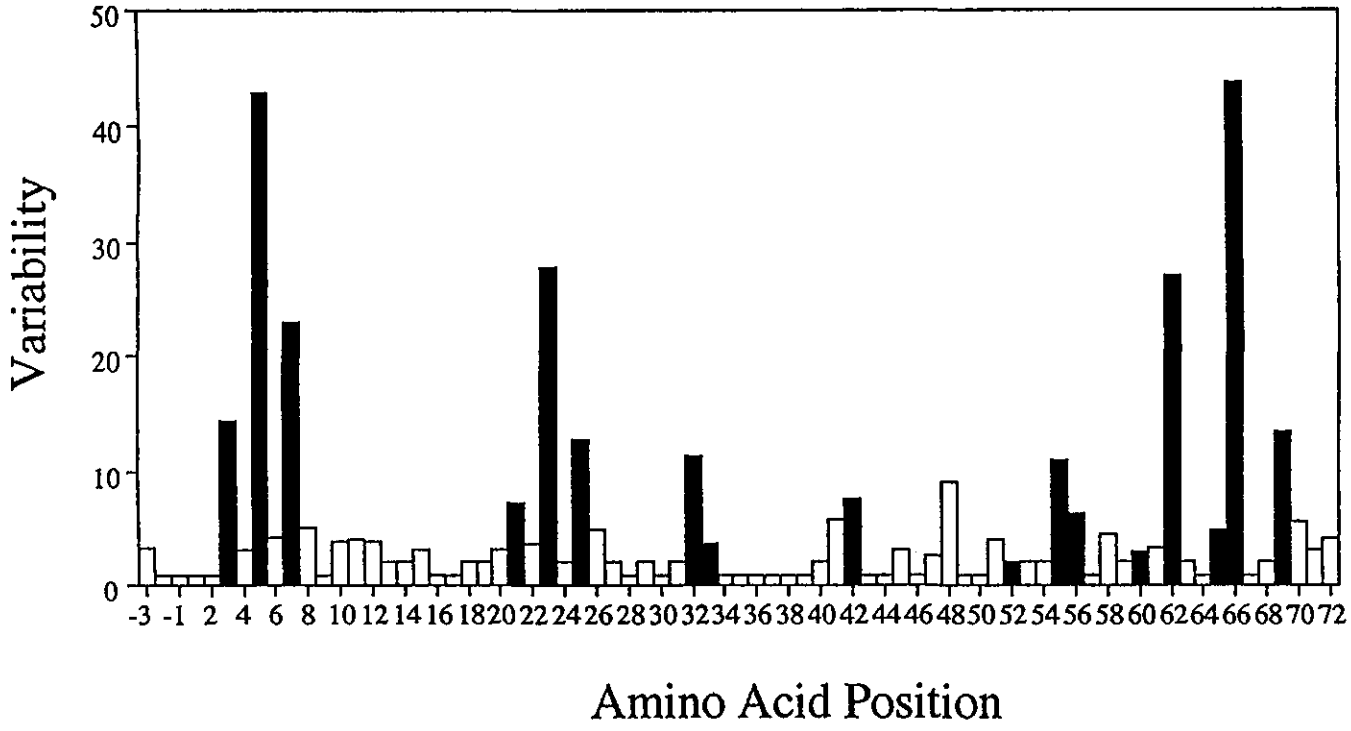

Figure 6. Wu-Kabat plot of MHC class II beta1-domains of *Barbus intermedius (Bain-DAB)*. Variability is expressed as the ratio of the number of different residues at a given position over the frequency of the most common residue at that position. Black bars indicate positions involved in peptide binding.

3. Rationale and aims of this thesis

The Cyprinidae is the group of teleost fish from which the largest amount of information about the MHC genes has been gathered in the past few years. In the common carp (*Cyprinus carpio*) representatives of the β_2m, class I and class II genes have been identified and to a certain extent characterized. However, the functionality of the molecules encoded by these genes is still a question. Up until this point the only way to address this question was to study the genomic organisation, and the expression into mRNA of these genes, and their transcription products, to predict, based on the nucleotide sequence, if they would be transcribed and translated into functional molecules. However, this approach has obvious limitations. An alternative is to get more information about MHC genes by studying their transcription patterns. By comparison with other vertebrates, it can be expected that MHC class I and class II genes may be transcribed in a tissue and cell type specific way. The first candidate for these type of analyses was the class II *Cyca-DAB* gene, from which the full-length cDNA sequence was known. In all animals studied so far, the expression of class II molecules, is known to be restricted to certain cell types. It is a property of MHC class II-positive cells to process and present exogenous antigen to T lymphocytes, and these processes have been indirectly demonstrated in fish. Therefore, the transcription of the *Cyca-DAB* in several lymphoid tissues and cell types was studied in order to obtain an insight into the function of these genes in fish (**Chapter 2**). Although such analysis provided information on the tissue and cell type specific distribution of the transcription of the carp class II gene, it did not address the question of the function of the molecule proper. No class II antibodies are available yet to study the function in more detail. However, with the successful production of polyclonal antibodies against recombinant Cyca-B2m and Cyca-UA proteins, it was possible to evaluate the levels of expression of carp class I molecules. Ectothermic animals such as carp do not regulate their body temperature, and it has been shown that the immune response of fish kept at low temperature is severely impaired. However, the mechanisms of temperature-dependent suppression of the T-cell response has never been studied at the MHC level. Therefore, the study of the cellular expression of Cyca-B2m and Cyca-UA molecules, in combination with analyses of *Cyca-B2m* and *Cyca-UA* transcription in carp kept at different temperatures, was carried out (**Chapter 3**). The expression and tissue distribution of MHC molecules during ontogeny is known to differ from the adult situation. For the normal development of the cytotoxic T-cell repertoire during ontogeny, the distribution of class I appears to be essential. The early appearence of MHC molecules in carp was supported by allograft rejection observed in 3 week old larvae. With the knowledge of class I molecules cellular distribution in the adult carp, the expression of Cyca-UA and Cyca-B2m

molecules on several cell types present in carp lymphoid organs was evaluated during ontogeny (**Chapter 4**). Since no antibody was available for class II ß and the class II α sequences it was only possible to study the expression of both class II (*Cyca-DAB*, *Cyca-DXA*) and class I molecule encoding genes (*Cyca-UA*, *Cyca-B2m*) during ontogeny at the transcription level. By these means it was also possible to investigate the transcription of carp MHC genes at very early stages during the development of the larvae (**Chapter 5**). In the last chapter (**Chapter 6**) results are brougth togther in a comprehensive discussion on the putative role of carp MHC molecules in initiating an immune response, which is critically dependent on developmental aspects and cellular distribution creating adequate microenvironments, and environmental factors such as temperature.

References

Bahram, S., Bresnahan, M., Geraghty, D. E. and Spies, T. (1994) A second lineage of mammalian major histocompatibility complex class I genes. *Proc. Nat. Acad. Sci. USA* **91**: 6259-6263.

Bartl, S. and Weissman, I. (1994) Isolation and characterisation of major histocompatibility complex class IIB genes from the nurse shark. *Proc. Nat. Acad. Sci. USA* **91**: 262-266.

Betz, U.A.K., Mayer, W.E. and Klein, J. (1994) Major histocompatibility complex genes of the coelacanth *Latimeria chalumnae*. *Proc. Nat. Acad. Sci. USA.* **91**: 11065-11069.

Bjorkman, P.J. and Parham P. (1990). Structure, function, and diversity of class I major histocompatibility complex molecules. *Ann. Rev. Biochem.* **59**, 253-288.

Bjorkman, P.J., Saper, M.A., Samraoui, B., Bennet, W.S., Strominger, J.L. and Wiley, D.C. (1987). Structure of the human class I histocompatibility antigen, HLA-A2. *Nature, London* **329**: 506-512.

Brown, J.H., Jardetzky, T.S., Gorga, J.C., Stern, L.J., Urban, R.G., Strominger, J.L. and Wiley, D.C. (1993). Three-dimensional structure of human class II histocompatibility antigen HLA-DR1. *Nature, London* **364**: 33-39.

Campbell, R. D. and Trowsdale, J. (1993) Map of the human MHC. *Immunol. Today* **14**: 349-352.

Cosson, P. and Bonifacino, J.S. (1992). Role of transmembrane domain interactions in the assembly of class II MHC molecules. *Science* **258**: 659-662.

Daggfeldt, A., Bengtén, E. and Pilström, L. (1993) A cluster-type organization of the loci of the immunoglobulin light chain in Atlantic Cod (*Gadus morhua* L.) and rainbow trout (*Oncorhynchus mykiss* Walbaum) indicated by nucleotide sequences of cDNAs and hybridization analysis. *Immunogenetics* **38**: 199-209.

Dixon, B., Stet, R.J.M., Van Erp, S.H.M. and Pohajdak, B. (1993). Characterization of β_2-microglobulin transcripts from two teleost species. *Immunogenetics* **38**: 27-34.

Dixon, B., Van Erp, S.H.M., Rodrigues, P.N.S., Egberts, E. and Stet, R.J.M. (1995*a*). Fish major histocompatibility complex genes: an expansion. *Dev. Comp. Immunol.* **19**: 109-133.

Dixon, B., Egberts, E. and Stet, R.J.M. (1995*b*) Unpublished. EMBL accesion no: Z54151.

Fremont, D.H., Matsumura, M., Stura, E.A., Peterson, P.A., and Wilson, I.A. (1992). Crystal structures of two viral peptides in complex with murine MHC class I H-2K^b. *Science* **257**: 919-927.

Germain, R.N. and Margulies, D.H. (1993). The biochemistry and cell biology of antigen processing and presentation. *Ann. Rev. Immunol.* **11**: 403-450.

Glamann J. (1995). Complete coding sequence of rainbow trout *Mhc* II β chain. *Scandi. J. Immunol.* **41**: 365-372.

Grimholt, U., Hordvik, I., Fosse, V.M., Olsaker, I., Endresen, C. and Lie, Ø. (1993). Molecular cloning of major histocompatibility complex class I cDNAs from Atlantic salmon (*Salmo salar*). *Immunogenetics* 37: 469-473.

Grimholt, U., Olsaker, I., De Vries Lindstrøm, C. and Lie, Ø. (1994). A study of variability in the MHC class II β1 and class I α2 domain exons of Atlantic salmon, *Salmo salar* L. *Anim. Genet.* 25: 147-153.

Güssow, D., Rein, R., Ginjaar, I., Hochstenbach, F., Seeman, G., Kottman, A. and Ploegh, H. L. (1987) The human ß2-microglobulin gene: primary structure and definition of the transcriptional unit. *J. Immunol.* **139**: 3132-3138.

Hardee, J.J., Godwin, U., Benedetto, R. and McConnell, T.J. (1995). Major histocompatibility complex class II *A* gene polymorphism in the striped bass. *Immunogenetics* **41**: 229-238.

Hashimoto, K., Nakanishi, T. and Kurosawa, Y. (1990). Isolation of carp genes encoding major histocompatibility complex antigens. *Proc. Nat. Acad. Sci. USA* **87**: 6863-6867.

Hordvik, I., Grimholt, U., Fosse, V.M., Lie, Ø. and Endresen, C. (1993). Cloning and sequence analyses of cDNAs encoding the MHC class II β chain in Atlantic salmon (*Salmo salar*). *Immunogenetics* **37**: 437-441.

Kasahara, M., McKinney, E., Flajnik, M.F. and Ishibashi, T. (1993). The evolutionary origin of the major histocompatibility complex: Polymorphism of class II α chains in the cartilaginous fish. *Eur. J. Immunol.* **23**: 2160-2165.

Katagiri, T., Hirono, I., Aoki, T. and Sakai, M. (1995) Unpublished. EMBL accession nos: D58379-D58395.

Kaufman, J., Salomonsen, J. and Flajnik, M. (1994). Evolutionary conservation of MHC class I and class II molecules-different yet the same. *Semin. Immunol.* **6**: 411-424.

Klein, J. (1986) *Natural History of the Major Histocompatibility Complex*. New York: J. Wiley and Sons.

Klein, J., Unpublished. See table 1 for Genbank accession numbers.

Klein, J. and O'hUigin, C. (1994) The conundrum of nonclassical major histocompatibility complex genes. *Proc. Nat. Acad. Sci. USA* **91**: 6251-6252.

König R., Huang, L-Y. and Germain R.N. (1992). MHC class II interaction with CD4 mediated by a region analogous to the MHC class I binding site for CD8. *Nature, London* **356**: 796-801.

Kroemer, G., Zoorob, R. and Auffray, C. (1990) Structure and expression of a chicken MHC class I gene. Immunogenetics **31**: 405-409.

Kronenberg, M., Brines, R. and Kaufman, J. (1994) MHC evolution: a long term investment in defense. *Immunol. Today* **15**: 4-5.

Marchionni, M. and Gilbert, W. (1986) The triosephosphate isomerase gene from maize: introns antedate the plant-animal divergence. *Cell* **46**: 133-141.

Mashimo, H., Chorney, M. J., Pontarotti, P., Fisher, D. A., Hood, L. and Nathenson, S. G. (1992) Nucleotide sequence of the BALB/c H-2T region gene, T3d. *Immunogenetics* **36**: 326-332.

Nagelkerke, L.A.J., Sibbing, F.A., Van den Boogaart, J., Lammens, E.H.R.R. and Osse J.W.M. (1994). The barbs (*Barbus* spp.) of Lake Tana, a forgotten species flock? *Environmental. Biol. Fishes* **39**: 1-22.

Nakayama, K., Tokito, S., Pannetier, C, Nakauchi, H. and Gachelin, G. (1991) MHC gene Q8/9d of the BALB/cJ mouse strain cannot encode a Qa-2,3 class I antigen. *Immunogenetics* **33**: 225-234.

Okamura, K., Nakanishi, T., Kurosawa, Y and Hashimoto, K. (1993). Expansion of genes that encode MHC class I molecules in cyprinid fish. *J. Immunol.* **151**: 188-200.

Ono, H., Klein, D., Vincek, V., Figueroa, F., O'hUigin, C., Tichy, H. and Klein, J. (1992). Major histocompatibility complex genes of the zebrafish. *Proc. Nat. Acad. Sci. USA* **89**: 11886-11890.

Ono, H., Figueroa, F., O'hUigin, C. and Klein, J. (1993*a*). Cloning of the β_2-microglobulin gene in the zebrafish. *Immunogenetics* **38**: 1-10.

Ono, H., O'hUigin, C., Vincek, V., Stet, R.J.M., Figueroa, F. and Klein, J. (1993*b*). New β chain encoding Mhc class II genes in the carp. *Immunogenetics* **38**: 146-149.

Ono, H., O'hUigin, C., Vincek, V. and Klein J. (1993*c*). Exon-intron organization of fish major histocompatibility complex class II B genes. *Immunogenetics* **38**: 223-234.

Parham, P., Adams, E.J. and Arnett, K.L. (1995). The origins of HLA A,B, C polymorphism. *Immunol. Rev.* **143**: 139-180.

Pohajdak, B., Dixon, B. and Stuart, G. Immune System. In: Hochachka, P., Mommsen, T. eds. Biochemistry and Molecular Biology of Fishes, Vol. 2. Amsterdam, Elsevier Science Inc., 1993:191-205.

Salter., R.D., Benjamin, R.J., Wesley, P.K., Buxton, S.E., Garrett, T.P.J., Clayberger, C., Krensky, A.M., Norment, A.M., Litman, D.R. and Parham, P. (1992) A binding site for the T-cell co-receptor CD8 on the α_3 domain of HLA-A2. *Nature, London* **345**: 41-46.

Saper, M.A., Bjorkman, P.J. and Wiley, D.E. (1991). Refined structure of the human histocompatibility antigen HLA-A2 at 2.6 Å resolution. *J. of Molec. Biol.* **219**: 277-319.

Shawar, S., Vyas, J.M., Rogers, J.R. and Rich, R.R. (1994). Antigen presentation by major histocompatibility complex class I-b molecules. *Ann. Rev. Immunol.* **12**: 839-880.

Steinmetz, M., Moore, K. W., Frelinger, J. G., Sher, B. T., Shen, F.-W., Boyse, E. A. and Hood, L. (1981) A pseudogene homologous to mouse transplantation antigens: transplantation antigens are encoded by eight exons that correlate with protein domains. *Cell* **25**: 683-692.

Stet, R.J.M. and Egberts E. (1991). The histocompatibility complex in teleostean fishes: from multiple histocompatibility loci to a major histocompatibility complex. *Fish Shellfish Immunol.* **1**: 1-16.

Sültmann, H., Meyer, W.E., Figueroa, F., O'hUigin, C. and Klein, J. (1993) Zebrafish *Mhc* class II α chain-encoding genes: Polymorphism, expression, and function. *Immunogenetics* **38**: 408-420.

Sültmann, H., Mayer, W.E., Figueroa, F., O'hUguin, C. and Klein, J. (1994) Organisation of MHC class II ß genes in the zebrafish (*Branchydanio rerio*). *Genomics* **23**: 1-14.

Takeuchi, H., Figueroa, F., O'hUigin, C. and Klein, J. (1995). Cloning and characterization of class I *Mhc* genes of the zebrafish, *Brachydanio rerio* . *Immunogenetics* **42**: 77-84.

Townsend, A., Elliott, T., Cerundolo, V., Foster, L., Barber, B. and Tse A. (1990). Assembly of MHC class I molecules analyzed in vitro. *Cell* **62**: 285-295.

Trowsdale, J. (1995). Both man, bird and beast: comparative organization of MHC genes. *Immunogenetics* **41**: 1-17.

Van Erp, S.H.M., Dixon, B., Figueroa, F., Egberts, E. and Stet, R.J.M. (1995*a*). Identification and characterization of a novel major histocompatibility complex class I gene from carp (*Cyprinus carpio* L.). *Immunogenetics* (in press) EMBL accesion no: X91015.

Van Erp, S.H.M., Egberts, E. and Stet, R.J.M. (1995*b*) Characterization of major histocompatibility complex class II A and B genes in a gynogenetic carp clone.(submited) EMBL accesion no: Z47730.

Walker, R.A. and McConnell, T.J. (1994). Variability in an MHC *Mosa* class II β chain-encoding gene in striped bass (*Morone saxatilis*). *Dev. Comp. Immunol.* **18**: 325-342.

Wang, C.-R. and Fischer-Lindahl, K. (1993) Organisation and structure of the H-2M4-M8 class I genes in the mouse major histocompatibility complex. *Immunogenetics* **38**: 258-271.

Watts, S, Davis, A. C., Gaut, B., Wheeler, C., Hill, L. and Goodenow, R.S. (1989) Organisation and structure of the Qa genes of the major histocompatibility complex of the C3H mouse: implications for Qa function and class 1 evolution. *EMBO J.* **8**: 1749-1759.

Zoorob, R., Béhar, G., Kroemer, G. and Auffray, C. (1990) Organization of a functional chicken class II B gene. *Immunogenetics* **31**: 179-187.

Chapter 2

Detection of MHC class II transcripts in lymphoid tissues of the common carp (*Cyprinus carpio* L.).

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Summary

In all vertebrates studied to date, the expression of Mhc class II genes is known to be restricted to a limited number of tissues and cell types. In order to have a better understanding of the function of the equivalent genes in teleost fish, the distribution of Mhc class II ß transcripts (*Cyca-DAB*) in the common carp (*Cyprinus carpio* L.) was investigated. RNA was isolated from tissues and leucocytes, cDNA was produced, and amplification of the *Cyca-DAB* genes was carried out by PCR. Of the organs with known immunological function, the highest level of *Cyca-DAB* transcription was found in the thymus. Despite their expected different cellular organization, total blood, head kidney, spleen and the second segment of the gut had similar *Cyca-DAB* expression levels. No class II transcripts were detected in the skeletal muscle. The studies carried out with leucocytes isolated from the lymphoid tissues point to a direct correlation between the levels of expression and the numbers of surface immunoglobulin positive (sIg^+) cells present in the different cell fractions. However, thymus leucocytes did not follow this correlation since the highest level of class II expression was found in a thymocyte fraction that contained very low numbers of Ig^+ cells. In PBL the Ig^+ cells were highly positive whereas the Ig^- were weakly positive. Adherent leucocytes were shown to be class II positive, although adherent cells from PBL show a lower level of expression compared to those from the spleen and head kidney.

Key Words: Carp MHC, Class II, tissue distribuition, *Cyca-DAB*, cDNA, PCR

Introduction

The structure and function of Mhc class I and II proteins is well documented and has been extensively studied especially for the few higher vertebrates generally used as experimental animals (reviewed by Klein, 1986). The importance of Mhc molecules is their involvement in antigen presentation (Bjorkman *et al.*, 1987; Brown *et al.*, 1993), and it is widely accepted that in general the Mhc class I molecule is involved in presentation of endogenous antigen to cytotoxic T cells whereas Mhc class II presents exogenous processed antigens to T helper cells (McCluskey, 1991).

Experimental data on acute allograft rejections, mixed lymphocyte reactions and in vitro antibody responses have long supported the existence of Mhc molecules in teleost fish (Stet *et al.*, 1991). However, attempts to isolate Mhc proteins have not been successful so far. With the adoption of another strategy, based on the comparisons of known Mhc sequences and the use of polymerase chain reaction (PCR), evidence for genes encoding Mhc antigens in fish was first reported by Hashimoto *et al.* in 1990. Later, due to the use of similar molecular techniques, genes encoding the α chain of MHC class I and the $ß_2$-microglobulin, as well as the α and ß chain of MHC class II molecules were isolated and sequenced for a number of

teleost species (reviewed by Dixon *et al.*, 1995).

Several of the known teleost Mhc genes seem to be functional since mRNA sequences encoding the leader peptide, the extracellular domains, the connecting peptide, the transmembrane region and cytoplasmatic tail of the MHC molecules have been reported. This organization of teleost Mhc proteins seems to be similar to those of mammals supporting the general view that all vertebrate immune systems may use these molecules for the same basic functions. Presentation of exogenous peptides to T cells is a property of several cell types, commonly named antigen presenting cells (APC). The ability of APCs to process and present antigen has been demonstrated in fish (Vallejo *et al.*, 1992). This function is a fundamental property of Mhc class II positive cells.

Carp (*Cyprinus carpio* L.) is one of the few teleost species for which a considerable amount of data about Mhc genes is available (Hashimoto *et al.*, 1990; Dixon *et al.*, 1993; Ono *et al.*, 1993; Van Erp *et al.*,1996; Van Erp et *et al., in prep*). The expression of Mhc class II genes is known to be restricted to a limited number of cell types (McCluskey, 1991). With the knowledge of the carp full-length Mhc class II ß chain (*Cyca-DAB*) gene, it is possible to study Mhc class II expression in different cell types and tissues. An understanding of the distribution of the Mhc class II transcripts will give some insight into the function of these genes in fish. In this study we investigated the level of *Cyca-DAB* transcription in different cells of the immune system and in several lymphoid tissues, using a number of molecular techniques.

Materials and methods

Animals

Common carp (*Cyprinus carpio L.*) were reared at 23° C in recirculating UV-sterilized water, and fed pelleted dry food (K30; Provimi, Roterdam, The Netherlands) at a ration of 2% of the body weight per day. Animals from the single F1 hybrid family, R3 x R8, weighing between 150-250g (10-16 months) were used. R3 and R8 are partly inbred strains of common carp originating from Poland and Hungary, respectively. Both R3 and R8 parental fish carry identical Cyca-DAB alleles (Wiegertjes *et al.*, in prep).

Cell isolation

The animals were anaesthetized in tricaine methane sulphonate (TMS; Crescent Research Chemicals, Phoenix, USA) at 3g/10 L , and heparinized blood was collected from the dorsal aorta. This was diluted 1:1 in cRPMI (RPMI 1640 adjusted to 270 mOsm), and peripheral blood leucocytes (PBLs) were separated on Lymphoprep (Nycomed, Oslo, Norway) by centrifugation at 680 x g for 30 min. at 4°C. Head kidney, spleen, and thymus cell suspensions were prepared by forcing the tissues through a 50 mesh nylon gauze filter while adding cRPMI. Gut leucocytes were isolated by scraping the gut mucosa from the serosa using a scalpel. The mucosal cells obtained were resuspended before being forced through the nylon filter. After having been washed in cRPMI (680 x g for 10 min. at 4° C) and resuspended, the cell suspensions were separated on a Percoll (Pharmacia, Uppsala, Sweden) discontinuous density gradient of 1.020, 1.060, 1.070 and 1.083 g/cm^3, respectively, by centrifugation at 840 x g for 30 min. at 4°C. The cells were harvested from the three interfaces, washed twice and resuspended at a concentration of 10^7 cells/ml in cRPMI. Cell fractions from blood, head kidney, thymus, gut and spleen of five individuals from the R3xR8 family were snap frozen in liquid nitrogen and kept at -80° C for future use. In order to obtain adherent cells from the spleen and head kidney, leucocytes from these organs were isolated on Percoll discontinous density gradient (as described above) and the second fraction was allowed to adhere to the surface of plastic 25cm^2 tissue culture plates (Costar, Cambridge, USA) for 1 h at 27°C (5% CO_2) in cRPMI. The same procedure was used with Lymphoprep-isolated PBL. After this step the plates were washed twice in cRPMI and the non-adherent cells discarded. The remaining adherent leucocytes were snap frozen and kept for future use.

Flow cytometry (FACS)

Cell fractions (10^6 cells) obtained were incubated for 30 min on ice in 0.5 ml of appropriately diluted (usually 1:100) WCI 12 monoclonal antibody, which detects carp surface immunoglobulin (sIg) (Secombes *et al.*, 1983). For all the incubation and washing steps FACS medium containing cRPMI, 1% BSA and 0.1% NaN_3 was used. After washing, binding of WCI 12 was detected by incubating the cells for 15 min on ice with fluorescein isothiocynate (FITC)-conjugated rabbit anti-mouse (RAM) antibody (Dakopatts, Glostrup, Denmark), diluted 1:100, in FACS medium containing 1% of pooled carp serum. Cells were washed and analyzed using a FACStar (Beckton-Dickinson Immunocytometry Systems, Mountain View, USA) with an argon laser tuned at 488 nm. The Consort 30 data analysis package was used to plot the forward (FSC) and side (SSC) scatter patterns and to determine the percentage of WCI 12-positive cells (FL1).

Magnetic cell sorting (MACS)

Peripheral blood leucocytes (PBL) were isolated as described above. During the procedure TBS (20 mM Tris/HCl pH 7.4, 150 mM NaCl, 0.8 mM $MgCl_2$, 0.2 mM $CaCl_2$) with 0.01% NaN_3 was used instead of cRPMI. RPMI contains biotin which may disturb the labelling. PBL (10^8) were washed in TBS, resuspended and incubated with biotin-conjugated WCI 12 (1:50) for 30 min on ice. Subsequently, cells were incubated with 1:5 diluted FITC-conjugated avidin (Beckton-Dickinson, Mountain View, USA), washed and resuspended in the presence of biotinylated superparamagnetic spheres. Cells were separated using the BS columns (Miltenyi Biotec GmbH, Germany), fitted onto the MACS. Positively (sIg^+) and negatively (sIg^-) separated cell populations were recovered and analysed using FACS analyses.

RNA and cDNA preparation

Cells were thawed out in lysis buffer (4 M guanidium thiocyanide, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M 2ß-ME) followed by phenol/chloroform extractions. Total RNA was precipitated in ethanol, washed and dissolved in water. Concentrations were measured by spectrophotometry (DU-62 spectrophotometer, Beckman) and the RNA stored at -80°C. Samples containing10μg of total RNA were converted into cDNA using the Riboclone cDNA Synthesis System (Promega, Madison WI, USA), according to the manufacture's specifications. Efficiency of cDNA synthesis was traced by determining the incorporation of ^{32}P-dCTP in a parallel reaction. The radiolabeled cDNA was precipitated and cpm measured using a liquid scintillation counter. The cDNA quality was assessed by using dixogenin-labelled uridine-triphosphate (DIG- dUTP; Boehringer, Mannheim, Germany) incorporation during

the cDNA synthesis. The DIG-labelled cDNA was separated on a 1% agarose gel and blotted onto a nylon filter (Hybond N+, Amersham, Amersham, UK). Detection was carried out by an enzyme-linked immunoassay using an anti-DIG alkaline phosphatase conjugated antibody and lumigen/PPD as the substrate, according to the standard Boehringer (Mannheim, Germany) protocol. The blots were autoradiographed with XAR5 film (Kodak, Rochester MY, USA) for 4 h at room temperature.

Polymerase chain reaction (PCR)

In order to amplify exon 2 of the Cyca-DAB transcripts, two oligonucleotides 5'-CTG ATG CTG TCT GCT TTC ACT GGA GCA-3', starting at codon -6 and 5'-GAG TCA GCG ATC CGT GAT AAA ACA G-3' ending at codon 95 were produced based on *Cyca-DAB* cDNA sequences (Ono *et al.*, 1993). The expected size of the PCR fragment was 304 bp. In addition, two sets of primers specific for the amplification of *Cyca-DYB*, formerly designated *TLAIIß-1* (Hashimoto *et al.*, 1990), with each set positioned at the start and the end of the exons encoding ß1 and ß2 domains were used. The amplification was performed in *Taq* buffer (Promega 10X *Taq* buffer: 500 mM KCl, 100 mM Tris-HCl pH 9.0, 1% triton X-100), using 1 unit of *Taq* polymerase (Promega), supplemented with 1.5 mM $MgCl_2$, 0.2 μM of each primer and 200 μM of each dNTP in a final volume of 100 μl. Template concentrations were balanced according to the number of cpm incorporated into cDNA in a parallel reaction containing labelled nucleotide (see under RNA and cDNA preparation). A control PCR product from sure clone kit (Pharmacia) was used as internal control. The mixtures were subjected to a thermal cycle profile (1 min 94° C, 2 min 55° C, 1 min 72° C) for 25 cycles, with an additional final extension step at 72°C for 10 min. All PCRs were carried out on a Techne PH-3 (Techne, Cambridge, UK) thermocycler. A PCR under the same conditions as described above was performed using a radioactive nucleotide (^{32}P-dCTP). The ^{32}P-dCTP incorporation over 40 cycles showed that the conditions described above (*i.e.*, 25 cycles) were well below the saturation level of amplification.

***Cyca-DAB* probes**

For the detection of mRNA (northern dot blot) and of the serially diluted PCR products (southern dot blot), a DIG-labelled and fluorescein-labelled *Cyca-DAB* probe was prepared. The full-length *Cyca-DAB**01 clone (Ono *et al.*, 1993) was excised from the pBluescript SK+ plasmid, using Eco RI-restriction digestion. The cDNA fragment was separated from the plasmid on agarose gel, recovered and precipitated. The DIG labelling of the fragment was performed according to the standard Boehringer protocol, and the fluorescein labelling of the cDNA was carried out by following the manufacture's specifications (Amersham, UK).

Northern dot blot

Lymphoid organs (blood, head kidney, spleen, thymus and gut second segment) were removed and snap frozen. Whole organs were homogenized in lysis buffer (see under section RNA and cDNA preparation). The total RNA was phenol/chloroform extracted, precipitated in ethanol, washed and dissolved in water. The concentrations were measured by spectrophotometry (DU-62 spectrophotometer, Beckman) and the RNA stored at -80°C. The quality and concentration of the samples were checked by separation on a 1.5% agarose gel before the dot blot procedure. Total RNA samples were diluted in equal volumes of RNA dilution buffer (H_20:20X SSC:formaldehyde = 5:3:2) and subsequently serially diluted. The RNA samples were transferred to a dot-blot apparatus fitted with nylon filter (Hybond N^+) presoaked in 10X SSC. The blots were dried for 45 min and exposed to UV light for 5 min. The nylon filters were incubated for 5-6 h at 42° C in a prehybridisation solution containing 45% formamide, 2.5% blocking solution (Boehringer) 5X SSC, 0.02% SDS, 0.1% NaCl, with 100μg of denatured *E. coli* DNA. The hybridisation was carried out overnight at 42°C by adding the probe to a fresh hybridisation solution. The filters were washed under high stringency conditions (0.1X SSC and 0.1% SDS for 15 min at 65° C). The detection was carried out by an enzyme-linked immunoassay, according the standard Boehringer protocol, using an anti-DIG alkaline phosphatase antibody conjugate (anti-DIG-AP), and a subsequent enzyme-catalysed color reaction with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitroblue tetrazolium salt (NBT).

Southern dot blot

The PCR yields were visualized on a 1.5% agarose gel. Aliquots of the PCR products were serially diluted in TE buffer, and an equal volume of 20X SSC (SSC; 150 mM NaCl, 150 mM sodium-citrate pH 7.0) was added. The PCR samples were transferred to a dot-blot apparatus fitted with a nylon filter (Hybond N+). The blots were denatured (5 min in 1.5 M NaCl, 0.5 M NaOH), neutralized (1.5 M NaCl, 0.5 M Tris-HCl pH 7), alkali fixed (0.4 M NaOH), briefly rinsed in 5X SSC, and dried. The nylon filters were incubated for 5-6 h at 42°C in a prehybridisation solution containing 45% formamide, 5% blocking solution (Amersham), 5X SSC, 0.02% SDS, 0.1% NaCl, with 100μg of denatured *E.coli* DNA. The hybridisation was carried out overnight at 42°C by adding the probe to a fresh hybridisation solution. The filters were washed under high stringency conditions (0.1X SSC and 0.1% SDS for 15 min at 65°C).The detection was carried out with the addition of a chemiluminescent detection reagent according to manufactures specifications (Amhersham) and exposure to X-ray film.

Results

Tissue *Cyca-DAB* mRNA expression

In order to study the carp Mhc class II (*Cyca-DAB*) expression in the established lymphoid organs, RNA was isolated from thymus, spleen, head kidney and second segment of the gut. In addition RNA was extracted from whole blood and from erythrocytes only. A non- lymphoid tissue, namely skeletal muscle, was used for comparison. Quality of the RNA preparations were assessed by gel electrophoresis, and subsequently equal amounts (20μg) of total undegraded RNA extracted from whole organs was serially diluted and blotted onto nylon filters. The presence of *Cyca-DAB* mRNA was detected by analyzing these filters with a *Cyca-DAB* DIG-labelled probe under high stringency conditions.

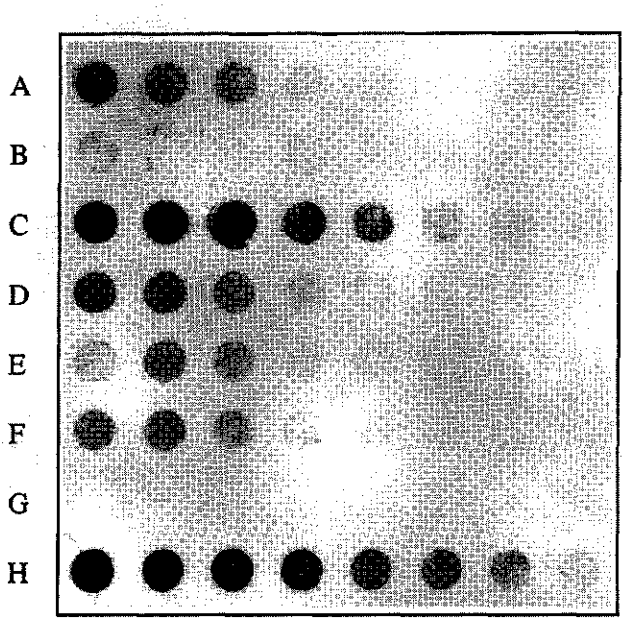

Figure 1. The levels of *Cyca-DAB* expression in different organs was analysed by Northern dot blot. From the organs total RNA was extracted and the concentrations were measured by spectrophotometry. The RNA quality was checked on agarose gel. From each sample, 20 μg of total RNA was serially diluted and blotted onto nitrocellulose. The blot was hybridised with a carp Mhc class II DIG-labeled probe. A-total blood; B-erythrocytes; C-thymus; D-head kidney; E-spleen; F-second segment of the Gut; G-skeletal muscle; H-positive control (probe)

A positive signal was obtained with RNA from whole blood, thymus, spleen, head kidney, and intestine (Fig 1). However, the steady state levels of RNA found between the several organs were different. The *Cyca-DAB* expression was relatively high in the thymus, and intermediate in peripheral blood, spleen, head kidney and the second segment of the gut. No detectable signal was obtained with RNA from skeletal muscle and erythrocytes.

Leucocyte characterisation

As several of the lymphoid organs express considerable amounts of *Cyca-DAB* mRNA, the cell types present in these tissues which might be responsible for the expression were investigated. In order to establish the nature of the cell types that were used for the RNA extraction, the cells, after density separation, were characterized by FACS analysis for both size and structure and for expression of surface immunoglobulin (sIg).

Several leucocyte fractions were obtained from the discontinuous Percoll gradient centrifugation of cell suspensions from different lymphoid organs. Fraction 1 corresponded to the 1.02-1.07 g/cm^3, fraction 2 to 1.06-1.07 g/cm^3, and fraction 3 to the 1.07-1.083 g/cm^3 density interface, respectively. PBLs were separated from erythrocytes using Lymphoprep and were not further density fractionated.

As Ig is the only lymphocyte marker available at the present for carp, and it is hypothesized that B lymphocytes are class II positive, and therefore the number of sIg$^+$ leucocytes was investigated. In order to determine the number of B cells in each fraction from the different tissues, cells were stained for surface immunoglobulin (sIg) using WCI 12 and analyzed by FACS. In addition, from the different cell populations, plots of the FSC/SSC, indicative of size and granularity, were analyzed. These analyses enabled the identification of lymphocytes and granulocytes, similar to previously reported observations (Verburg-Van Kemenade *et al.*, 1994; Koumans-Van Diepen *et al.*, 1994). From a number of cell suspensions the percentage of B cells was determined based on FACS analyses (Table 1).

The head kidney leucocytes were separated into three different fractions. Fraction 1 contained 15.9 % B lymphocytes, in the second fraction the B cells represented 5.6 %, and in the third fraction 3.1 % of Ig$^+$ cells were found. The splenocytes were also recovered from three interfaces and showed a similar distribution of B cells when compared with head kidney leucocytes. The thymocytes were separated in fraction 1 and 2, where the first fraction contained 3.8 % of Ig$^+$ cells, and in the second fraction only 0.7 % of B lymphocytes were found. All gut leucocytes were located in fraction 1, with 3.9 % of Ig$^+$ cells. The FACS analysis of peripheral blood leucocytes (PBL) separated on Lymphoprep showed that these contained 36.2 % B lymphocytes.

Table 1. The percentage of Ig^+ ($WCI\ 12^+$) leucocytes isolated by discontinuous percoll from each lymphoid tissue.

	Fraction 1†	Fraction 2†	Fraction 3†
Gut Leucocytes	3.9 ± 0.6	*	*
Thymocytes	3.8 ± 0.3	0.7 ± 0.1	*
Kidney Leucocytes	15.9 ± 3.4	5.6 ± 1.2	3.1 ± 0.8
Splenocytes	23.7 ± 4.6	9.7 ± 2.1	1.6 ± 0.1

†Each value represents the average (±SD) of six individual carp.
*No cells were recovered in this density interface.
The average of sIg^+ cells in PBL separated by lymphoprep was 36.2 ± 5.2.

Cyca-DAB expression in leucocytes isolated by density fractionation

To investigate the level of class II expression in different cell types, RNA was extracted according to the standard protocol. Equal concentrations of total RNA were analyzed on agarose gel and converted into cDNA. To assess efficiency of reverse transcriptase (RT) activity a trace reaction was carried out. Semi-quantitative PCR relies critically on similar amounts of template being present in each individual reaction. In order to compensate for this, equal amounts of cDNA, based on cpm from the trace reaction, were used as template for the PCR amplification. In addition variability of each individual PCR was controlled by the addition of a control mixture to the PCR reaction. This mixture containing a known template, and respective primers amplified a PCR product of a predicted size, independently of the *Cyca-DAB* sequence amplification. Invariably in each of the separate PCR experiments similar amounts of control template were generated based on estimation after gel electrophoresis (data not shown). Amplification of cDNA was carried out on PBLs, thymocytes (fraction 1 and 2), head kidney leucocytes (fraction 1, 2, and 3), and gut leucocytes (fraction 1 only). Aliquots of the PCR products were serially diluted and blotted onto nitrocellulose. The blot was hybridized with the *Cyca-DAB* fluorescein-labelled probe (Fig. 2).

The level of *Cyca-DAB* transcripts found in several lymphoid cells from different organs varied. Class II expression was high in peripheral blood leucocytes, thymocyte fraction 2, and the fraction 1 and 2 of head kidney leucocytes. Very low expression was seen in gut leucocytes. The thymocyte fraction 1 and head kidney fraction 3 showed intermediate expression. Amplification of *Cyca-DYB* cDNA fragments using specific primers however, could not be detected.

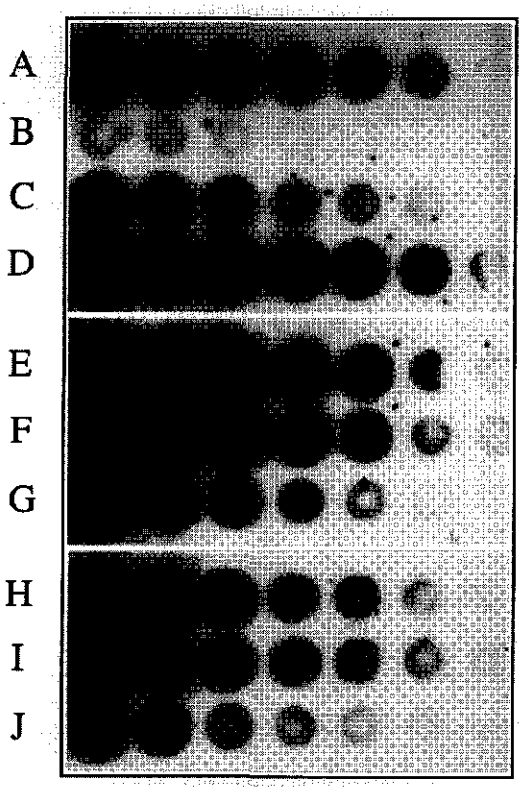

Figure 2. The level of class II *Cyca-DAB* transcripts in several lymphoid cells from different organs was analysed by PCR followed by Southern dot blot. Aliquots of the PCR products were serially diluted and blotted onto nitrocellulose. The blot was hybridised with a carp Mhc class II fluorescein-labeled probe. A-peripheral blood leucocytes; B-gut leucocyte fraction 1; C-thymocyte fraction 1; D-thymocyte fraction 2; E-head kidney leucocyte fraction 1; F-head kidney leucocyte fraction 2; G-head kidney leucocyte fraction 3; H-splenocyte fraction 1; I-splenocyte fraction 2; J-splenocyte fraction 3.

Cyca-DAB expression in adherent cells

Spleen and head kidney leucocytes were used to study the Mhc class II expression in adherent cells. Cells were isolated by density gradient centrifugation and the cells from fraction 2 containing mainly macrophages (Verburg-Van Kemenade *et al.*, 1994) were adhered to plates. The same adherence procedure was carried out for PBL. From each sample total RNA was extracted, and equal concentrations were converted into cDNA. Equal amounts of template were used for PCR amplification. Aliquots of the PCR products were serially diluted and blotted onto nitrocellulose. The presence of *Cyca-DAB* mRNA was detected by analysing these filters with a *Cyca-DAB* fluorescein labelled probe under high stringency conditions (Fig. 3.1).

Different levels of *Cyca-DAB* transcripts were detected in adherent leucocytes from different organs, with the lowest class II expression found in adherent cells from peripheral blood leucocytes. The adherent cells from fraction 2 of spleen and head kidney leucocytes, however, showed similar levels of expression.

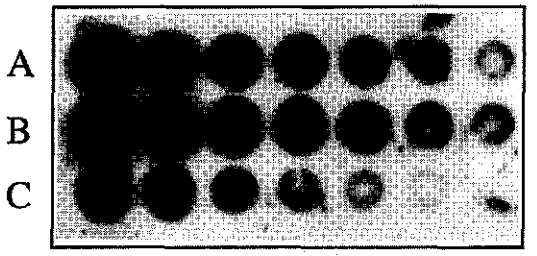

Figure 3.1 The levels of *Cyca-DAB* expression in adherent cells from the spleen head kidney and PBL were analysed by PCR followed by Southern dot blot. Cell suspensions were separated by density fractionation and adhered. PCR aliquots of the PCR products were serially diluted and blotted onto nitrocellulose. The blot was hybridised with a carp Mhc class II fluorescein-labeled probe. A-adherent PBL; B-adherent head kidney leucocytes; C-adherent splenocytes.

Cyca-DAB expression in sIg^+ and sIg^- cells

PBL, which contained 38.8 % B cells, were separated into two fractions based on sIg expression using a MACS. sIg^- and sIg^+ fractions were obtained with a purity of 98.0% and 93.4%, respectively (Fig. 4). Amplification by PCR of cDNA from PBLs, sIg^+ and sIg^- cells was carried out, as described above. Aliquots of the PCR products were serially diluted and blotted onto nitrocellulose. The blot was hybridized with the *Cyca-DAB* fluorescein labelled probe (Fig. 3.2).

Cyca-DAB expression in PBL used to obtained the sIg^+ and sIg^- populations was as expected (see Fig 2 and 4). However, mRNA isolated from sIg^- cells showed small amounts of *Cyca-DAB* transcripts compared to that of sIg^+ cells. The latter population contained comparable amounts as seen with unfractionated PBL (Fig. 3.2).

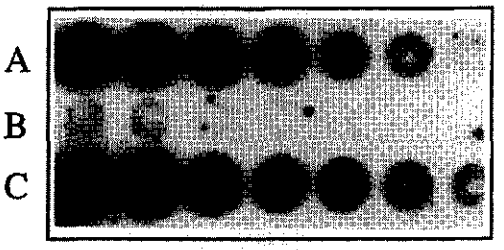

Figure 3.2 The levels of *Cyca-DAB* expression in PBL subpopulations was analysed by Southern dot blot (see above). PBL were isolated by density fractionation and separated by MACS into sIg^- and sIg^+ cells. A-sIg^+ fraction; B-sIg^- fraction; C-unseparated PBL.

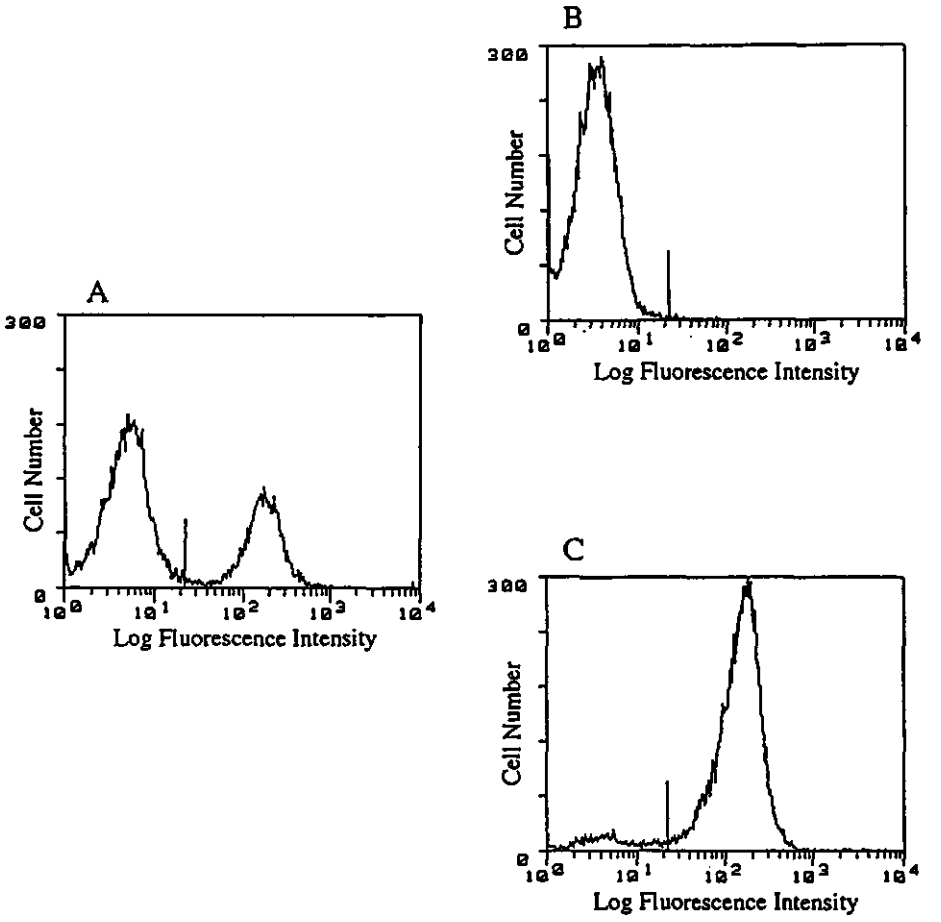

Figure 4. PBL separation by the use of Magnetic Activated Cell Sorter (MACS). Peripheral blood leucocytes were stained with a monoclonal antibody WCI 12, recognizing carp membrane immunoglobulin. Fluorescence intensity histograms from the unsorted and sorted cell populations are depicted. A-unfractionated PBL (36.7% of sIg^+ cells); B-sIg^- fraction (0.7% of sIg^+ cells); C-sIg^+fraction (94.7% of sIg^+ cells).

Discussion

Although it has been well established that Mhc class I and class II molecules are involved in binding of self and non-self peptides (Bjorkman *et al.*, 1987; Brown *et al.*, 1993), such expression can only be fully implicated in the immune response in the context of a given microenvironment. Therefore, it is necessary to study the expression of class II molecules in particular. In general studies on Mhc expression carried out so far, it has been shown that Mhc class I and II molecules have a different tissue distribution (Klein, 1986). Mhc class I genes are expressed in most somatic cells, whereas class II molecules are known to have a restricted tissue distribution, being expressed predominantly on cells of the immune system.

Previous qualitative studies using a Northern analysis have shown expression of *Cyca-DAB* in spleen, head kidney, gut, and liver, but not in heart, skeletal muscle, brain and ovaries (Ono *et al.*, 1993). In this study this qualitative analysis of class II expression was extended. Concurrently it was shown that *Cyca-DYB* is not expressed in our carp. Moreover, no expression of this gene have been reported so far (Dixon *et al.*, 1993). The first analysis comprised a Northern dot blot of total RNA isolated from whole organs with established immunological functions, i.e. thymus, head kidney, spleen, intestine and blood (Manning, 1994), and as a control muscle tissue was included. This revealed that the thymus expressed the highest level of class II transcripts, followed by similar levels detected in all other organs studied, except muscle tissue, which was negative (Fig. 1). This distribution of class II expression is in agreement with that described for rainbow trout (Juul-Madsen *et al.*, 1992). The lack of expression in muscle is consistent with the fact that this tissue clearly does not contain abundant lymphoid cells, nor epithelial and endothelial cells. Levels of class II expression detected in spleen, head kidney, gut, and blood could be attributed to a number of different cell types; leucocytic, endothelial and epithelial cells, based on observations in other vertebrates (Turner, 1994). Although the proportion of these cell types is clearly different in spleen, head kidney, gut, and blood, levels of *Cyca-DAB* transcripts seem to be similar. No information, however, is generated on the cell type responsible for this expression using this approach. The level of class II expression in the thymus as observed in the analysis of total RNA obtained from organ homogenates is consistent with that seen in chicken (Bourlet *et al.*, 1988), and mammals (Klein, 1986). In comparison to other lower vertebrates, general patterns of class II expression in the thymus as detected with antibodies are only available from *Xenopus* (Du Pasquier *et al.*, 1990; Flajnik *et al.*, 1990; Rollins-Smith *et al.*, 1990) and to a lesser degree for chicken (Ewert *et al.*, 1984). These studies indicated that not only epithelial cells of the cortex and

medullary antigen presenting cells (APC) express class II molecules, but more importantly also thymocytes. Similarily, in some mammalian species thymocytes can express class II molecules as well . The fact that especially in the thymus more than one cell type can express class II transcripts warrants a more detailed analyses of the cells responsible for the observed *Cyca-DAB* expression.

Cell characterization is critically dependent on the availability of cell surface markers, morphological parameters and functional assays. In carp the only cell marker available is surface immunoglobulin (sIg) which identifies leucocytes of the B-cell lineage (Secombes *et al.*, 1983). Other leucocytes are mainly described by morphological and/or functional characteristics (Verburg-Van Kemenade *et al.*, 1994; Rombout *et al.*, 1993; Van Diepen *et al.*, 1991; Koumans-Van Diepen *et al.*, 1994). In the current study cells were separated by density, and identified on the basis of expression of sIg and morphology using the FACS. It was hypothesized that, similar to the situation in other vertebrates (Turner, 1994), carp B cells constitutively express class II molecules, which may be reflected in the amount of mRNA transcripts detected in those cells.

In PBLs the high expression of class II transcripts, as detected by southern analyses of PCR amplified cDNA (Fig. 2), correlated well with the number of B cells as established by FACS (Table 1). The high levels of class II expression found in the gut were not detected in isolated gut leucocytes. This observation seems to indicate that other cells, possibly epithelial cells, rather than gut leucocytes are mainly responsible for the amount of class II transcripts detected in this tissue. Gut leucocytes on average only contain 3.9% sIg^+ cells, which is reflected in the low level of expression of *Cyca-DAB* found. Moreover, as it has been demonstrated (Rombout *et al.*, 1993) that in the gut second segment the majority of sIg^+ cells are not B cells, but most likely intraepithelial macrophages, which have bound exogenous Ig. This observation seems to lend support to the hypothesis that there might be a correlation between B cells and Mhc class II expression. The levels of class II expression in head kidney fractions 1 and 3 indeed follows that of the relative representation of the number sIg^+ cells (Table 1). Fraction 1 contains mainly lymphocytes, whereas in fraction 3 the majority of the cells are granulocytes. Similar levels of expression, however, were found in head kidney fraction 1 and 2, although much fewer B cells were present in fraction 2. A possible explanation for this observation could be the fact that this fraction contains considerably more macrophages than fraction 1 and 3 (Verburg-Van Kemenade *et al.*, 1994), which may contribute to the observed increased expression of class II mRNA. The technique used to detect the expression was carefully controlled by using equal amounts of undegraded total RNA assessed both by gel electrophoresis and UV spectrophotometry. Subsequently, cDNA synthesis was traced in a parallel reaction and PCR performed in the presence of an internal control. An internal RNA

control is essential for an accurate semi-quantitative analysis of low-copy number messengers in a limited cell sample (Murphy *et al.*, 1993). However, it was expected that the amount of class II transcripts was of a level similar to that in other vertebrates, which allowed a relatively simple semi-quantative approach. In our experiments we controlled the amount of RNA and efficacy of the cDNA synthesis, instead of using a house-keeping genes as control, as it has been indicated that the expression of these genes can also vary to some extent (Siebert *et al.*, 1992). Cellular composition of density fractionated splenocytes is comparable to that of head kidney cells, and therefore the mRNA class II expression was expected to be similar.

Thymocytes were density fractionated into two fractions which showed very different levels of class II expression. Similar to the other organs, the possibility of a correlation between the number of sIg^+ cells and class II expression was investigated. In this case fraction 2, which contained only 0.7% of B cells, was found to express the highest amount of class II transcripts. FACS analysis of this fraction revealed that it only contained small leucocytes, and therefore is it likely that sIg^- thymocytes express class II transcripts.

In mammals class II expression in the thymus is mainly confined to epithelial cells, dendritic cells and macrophages , whereas both cortical and medullary thymocytes are class II negative (Klein, 1986). In chicken using Northern blot analysis, high expression of *B-Lβ* genes was found in the thymus (Bourlet *et al.*, 1988), but it was shown that this is probably due to class II expression in epithelial cells, and not to class II positive thymocytes (Ewert *et al.*, 1984). However, studies carried out in *Xenopus* using monoclonal antibodies showed that an age-dependent proportion (32% at 5-6 months) of thymocytes express class II molecules in postmetamorphic frogs (Rollins-Smith *et al.*, 1990). Thus, in carp a similar situation is found in which an unknown proportion of thymocytes express class II molecules. Further investigations are necessary to establish a possible age-dependent expression. Between thymus fraction 1 and 2 there is a clear difference in the level of class II expression. The cellular composition of each fraction could be an explanation for this difference. In *Xenopus* it has been shown that medullary thymocytes express more class II molecules than thymocytes in the cortex (Du Pasquier *et al.*, 1990). However, in carp the thymus is not organized into a distinct cortex and medulla (Botham and Manning, 1981), although different microenvironments can be expected, resulting in thymocytes expressing different developmental markers. In the present study FACS analyses could detect, apart from a difference in the number of sIg^+ cells, no differences in the cellular contents of the two fractions. However, these fractions may still contain different maturation stages of T cells, responsible for the difference in their class II expression, similar to the situation in *Xenopus*.

Cyca-DAB is expressed in adherent cells from PBL, spleen and head kidney. However, the amount of transcripts found in adherent PBL was lower then that found in spleen and head

kidney. This may be explained by a different cell composition in the adherent cells from PBL, spleen and head kidney. Adherent cells from spleen and head kidney are most likely to be enriched in adult macrophages (Verburg-Van Kemenade *et al.*, 1994), whereas adherent cells found in PBL are most likely to be enriched in monocytes. Undifferentiated macrophages do not always express class II genes (Klein, 1986). While it is possible that the *Cyca-DAB* expression seen in all these adherent cells might be due to the presence of adherent B lymphocytes, the numbers of Ig^+ cells are higher in PBL that in the spleen and head kidney (see Table 1), which would give a different relative proportion of transcripts than that observed (Fig 3.1). Thus the amount of *Cyca-DAB* transcripts depicted most likely reflects the actual expression pattern of the gene. This observation is reinforced by the fact that a similar distribution is seen in chicken and *Xenopus* (Peck *et al.*, 1982; Ewert *et al.*, 1984).

The amount of Cyca-DAB transcripts found in the PBL Ig^+ cells was much higher them the Ig^- cells. Due to this large difference of class II expression it seems evident that the main cell type responsible for the levels of *Cyca-DAB* expression observed in unseparated PBL is the B lymphocyte. The very low class II expression levels found in Ig^- PBL, may be due to some residual adherent leucocytes, which express *Cyca-DAB* (see Fig 3.1), or a small percentage of Ig^+ cells (Fig. 4B). It is clear, however that the majority of the non-adherent Ig^- PBL probably do not express *Cyca-DAB*, which agrees with studies carried out in other vertebrate species (Klein, 1986; Turner, 1994).

In conclusion,the expression pattern of *Cyca-DAB* follows the pattern observed in other lower vertebrates. Transcripts of class II genes are found only in tissues that play a role in the teleost's immune system. The expression of *Cyca-DAB* found in these tissues can be attributed to leucocytes that have known immunological functions. Within the thymus, *Cyca-DAB* expression is highest in thymocytes. Within spleen and head kidney, where antigen presentation is known to occur, class II mRNA expression is high in adherent cells. These adherent cells are most probably adult macrophages which are known to present antigen. Class II expression in PBL is high in Ig^+, and occurs at very low levels in Ig^- cells. This suggests that *Cyca-DAB* is probably constitutively expressed in B lymphocytes. The cell type responsible for the low class II expression in the Ig^- fraction could not be positively determined, but could be attributed to low level of contaminant B cells or adherent cells. However, it seems likely that non-adherent Ig^- will be class II negative. The latter conclusion awaits positive identification of circulating mature T cells.

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References

Bjorkman, P.J., Saper, M.A., Samraoui, B., Bennett, W.S., Strominger J.L. and Wiley, D.C.(1987) The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature* **329**: 512-518.

Bourlet, Y., Béhar, G., Guillemot, F., Fréchin, N., Chaussé, A.M., Zoorob, R. and Auffray, C.(1988)Isolation of chicken major histocompatibiitly complex class II (B-) ß chain sequences: comparison with mammalian ß chains and expression in lymphoid organs. *EMBO J.* **7**: 1031-1039.

Botham, J.W. and Manning, M. J. (1981) The histogeneses of the lymphoid organs in the carp *Cyprinus carpio* L. and ontogenic development of allograft reactivity. *J. Fish Biol.* **19**: 403-414.

Brown, J. H., Jardetzky, T.S., Gorga, J.C., Stern, L. J., Urban, R.G., Strominger, J. L. and Wiley, D. C. (1993) Three-dimensional structure of human class II histocompatibility antigen HLA-DR1. *Nature* **364**: 33-39.

Dixon, B., Van Erp, S.H.M., Rodrigues, P.N.S., Egberts, E. and Stet, R.J.M. (1995) Fish major histocompatibility complex genes: an expansion. *Dev. Comp. Immunol.* **19**: 109-135.

Dixon, B, Stet, R.J.M., Van Erp, S.H.M. and Pohajdak, B. (1993) Characterization of ß2-microglobulin transcripts from two teleost species. *Immunogenetics* **38**: 27-34.

Du Pasquier, L. and Flajnik, M.F. (1990) Expression of MHC class II antigens during *Xenopus* development. *Dev.Immunol.* **1**: 85-95.

Ewert, D.L., Munchus, M.S., Cheng, C.H. and Cooper M.D. (1984) Analysis of structural properties and cellular distribution of avian Ia antigen by using monoclonal antibody to monomorphic determinants. *J. Immunol.* **132**: 2524-2530.

Flajnik, M.F., Ferrone, S., Cohen, N. and Du Pasquier, L. (1990) Evolution of the MHC: antigenicity and unusual tissue distribution of *Xenopus* (frog) class II molecules. Molecular Immunol. **27**: 451-462.

Hashimoto, K., Nakanishi, T. and Kurosawa, Y. (1990) Isolation of carp genes encoding major histocompatibility complex antigens. *Proc. Nat. Acad. Sci. USA* **87**: 6863-6867.

Juul-Madsen, H.R., Glamann, J., Madsen, H.O. and Simonsen, M. (1992) MHC class II beta-chain expression in the rainbow trout. *Scand.J.Immunol.* **35**:687-694

Klein, J. (1986) *The Natural History of the Major Histocompatibility Complex.* John Wiley and sons: New York

Koumans-Van Diepen, J.C.E., Harmsen, E.G.M. and Rombout, J.H.W.M. (1994) Immunocytochemical analysis of mitogen responses of carp (*Cyprinus carpio* L.) peripheral blood leucocytes. *Vet. Immunol. and Immunopathol.* **42**: 209-219.

Koumans-Van Diepen, J.C.E., Taverne-Thiele, J.J., Van Rens, B.T.T.M. and Rombout, J.H.W.M. (1994) Immunocytochemical and flow cytometric analysis of B cells and plasma cells in carp (*Cyprinus carpio* L.); an ontogenetic study. *Fish and Shellfish Immunol.* **4**: 19-28.

Manning, M.J. (1994) Fishes. In: *Immunology a comparative approach*. Turner ed., John Wiley and sons, New York, pp 69-100.

McCluskey J.(1991) *Antigen Processing and Recognition*. CRC Press: Boca Raton, Florida.

Murphy, E., Hieny, S., Sher, A. and O'Garra, A. (1993) Detection of in vivo expression of interleukin-10 using a semi-quantitative polymerase chain reaction method in *Schistosoma mansoni* infected mice. *J. Immunological Methods* **162**: 211-223.

Okamura, K., Nakanishi, T., Kurosawa, Y. and Hashimoto, K. (1993) Expansion of genes that encode MHC class I molecules in cyprinid fishes. *J. Immunol.* **151**: 188-200.

Ono, H., O'hUigin, C., Vincek, V., Stet, R. J. M., Figueroa, F. and Klein, J. (1993) New ß chain-encoding Mhc class II genes in the carp. *Immmunogenetics* **38**: 146-149.

Peck, R., Murthy, K.K. and Vainio, O. (1992) Expression of *B-L* (Ia-like) antigens on macrophages from chicken lymphoid organs. *J. Immunol.* **129**: 4-5

Rollins-Smith, L.A. and Blair, P. (1990) Expression of class II major histocompatibility complex on adult T cells in *Xenopus* is metamorphosis dependent. *Dev. Immunol.* **1**: 97-104.

Rombout, J.H.W.M., Taverne-Thiele, A.J. and Villena, M.I. (1993) The gut-associated lymphoid tissue (GALT) of carp (*Cyprinus carpio* L.): an immunocytochemical analysis. *Dev. Comp. Immunol.* **17**: 55-66.

Secombes, C.J., Van Groningen, J.J.M. and Egberts E. (1983) Separation of lymphocyte subpopulations in carp by monoclonal antibodies: immunohistochemical studies. *Immunol.* **48**: 165-175.

Siebert, P.D. and Larrick, J.W. (1992) Competitive PCR. *Nature* **359**: 557-558.

Stet, R.J.M. and Egberts, E. (1991) The histocompatibility system in teleostean fishes: from multiple histocompatibility loci to a major histocompatibility complex. *Fish and Shellfish Immunol.* **1**: 1-16.

Turner, R.J. (1994) *Immunology a comparative approach*. John Wiley and sons, New York.

Vallejo, A.N., Miller, N.W. and Clem, L.W. (1992) Antigen presentation in teleost immune responses. *Annual Rev. of Fish Diseases*. pp 73-89.

Van Erp, S.H.M., Dixon B., Figueroa F., Egberts E. and Stet, R.J.M. Identification and characterisation of a new MHC class I gene from carp (*Cyprinus carpio* L.). *Immunogenetics* (in press)

Van Erp, S.H.M., Egberts, E. and Stet, R.J.M. Characterization of major histocompatibility complex class II *A* and *B* genes in a gynogenetic carp clone. *Immunogenetics* (in preparation)

Van Diepen, J.C.E., Wagenaar, G.T.M. and Rombout, J.H.W.M. (1991) Immunocytochemical detection of membrane antigens of carp leucocytes using light and electron microscopy. *Fish and Shellfish Immunol.* **1**: 47-57.

Verburg-Van Kemenade, B.M.L., Groeneveld, A., Van Rens, B.T.T.M. and Rombout, J.H.W.M. (1994) Characterization of macrophages and neutrophilic granulocytes from the pronephros of carp (*Cyprinus carpio* L.). *J. Exp. Biol.* **187**: 143-158.

Chapter 3

Expression and temperature dependent regulation of the beta$_2$-microglobulin (*Cyca-B2m*) gene in a cold-blooded vertebrate the common carp (*Cyprinus carpio* L.).

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Summary

The expression of beta$_2$-microglobulin (β_2m) in the common carp (*Cyprinus carpio* L) was studied using a polyclonal antibody raised against a recombinant protein obtained from prokaryotic expression of the *Cyca-B2m* gene. β_2m is expressed on peripheral blood Ig^+ and Ig^- lymphocytes, but not on erythrocytes and thrombocytes. In spleen and pronephros dull- and bright-positive populations could be identified correlating with the presence of erythrocytes, thrombocytes and mature leucocytes or immature and mature cells from the lympho-myeloid lineage, respectively. Thymocytes were shown to be comprised of a single bright-positive population. The Cyca-B2m polyclonal antiserum was used in conjunction with a similarly produced polyclonal antiserum to a class I (Cyca-UA) α-chain to investigate the expression of class I molecules on peripheral blood leucocytes (PBL) at different permissive temperatures. At 12°C a temporary down-regulation of class I molecules was demonstrated, which recovered to normal levels within three days. However, at 6°C a lasting absence of class I cell surface expression was observed, which could slowly be restored by transfer to 12°C. The expression of immunoglobulin molecules on B cells was unaffected by temperature changes. The absence of the class I cell surface expression was shown to be the result of a lack of sufficient *Cyca-B2m* gene transcription, although *Cyca-UA* mRNA was present at comparable levels at all temperatures. This suggests that class I expression is regulated by a temperature-sensitive transcription of the *Cyca-B2m* gene.

Key words: Carp, temperature, Cyca-B2m, Cyca-UA, cell surface expression, transcription

Introduction

The major histocompatibility complex (Mhc) class I heterodimers are cell surface glycoproteins comprising a heavy chain, normally referred to as α chain, which is associated noncovalently with β_2-microglobulin (β_2m) (Klein, 1986). In the common carp, the β_2m and class I α molecules are encoded by the *Cyca-B2m* and *Cyca-UA* genes, respectively. The Cyca-B2m mature protein has 97 amino acids and a deduced molecular weight of 11,174 Da (Dixon *et al.*, 1993). The Cyca-UA mature protein has 332 amino acids with three external domains, a transmembrane, and a cytoplasmic segment (Van Erp *et al.*, 1996). The inferred Cyca-UA molecular weight is 37,213 Da, excluding the putative glycosylation products.

Generally Mhc class I molecules are located on the surface of most cells and are involved in binding and presentation of antigen to a subset of T lymphocytes, the cytotoxic T cells (Bjorkman *et al.*, 1987; Salter *et al.*, 1990). For the efficient transport of properly folded class I molecules to the cell surface, the association of the class I α chain with both peptide and β_2m is required (Vitiello *et al.*, 1990). The peptide of a certain length as well as β_2m are essential not only to promote Mhc class I assembly, but also to give stability to the heterodimer (Townsend *et al.*, 1989; Townsend *et al.*, 1990). The free class I heavy chain that results from the dissociation of the β_2m from previously assembled heterodimers, appears to be unable

to present peptides to T cells (Rock *et al.*, 1991). Although the stability of the class I heterodimer is temperature dependent as shown in experiments with the RMA-S cell line (Ljunggren *et al.*, 1990), nothing is known of such requirements for class I trimolecular stability in poikilothermic animals such as teleostean fish.

The immune system of fish, although with some differences, seems to be comprised of the same basic features found in other vertebrates (Turner, 1994). For some time functional assays (*e.g.*, skin transplantation, MLR, *in vitro* antibody production) were considered to be evidence for the presence of an MHC in teleostean fish (Stet and Egberts, 1991). Recently, however, a large number of Mhc (class I α, β_2m, class II α, and class II β) and Mhc related, genes of different teleost species have been identified (Dixon *et al.*, 1995). As yet no data are available concerning the functional properties of the molecules encoded by these Mhc genes (Stet *et al.*, 1996). Studies on function require appropriate tools, such as antibodies. Due to the availability of carp β_2m (*Cyca-B2m*) and class I α-chain (*Cyca-UA*) full-length cDNAs, it became possible to produce recombinant proteins and raise polyclonal antibodies to such proteins as has recently been demonstrated for the Cyca-UA class I molecule (Van Erp *et al.*, 1996).

Experiments in the past have shown that the immune response of fish kept at the lower limit of their physiological temperature is severely impaired (Bly and Clem, 1992). These findings were further investigated by studies carried out in the channel catfish (*Ictalurus punctatus*), where it was demonstrated that *in vitro* teleost antigen-presenting cells are able to take up, process, and present exogenous antigen and trigger antibody production at low permissive temperatures (Vallejo *et al.*, 1992). It was concluded that the previously observed temperature-dependent suppression of primary T-cell responses in fish could not be attributed to impaired class II expression. However, the effect on Mhc class I molecule expression has never been investigated in fish.

In this study the expression of class I molecules is investigated with the aid of polyclonal antibodies raised against recombinant carp class I α-chain (Cyca-UA) and β_2m (Cyca-B2m) molecules, in carp kept at different permissive temperatures.

Materials and methods

Animals

Common carp (*Cyprinus carpio* L), were reared at 23 °C in recirculating UV-sterilized water, and fed pelleted dry food (Provimi, Rotterdam, The Netherlands) at a ration of 2 % of body weight per day. Animals from a single F1 hybrid family R3 x R8, weighing between 150-250 g (10-16 months), were used. R3 and R8 are partly inbred strains of common carp originating from Poland and Hungary, respectively (Wiegertjes *et al.*, 1994).

Production of recombinant protein and polyclonal antisera

A PCR fragment was produced by anchored PCR using the previously described *Cyca-B2m* cDNA clone in conjunction with the specific primer OL-85 (Dixon *et al.*, 1993). This fragment, which contains the last 73 codons and the 3' untranslated region of the clone, was cloned into the vector pCRII and sequenced using the Sequenase version 2.0 kit (U.S. Biochemicals, Cleveland OH, USA). Following sequence confirmation, this fragment was excised from the vector using *Eco*RI and ligated in frame into the vector pRSET (Invitrogen, Leek, The Netherlands). The plasmid was transformed into bacterial strain BL21-DE3 (Novagen, Madison WI, USA), and recombinant protein production was induced using 0.8 mM IPTG. The recombinant B2m protein was purified by nickel-affinity chromatography using the Xpress system (Invitrogen). Under denaturating conditions, SDS-PAGE was used to determine the purity and size of the recombinant protein. Four hundred micrograms of the protein were conjugated to 2 mg of KLH by glutaraldehyde, and injected i.m. into a rabbit at two sites. The animal was boosted after one month, and bled at two months post-injection. Serum was collected after clotting, and stored at -80 °C for future use.

Western blotting

The recombinant B2m protein was further purified over Sephadex G-100 to remove contaminating bacterial proteins, and separated on a denaturing 15 % SDS polyacrylamide gel. The protein was transferred to a nitrocellulose filter by electroblotting, and the filter was incubated with the polyclonal antisera at an appropriate dilution. Reactivity of the rabbit serum was determined using an alkaline-phosphatase-conjugated goat-anti rabbit immunoglobulin, and visualized using NBT and BCIP substrate buffer.

Cell isolation

Fish were anaesthetized in tricaine methane sulphonate (TMS; Crescent Research Chemicals, Phoenix, USA) at 3 g/10 L, and heparinized blood was collected from the dorsal aorta. The blood sample was diluted 1:1 in cRPMI (RPMI 1640 adjusted to 270 mOsm), and peripheral blood leucocytes were separated on Lymphoprep (Nycomed, Oslo, Norway) by centrifugation at 840 x g for 30 min at 4°C. The cells were harvested from the interface, washed twice and resuspended in cRPMI. The peripheral blood leucocytes were used for further analyses. Pronephros, spleen, and thymus cell suspensions were prepared by forcing the tissues through a 50 mesh nylon gauze filter while adding cRPMI. After having been washed in cRPMI (680 x g for 10 min at 4°C) and resuspended, the cell suspensions were separated on Lymphoprep by centrifugation at 840 x g for 30 min at 4°C. The cells were harvested, washed twice and resuspended at a concentration of 10^7 cells/ml in cRPMI.

Flow cytometry (FACS)

For the flowcytometry studies the following antibodies were used: the polyclonal antiserum described in this paper raised against recombinant Cyca-B2m protein; an anti-class I (Cyca-UA) polyclonal serum (Van Erp *et al.*, 1996); the WCI12 monoclonal antibody, which detects carp surface immunoglobulin (Secombes *et al.*, 1983); and the WCL6 monoclonal antibody specifically recognising carp thrombocytes (Rombout *et al.*, 1994). Peripheral blood leucocytes (PBL), isolated as described above, were incubated for 30 min on ice in 0.5 ml of appropriately diluted (usually 1:100) Cyca-UA or Cyca-B2m polyclonal antibodies. For all the incubation and washing steps FACS medium containing cRPMI, 1% BSA and 0.1% NaN_3 was used. After washing, binding of the polyclonal antibodies was detected by incubating the cells for 15 min on ice with phycoerythrine (PE)-conjugated goat-anti-rabbit immunoglobulin antibody (GAR-PE; Southern, Birmigham AL, USA), diluted 1:100 in FACS medium, and containing 1% of pooled carp serum. For the PBL double labelling, leucocytes were incubated with the Cyca-B2m polyclonal antiserum together with WCI12 or WCL6, followed by an incubation with GAR-PE and fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse immunoglobulin serum (Dakopatts, Glostrup, Denmark). Cells were analyzed using a FACStar (Becton Dickinson Immunocytometry Systems, Mountain View CA, USA) with an argon laser tuned at 488 nm. For analyzing the FACS results the Consort 30 data analysis package was used.

Temperature experiments

A group of eighteen carp from the same F1 generation (R3 x R8) were kept at three different temperatures. The control group of six individuals was maintained at normal rearing conditions, *i.e.*, at a temperature of 24°C throughout the experiment. The remaining twelve individuals

were kept at 12°C. After three days, six animals from the 12°C group were transferred to 6°C. The resulting three groups of six animals were kept at these temperatures for at least two weeks. After this period the carp at 12°C were transferred to 24°C and the animals at 6°C were placed at 12°C. Experimental fish were bled at regular intervals, and isolated PBL were labelled with different sera and analyzed by FACS. Small numbers of carp were kept at the three different temperatures for up to one month, after which they were sacrificed for further transcription analyses using RNA isolation procedures.

RNA and cDNA preparation

RNA and cDNA preparations for semi-quantitative PCR analyses were performed as described by Rodrigues and co-workers (1995). Briefly, cells were thawed out in lysis buffer (4 M guanidium thiocyanide, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2ß-ME), followed by phenol/chloroform extractions. Total RNA was precipitated in ethanol, washed and dissolved in water, and stored at -80°C. Samples containing 10μg of total RNA were converted into cDNA using the Riboclone cDNA Synthesis System (Promega, Madison WI, USA). Efficiency of cDNA synthesis was traced by determining the incorporation of fluorescein-dUTP in a parallel reaction. The labelled cDNA samples were serially diluted and blotted onto a nylon filter (Hybond N+, Amersham, UK). Detection was carried out by an enzyme-linked immunoassay using an anti-fluorescein alkaline phosphatase conjugate, subsequent addition of chemiluminescent detection reagent (Amersham, UK), and exposure to XAR5 film (Kodak) for 16 h at room temperature.

Polymerase chain reaction (PCR)

In order to detect *Cyca-B2m* transcripts by PCR, two oligonucleotides 5'-ATG AGA GCA ATC ATC ACT TTT GC-3' starting at codon 1, and 5'-TTA CAT GTT GGG CTC CCA AA-3' ending at codon 98, were produced based on the *Cyca-B2m* sequences (Dixon *et al.*, 1993). Similarly, for the detection of *Cyca-UA* transcripts, two oligonucleotides 5'-GGT GTT CAC TCA GTC CAG-3' starting at codon 1 of α_2 domain, and 5'-GCG CCT GCA GTT TTG ATC TTG TCC-3' ending at codon 96 of the α_3 domain, were produced based on the *Cyca-UA* cDNA sequences reported (Van Erp *et al.*, 1996). The amplification was performed in *Taq* buffer (Eurogentec, Seraing, Belgium), using 1 unit of Goldstar *Taq* polymerase (Eurogentec), supplemented with 1.5 mM $MgCl_2$, 0.2 μM of each primer and 200 μM of each dNTP in a final volume of 100 μl. Template concentrations were balanced according to the levels of Fl-dUTP incorporated into cDNA in a parallel reaction (see under RNA and cDNA preparation). The mixtures were subjected to a thermal cycle profile (1 min 94°C, 2 min 55°C, 1 min 72°C) for the indicated number of cycles, and analyzed by agarose gel electrophoresis.

Results

Characterization of the carp ß$_2$-microglobulin antiserum

We have previously reported the cloning of a full-length cDNA encoding carp ß$_2$-microglobulin (*Cyca-B2m*) (Dixon *et al.*, 1993). Part (73 codons) of the *Cyca-B2m* cDNA was cloned in frame into the pRSET vector, and after expression of the clone in bacteria, the resultant recombinant protein was used to immunise rabbits. The antiserum obtained was tested against the purified recombinant protein in a western blot. The serum reacted with a band of relative molecular mass (M_r) of 13.7 kD from the recombinant protein extraction (Fig. 1).

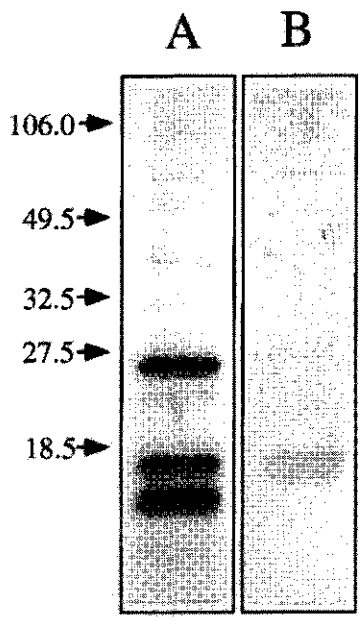

Figure 1 A recombinant Cyca-B2m protein was transferred to a nitrocellulose filter, and the western blot was incubated with the Cyca-B2m antiserum. Reactivity of the rabbit serum was determined using an alkaline-phosphatase-conjugated goat-anti rabbit immunoglobulin, and visualized using NBT and BCIP substrate buffer. (**A**) Purified recombinant Cyca-B2m separated by SDS-PAGE. (**B**) Western blot of the recombinant Cyca-B2m detected with the rabbit antiserum.

The antiserum was tested on live cells by FACS analyses (Fig. 2A). The pre-immune serum staining was negative, being close to the range of the conjugate only. The specific antiserum recognized an antigen on the cell surface of carp peripheral blood leucocytes (PBL), labelling strongly the majority (66.5%) of the leucocytes (Fig. 2A).

Detailed FACS analyses, using double staining of the Cyca-B2m antiserum combined with monoclonal antibodies to Ig^+ B cells (WCI12) or to thrombocytes (WCL6), was carried out on carp PBL (Fig. 2B and 2C). The different percentages of the labelled PBL were determined from the FACS experiments. For the double staining with the monoclonal antibody against surface Ig the percentages were: Cyca-$B2m^+$/$WCI12^+$ (42%), Cyca-$B2m^+$/$WCI12^-$ (17%), Cyca-$B2m^-$/$WCI12^+$ (2%) and Cyca-$B2m^-$/$WCI12^-$ (37%). In the case of the thrombocyte marker, the following proportions were found: Cyca-$B2m^+$/$WCL6^+$ (7%), Cyca-$B2m^+$/$WCL6^-$ (57%), Cyca-$B2m^-$/$WCL6^+$ (17%) and Cyca-$B2m^-$/$WCL6^-$ (19%).

In order to study the expression of the Cyca-B2m in the major lymphoid organs of the carp (*i.e.*, thymus, spleen and pronephros), leucocytes were isolated, labelled with the polyclonal anti-carp B_2m serum and analyzed by FACS (Fig 3). No expression was detected on erythrocytes, as indicated by the fact that the staining of these cells was similar to the negative control, *i.e.*, conjugate only (Fig. 3A). The antiserum recognized leucocytes isolated from the thymus, pronephros, and spleen, but the level of expression differed between these cell populations. Pronephrocytes and splenocytes could be divided into three populations; a negative, a dull-, and a bright-positive population. In the pronephros equal representation of negative and bright-positive populations was observed, whereas with splenocytes the majority of the cells were found in the negative and dull-positive populations (Fig. 3A and 3B). Thymocytes comprised only bright-positive cells (Fig. 3B).

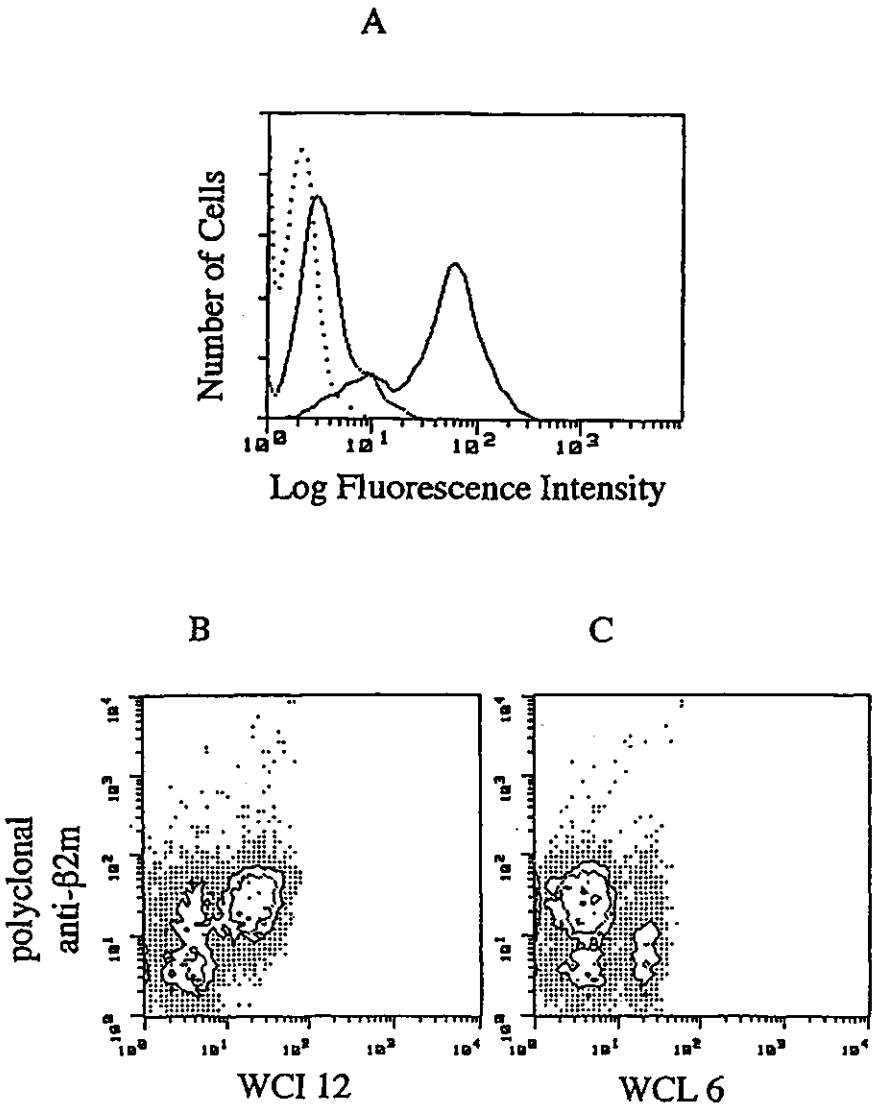

Figure 2. Peripheral blood leucocytes (PBL) were labelled with the anti-b2m recombinant protein serum and the binding of the polyclonal was detected with goat-anti-rabbit IgG conjugated with PE (GAM-PE). (**A**) The fluorescence intensity histogram depicted represents: (·····) conjugate only, (——) pre-immune serum, (—) immune serum. (**B** and **C**) Fluorescence intensity contour graphs of double labelled peripheral blood leucocytes (PBL) with the Cyca-B2m antiserum, combined with (**B**) the monoclonal antibody to B cells (WCI12) and (**C**) to thrombocytes (WCL6). For the detection of the Cyca-B2m antiserum and the monoclonal antibodies, goat anti-rabbit IgG conjugated with PE (GAR-PE) and goat-anti-mouse Ig conjugated to FITC (GAM-FITC) were used, respectively.

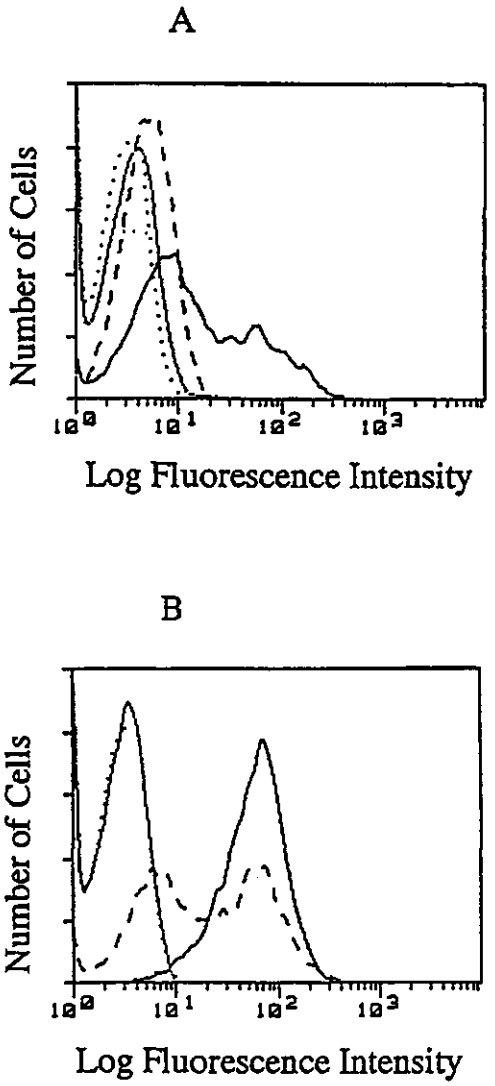

Figure 3. Leucocytes were isolated from cell suspensions of thymus, pronephros, and spleen. Erythrocytes were isolated from peripheral blood. Cells were labelled with the Cyca-B2m antiserum, and the binding of the polyclonal was detected with GAR-PE. Fluorescence intensity histograms of cells labelled with the Cyca-B2m antiserum are shown. (**A**) (·····) Erythrocytes control, (- - -) Erythrocytes, (-----) Splenocytes control, (—) Splenocytes. (**B**) (·····) Pronephrocytes control, (- - -) Pronephrocytes, (-----) Thymocytes control, (—) Thymocytes.

Temperature effect on ß2-microglobulin and Mhc class I expression on carp leucocytes

Mhc class I expression has been shown to be critically dependent on association with $ß_2$m and peptide, and in the RMA-S cell line this expression can be modulated by temperature (Ljunggren *et al.*, 1990). In this study the effect of three different temperatures (24°C, 12°C and 6°C) on *in vivo* cell surface expression of Mhc molecules was investigated. For this purpose the Cyca-UA and Cyca-B2m polyclonal antiserum were used in conjunction with the WCI12 monoclonal antibody as a control (Fig. 4). The antiserum raised against recombinant Cyca-UA, a locus known to be expressed, may not recognize all carp class I molecules, whereas the Cyca-B2m polyclonal antiserum is expected to give an indirect estimation of the levels of all class I cell surface expression.

Carp PBL were isolated at different time points from the different experimental groups and labelled with the polyclonal antiserum against Cyca-B2m and Cyca-UA or the monoclonal antibody WCI 12, specific for surface Ig. In the first experiment, carp were transferred from their standard rearing temperature of 24°C to 12°C. Subsequently they were exposed to this temperature for two weeks and then moved back to 24°C. Fish living at standard conditions of 24°C show the typical histograms for the three antibodies used as depicted in Figs. 4 and 5 at day 0. After 3 days of exposure to 12°C the levels of expression of Cyca-B2m and Cyca-UA were reduced, but recovered to control levels after 6 days. The expression of surface Ig during this period was constant. Subsequent transfer of carp from 12°C back to 24°C did not change the expression patterns studied to any significant degree (Fig. 4).

In the second experiment, the carp were moved from 24°C to 12°C, and finally to 6°C. The fish remained at this temperature for two weeks before being transfered back to 12°C. Fish kept at 6°C showed, after 3 days at this temperature, reduced levels of Cyca-B2m and Cyca-UA expression. However, after 6 days, all cell surface expression studied, but Ig, was lost, and remained absent for prolonged periods. After transferring the carp to 12°C both the Cyca-B2m and Cyca-UA expression recovered to normal levels within 6 days at this temperature. Cell surface expression of surface Ig again remained constant during the experimental period (Fig. 5). In contrast, the absence of Cyca-B2m and Cyca-UA cell surface expression at 6°C was observed for a period up to 4 weeks.

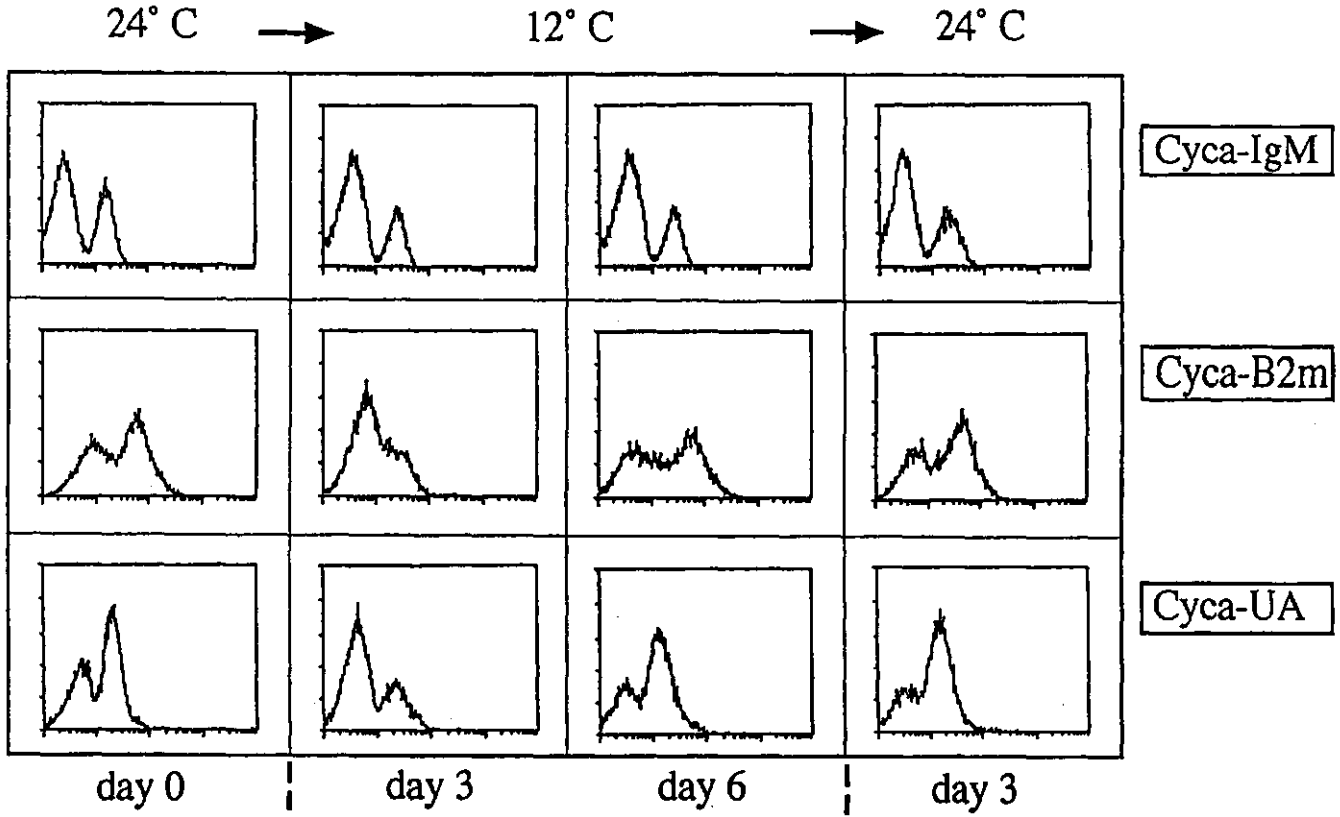

Figure 4. A group of carp were kept at to two different temperatures. Fish maintained at the normal temperature of 24°C were transferred to 12°C, and kept at this temperature for two weeks. After this period, the fish were transferred back to 24°C. Carp were bled at regular intervals, and PBL were isolated and labelled with the Cyca-B2m and the Cyca-UA antisera. As a control, WCI 12, a monoclonal antibody against carp Ig, was used. Labelled PBL from carp kept at different temperatures were analyzed by FACS, and the relevant fluorescence intensity histograms are depicted.

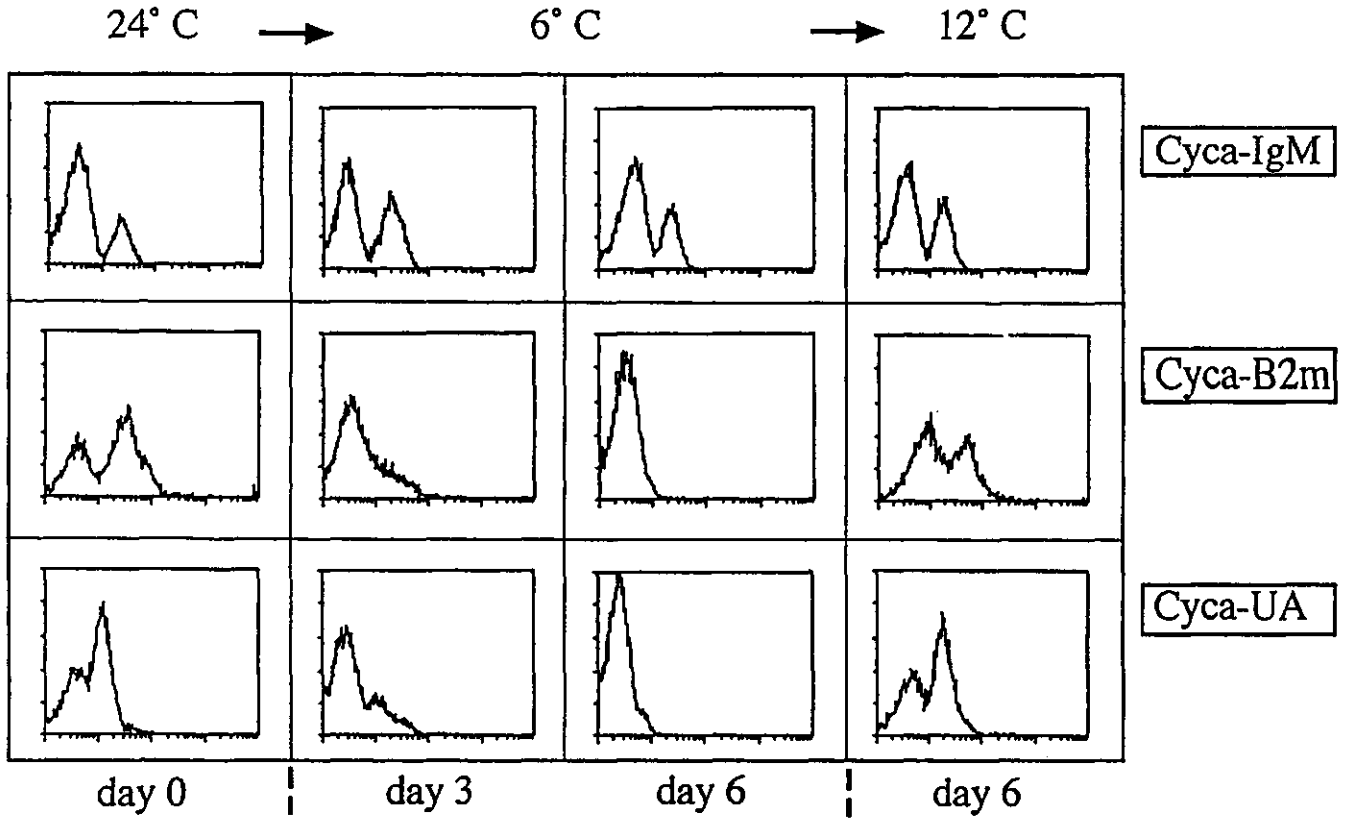

Figure 5. A group of carp was subjected to three different temperatures. Fish reared at the normal temperature of 24°C were transferred to 12°C and subsequently to 6°C, and were kept at this temperature for two weeks. After this period, the fish were transferred back to 12°C. Carp were bled at regular intervals, and PBL were isolated and labelled with Cyca-B2m and Cyca-UA antisera. As a control, WCI 12, a monoclonal antibody against carp Ig, was used. Labelled PBL from carp kept at different temperatures were analyzed by FACS, and the relevant fluorescence intensity histograms are depicted.

Temperature effect on $ß_2$-microglobulin and Mhc class I transcription in carp leucocytes

In the previous experiment a number of animals were kept at the three different temperatures (24°C, 12°C and 6°C) for 4 weeks, and samples for transcription analysis were taken. For each temperature group, PBL from two animals were isolated, RNA was extracted and cDNA prepared. A PCR using specific primers for *Cyca-B2m* and *Cyca-UA* genes was carried out. The concentrations of the *Cyca-B2m* and *Cyca-UA* transcripts in PBL were compared between groups by evaluating the yield obtained at different cycle numbers (Fig. 6).

Within temperature groups no clear difference in amplification was found both for *Cyca-B2m* and *Cyca-UA*. However, between groups a clear difference in yield of amplification was found for *Cyca-B2m* , but not for *Cyca-UA* . In the case of *Cyca-B2m* (Fig. 6A), the PCR products generated from the cDNA of the 6°C temperature group was not detectable within 20 cycles, and lower yields were seen with 25 and 30 cycles, when compared to the products generated in both the 24°C and 12°C temperature groups. No visible difference was found between the amount of PCR products from PBL of fish living at 24°C and 12°C. In contrast, in all temperature groups the yield of *Cyca-UA* (Fig. 6B) generated by the PCR amplification increased proportionally with the number of cycles (20, 25, and 30).

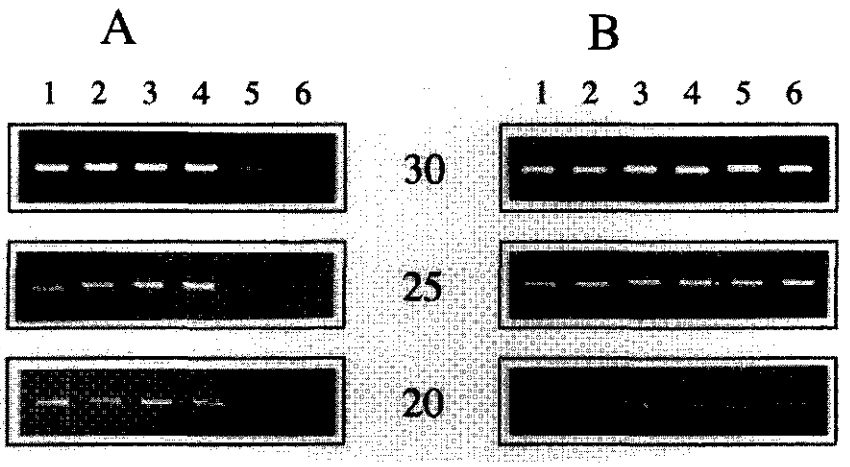

Figure 6. Carp were kept for four weeks at three different temperatures (24, 12 and 6°C). After this period PBL were isolated from the three temperature groups. RNA was extracted, cDNA prepared and a PCR with specific primers for *Cyca-B2m* and the *Cyca-UA* was carried out and yields visualized by agarose gel electrophoresis. (**A**) PCR products for the *Cyca-B2m* amplification after different cycle numbers. (**B**) PCR products for the *Cyca-UA* amplification. Lanes 1 to 6 depict PCR yields from individual carp kept at different temperatures: 1 and 2 (24°C); 3 and 4 (12°C); and 5 and 6 (6°C).

Discussion

The expression of Mhc molecules and their tissue distribution can not be dissociated from age and immunological state of the species considered.However, it is generally accepted that class I molecules are present on the cell surface of most somatic cells, whereas class II expression occurs mainly on the cells of the immune system (Klein, 1986). With the recent isolation of the carp B2m gene (*Cyca-B2m*) from a spleen/pronephros cDNA library (Dixon *et al.*, 1993), it became possible to study the expression levels and tissue distribution of transcripts from this gene. PCR amplification of cDNA obtained from leucocytes from spleen , pronephros, blood and thymus confirmed the presence of transcripts in these organs (Rodrigues *et al.*, in preparation). However, due to the limitations of this type of analysis a polyclonal antiserum was produced against a Cyca-B2m recombinant protein.

The Cyca-B2m antiserum showed substantial reactivity against the recombinant protein, as well as to cell surface determinants on leucocytes. Since it is was hypothesized that Cyca-B2m is associated with all carp class I gene products, it was expected that all nucleated cells would be positive with the serum against carp β_2m. However, although nucleated erythrocytes and thrombocytes were anti-Cyca-B2m negative, and thus also class I negative. This has been confirmed by using antibodies to Cyca-UA (Van Erp *et al.*, 1996). These findings are in disagreement with the distribution of *Xenopus* class I molecules which, at the time of metamorphosis to adult stage became apparent on leucocytes and erythrocytes (Flajnijk *et al.*, 1984; Flajnijk *et al.*, 1987). However, in chicken the *B-F* encoded molecules were also not detectable on nucleated erythrocytes by immunofluorescence (Pink *et al.*, 1985).

The fluorescence histogram of carp PBL labelled with the Cyca-B2m antiserum showed the presence of a dull and a bright-positive population. With the help of the only two monoclonal antibodies against carp leucocytes available, a B cell-specific (Secombes *et al.*, 1983) and a thrombocyte-specific (Rombout *et al.*, 1994) markers, the nature of the different populations was further investigated. Double labelling of PBL revealed that the $WCL6^+$ cells stained only weakly with the Cyca-B2m antiserum. These findings are in are contrast to what is known of expression of class I for the mammalian counterparts of thrombocytes, the platelets (Klein, 1986), and the chicken thrombocytes (Pink *et al.*, 1985). It was also apparent from the PBL double labelling, that almost all Ig^+ lymphocytes ($WCI12^+$) are Cyca-B2m bright-positive. There was also an Ig^- population observed which was bright-positive, which account for 17% of the PBL, and most probably representing putative peripheral T cells.

To investigate the tissue distribution of Cyca-B2m molecules, cells from lymphoid organs were labelled with the Cyca-B2m antiserum. Since erythropoiesis and trombopoiesis occur

mainly in the spleen (Rombout *et al.*, 1994), the proportions of thrombocytes and several stages of erythrocytes in the density separated cell fractions are probably high. Thus, the large proportion of dull-positive cells isolated from the spleen is most likely accounted for by the presence of thrombocytes and erythrocytes. The pronephros is thought to be the haemopoietic organ in fish, and therefore may be considered the equivalent of the mammalian bone marrow, but is also a peripheral immunological organ containing numerous antibody producing cells after antigenic stimulation. Since in such tissue a large proportions of undifferentiated cells are present, the dull-positive cells most likely represent the developmental stages of different leucocyte lineages, and the bright-positive cells are the mature Ig^+ and Ig^- leucocytes. In chicken bone marrow and spleen a similar distribution of class I (B-F) dull- and bright-positive cell populations has been reported (Dunon *et al.*, 1990). The fluorescence histogram of the thymocytes revealed just one type of cells, all bright-positive, which is consistent with what is known for mammalian thymocytes (Klein, 1986).

In the next set of experiments we addressed the effect of temperature on the expression of the β_2m and Cyca-UA class I molecules. It has been demonstrated that both β_2m and peptide are instrumental in the proper folding of the trimolecular structure of class I molecules (Elliot, 1990). There are, however, instances in which either of the two requirements can be circumvented. One of the best known examples is the RMA-S cell line, where, in the absence of peptides, class I expression can be rescued by lowering the temperature (Ljunggren *et al.*, 1990). The β_2m molecule seems to be more essential for a proper class I expression (Zijlstra *et al.*, 1990), although low levels of re-folding with exogenous β_2m and peptide have been observed (Vitiello *et al.*, 1990).

The experiments described in this study clearly show that lowering the ambient temperature of the carp results in a decreased level of β_2m and class I expression, which remained undectable when carp are kept at 6°C. Recovery to normal expression levels after transfer to a higher temperature was achieved in all groups, albeit with a slower rate in the 6°C group. In contrast, no changes were observed in the expression of surface Ig on the B cells. The latter observation is consistent with the fact that temperature effects in fish have been noted for T cell but not B cell functions (Bly and Clem, 1992).

The question that arises is whether the observed down-regulation of class I molecule expression is due to: 1. a failure to transport the molecules to the cell surface; or an 2. inability to fold the trimolecular structure due to the absence of one or more of the constituents. The transport system of macromolecules does not seem to be impaired at low permissive temperatures, based on the fact that normal levels of Ig molecules have been observed on the surface of B cells in the different temperature groups. Thus, the absence of either peptide, β_2m or class I molecules seems to be a likely cause. Although peptide is required to fold a

functional class I molecule, a low temperature would allow for the expression of empty class I molecules (Ljunggren *et al.*, 1990). In addition, the fact that antigen uptake and processing have been observed in fish at low permissive temperatures, although at a slower rate, would argue against an absence of protein processing activities (Vallejo *et al.*, 1992). The latter study also showed that exogenous antigens are being presented, which seems to suggest in fish a different effect of temperature on expression of class II molecules.

The remaining possibility of absence of either the β_2m, or the class I α chain molecule was investigated by semi-quantative PCR. This experiment clearly indicated a down-regulation of *Cyca-B2m* gene transcription only at 6°C. *Cyca-UA* gene transcription is unaffected at all temperatures. Therefore, the absence of normal transcription levels of the *Cyca-B2m* may well gene accounts for the down-regulation of class I molecules on the cell surface. This observation is reminiscent of β_2m knock-out mice, where also normal levels of class I transcripts can be found in the absence of class I cell surface expression (Zijlstra *et al.*, 1990).

In conclusion, temperature changes within permissive ranges can result in temporal or long lasting changes in class I expression, which is determined by a temperature-sensitive transcription regulation mechanism of the β_2m gene. This observation could account, in aquacultural practices, for the observed "immunological disasters" after severe and sudden temperature changes (Bly and Clem, 1992).

Acknowledgments

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References

Bjorkman, P.J., Saper, M.A., Samraoui, B., Bennett, W.S., Strominger J.L. and Wiley, D.C. (1987) The foreign antigen binding site and T cell recognition regions of class I histocompatibilty antigens. *Nature* **329**: 512-518.

Bly, J.E. and Clem, L.W. (1992) Temperature and teleost immune functions. *Fish Shellfish Immunol.* **2**: 159-171.

Dixon, B., Stet, R.J.M., Van Erp, S.H.M. and Pohajdak, B. (1993) Characterization of ß2-microglobulin transcripts from two teleost species.*Immunogenetics* **38**: 27-34.

Dixon, B., Van Erp, S.H.M., Rodrigues, P.N.S., Egberts, E. and Stet, R.J.M. (1995) Fish major histocompatibility complex genes: an expansion. *Dev. Comp. Immunol.* **19**: 109-135.

Dunon, D., Salomonsen, J., Skjødt, K., Kaufman, J. and Imhof, B.A. (1990) Ontogenic appearance of MHC class I (B-F) antigens during chicken embryogenesis. *Dev. Immunol.* **1**: 127-135.

Elliott, T. (1991) How do peptides associate with MHC class I molecules. *Immunol. Today* **12:** 386-388.

Flajnik, M.F., Kaufman, J.F., Riegert, P. and Du Pasquier, L. (1984) Identification of class I major histocompatibility complex encoded molecules in the amphibian *Xenopus. Immunogenetics* **20**: 433-442.

Flajnik, M.F., Hsu, E., Kaufman, J.F. and Du Pasquier, L. (1987) Changes in the immune system during metarmorphosis of *Xenopus. Immunol. Today* **8**: 58-64.

Ljunggren, H.G., Stam, N.J., Ohlen, C., Neefjes, J.J., Hoglund P., Heemels, M.T., Bastin, J., Schumacher, T.N.M., Townsend, A., Karre, K. and Ploegh, H.L. (1990) Empty MHC class I molecules come out in the cold. *Nature* **346**: 476-480.

Klein, J. (1986) *The Natural History of the Major Histocompatibility Complex.* John Wiley and sons, New York.

Pink, J.R.L., Kieran, M.W., Rijnbeek, A.M. and Longenecker, B.M. (1985) A monoclonal antibody against chicken MHC class I (B-F) antigens. *Immunogenetics* **21**: 293-297.

Rock, K.L., Gamble, S., Rothstein, L., Gramm, C. and Benacerraf, B. (1991) Dissociation of ß$_2$-microglobulin leads to a substantial pool of inactive class I MHC heavy chains on the cell surface. *Cell* **65**: 611-620.

Rodrigues, P.N.S., Hermsen, G.T., Rombout, J.H.W.M., Egberts, E. and Stet, R.J.M. (1995) Detection of MHC class II transcripts in lymphoid tissues of the common carp (*Cyprinus carpio* L.). *Dev. Comp. Immunol.* **19**: 483-496.

Rombout, J.H.W.M., Koumans-Van Diepen, J.C.E., Taverne, N. and Taverne-Thiele, J.J. (1994) Analysis of blood leucocytes and thrombopoiesis with monoclonal antibodies specific for carp thrombocytes. *Dev. Comp. Immunol.* **18** Suppl. 1, S61.

Salter, R.D., Benjamin, R.J., Wesley, P.K., Buxton, S.E., Garrett, T.P.J., Clayberger, C., Krensky, A.M., Norment, A.M., Littman, D.R. and Parham, P. (1990) A binding site for the T-cell co-receptor CD8 on the a_3 domain of HLA-A2 histocompatibility antigens. *Nature* **345**: 41-46.

Secombes, C.J., Van Groningen, J.J.M. and Egberts, E. (1983) Separation of lymphocyte subpopulations in carp by monoclonal antibodies: immunohistochemical studies. *Immunology* **48**: 165-175.

Stet, R.J.M. and Egberts, E. (1991) The histocompatibility system in teleostean fishes: from multiple histocompatibility loci to a major histocompatibility complex. *Fish Shellfish Immunol.* **1**: 1-16.

Stet, R.J.M., Dixon, B., Van Erp, S.H.M., Van Lierop, M.C., Rodrigues, P.N.S. and Egberts, E. (1996) Inference of structure and function of fish major histocompatibility complex (MHC) molecules from expressed genes. *Fish Shellfish Immunol.*, (in press)

Townsend, A., Ohlen, C., Bastin, J., Ljunggren, H.G., Foster, L. and Karre, K. (1989) Association of class I major histocompatibility heavy and light chains induced by viral peptides. *Nature* **340**: 443-448.

Townsend, A., Elliott, T., Cerundolo, V., Foster, L., Barber, B. and Tse, A. (1990) Assembly of MHC class I molecules analysed in vitro. *Cell* **62**: 285-295.

Turner, R.J. (1994) *Immunology; a comparative approach.* John Wiley and sons, New York.

Vallejo, A.N., Miller, N.W. and Clem, L.W. (1992) Cellular pathway(s) of antigen processing in fish APC: effect of varying *in vitro* temperatures on antigen catabolism. *Dev. Comp. Immunol.* **16**: 367-381.

Van Erp, S.H.M., Dixon B., Figueroa F., Egberts E. and Stet, R.J.M. (1996) Identification and characterisation of a new MHC class I gene from carp (*Cyprinus carpio* L.). *Immunogenetics* (in press)

Vitiello, A., Potter, T.A. and Sherman L.A. (1990) The role of β_2-microglobulin in peptide binding by class I molecules. *Science* **250**: 1423-1426.

Wiegertjes, G.F., Stet, R.J.M. and Van Muiswinkel, W.B. (1994) Divergent selection for antibody production in common carp (*Cyprinus carpio* L.) using gynogenesis. *Anim. Genet.* **25:** 251-257.

Zijlstra, M., Bix, M., Simister, N.E., Loring, J.M., Raulet, D.H. and Jaenisch, R. (1990) β_2-microglobulin deficient mice lack $CD4^-8^+$ cytolytic T cells. *Nature* **344**: 742-746.

Chapter 4

Cell surface expression of MHC class I and β_2-microglobunin in carp (*Cyprinus carpio* L.) lymphoid tissues during ontogeny.

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Submitted

Summary

The appearance of class I molecules during the ontogeny of carp (*Cyprinus carpio* L.) was investigated. In this study, polyclonal antisera against carp recombinant $ß_2$m (Cyca-B2m) and class I α chain (Cyca-UA) were used. Cells were isolated from the lymphoid organs at different time points during ontogeny, and labelled with the polyclonal antibodies. The time of appearance of Cyca-B2m and Cyca-UA determinants at the cell surface was clearly different for all tissues studied. In pronephros and spleen, during the first weeks (3-10), more cells were labelled with the Cyca-B2m than with the Cyca-UA polyclonal antiserum. After week 10, the number of cells labelled with both antisera were similar in pronephros and spleen. Almost all Ig^+ pronephrocytes and splenocytes were Cyca-B2m-positive at week 4, but for Cyca-UA the same percentages were only seen at week 12. The differences during the first weeks in the percentages of Cyca-B2m and Cyca-UA reactivity in pronephros and spleen could be largely attributed to the Ig^+ cells present in both organs. However, the fact that a large proportion of Ig^+ cells were Cyca-B2m-positive, but Cyca-UA-negative, raises the question with which molecule on these cells $ß_2$m is associated. In thymus a very different results from pronephros and spleen were observed. All thymocytes were labelled with Cyca-B2m at week 6, in contrast the number of cells labelled with Cyca-UA was low during ontogeny, reaching 45 % of positively labelled at week 13, as in the adult fish. It is suggested that durring development a class I α chain different from Cyca-UA is beeing used in associationn with Cyca-B2m. Obvious candidates are the molecule encoded by *Cyca*-TC16, that is only found on thymus, or an other carp non-classical molecule encoded by the *Cyca-Z* lineage. The use of a different class I α chain seems to remain present in the thymus of the adult carp, whereas the peripheral circulating Ig^+ and Ig^- lymphoid cells (including putative T cells) have been shown to use the Cyca-UA encoded α chain.

Key Words: Carp ontogeny, cellular expression, class I molecules, Cyca-B2m, Cyca-UA.

Introduction

The tissue distribution of products encoded by MHC class I and class II genes has been the subject of intensive study (Klein, 1986). The MHC molecules distribution pattern can not be dissociated from certain factors, like, age, immunological state of the animal, and species considered. However, as a matter of generalization,it is generally accepted that MHC class I molecules are ubiquitously expressed by most somatic cells, whereas MHC class II molecules show a more restricted distribution, mainly being present on cells of the hemopoietic system, including antigen presenting cells (APCs).

The MHC of fish has deserved in the passed years appropriate attention, and at the moment there is considerable amount of data concerning sequences of MHC genes accumulated (Dixon *et al.*, 1995). From carp the first MHC class I genes to be reported was the *Cyca-Z* partial genomic sequence (Hashimoto *et al.*, 1990). Later the knowledge about the *Cyca-Z* lineage

was expanded (Okamura *et al.*, 1993), but due to certain features of the sequences some doubts have been raised as to of their function. Carp $ß_2$-microglobulin ($ß_2$m) encoding sequence (*Cyca-B2m*) was identified (Dixon *et al.*, 1993), and recently, a carp MHC class I full-length cDNA and genomic sequences *Cyca-UA* have been reported (Van Erp *et al.*, 1996*a*). Concurrently, the production of polyclonal antibodies against recombinant proteins produced with these carp *Cyca-B2m* and *Cyca-UA* gene sequences, has made it possible to perform studies on the expression of the gene products (Rodrigues *et al.*, 1996; Van Erp *et al.*, 1996*a*). With the limitation concerning the availability of antibodies to carp cell markers, it was only possible to determine that in the adult stage Ig^+ and Ig^- lymphocytes, and also granulocytes, express MHC class I and $ß_2$m on the cell surface, whereas erythrocytes and thrombocytes were negative. Some aspects of the tissue distribution of Cyca-UA and Cyca-B2m expression can be interpreted in the context of the biological function of these molecules in carp. The histogenesis of the carp lymphoid organs point to a rapid development of the immune system. In carp larvae, the first lymphoid organ to appear within one week after hatching is the thymus, followed by the pronephros and later the spleen (Botham and Manning, 1981). In addition, the ontogenetic development of allograft reactivity is also fast, within 3 weeks lymphocytes are capable of effecting allograft rejection. The study of class I molecules expression during ontogeny is expected to provide further information on the development of the cytotoxic T-cell immune repertoire. In this study the appearance of carp class I molecules during ontogeny was investigated with the aid of polyclonal antibodies against carp recombinant $ß_2$m (Cyca-B2m) and class I α chain (Cyca-UA) proteins. The advantage of using a polyclonal antibody to Cyca-B2m is that it is assumed that it associates with all classical MHC class I molecules and, therefore can provide information of total class I molecule expression independent of the presence of different class I α chain. Usage of different class I α chains may indicate separate roles for the class I molecules during ontogeny in the lymphoid organs studied.

Materials and methods

Animals

Common carp (*Cyprinus carpio* L.), were grown at 25°C in recirculating, filtered U.V-treated water. After initial feeding on *Artemia nauplii* for a period of 3 weeks following yolk-sac absorption, the feeding regime was switched to pelleted dry food (Provimi, Rotterdam, The Netherlands) at a age-dependent daily ration. The carp were all reared in the same system starting post-hatch, and held in the same tank throughout the experiment. The experimental populations consisted of two offsprings. These were the result of two separate crosses between between individuals from different R3 and R8 parental fish stocks. The R3 and R8 stocks are partly inbred strains of common carp originating from Poland and Hungary, respectively (Wiegertjes *et al.*, 1994)

Dissection of lymphoid tissues and cell isolation

Fish were anaesthetized in tricaine methane sulphonate (TMS, Crescent Research Chemicals, Phoenix AZ, USA) at 3g/10 L. In order to remove the blood of the youngest fish (3 and 4 weeks) the tail was cut off and the larvae were placed in heparinized cRPMI (RPMI 1640 adjusted to 270 mOsm). From 6 weeks onwards the blood was collected from the dorsal aorta, and after having been diluted 1:1 in heparinized cRPMI the peripheral blood leucocytes were separated on Lymphoprep (Nycomed, Oslo, Norway) by centrifugation at 840 x g for 30 min. at 4°C. Subsequently, with the help of a binocular dissecting microscope the lymphoid tissues were removed. From 10 to 20 larvae, pronephros, spleen, and thymus were collected. Cell suspensions, from these ppoled organs, were prepared by forcing the tissues through a 50 mesh nylon gauze filter while adding cRPMI. After having been washed in cRPMI (680 x g for 10 min at 4°C) and resuspended, the cells were separated on Lymphoprep by centrifugation at 840 x g for 30 min at 4°C. The cells were collected from the interphase, washed twice and resuspended at a concentration of 10^7 cells/ml in cRPMI.

Flow cytometry (FACS)

For the flowcytometry studies, the following antibodies were used: a polyclonal antiserum against a recombinant Cyca-B2m protein (Rodrigues *et al.*, 1996); an anti-class I (Cyca-UA) polyclonal antiserum (Van Erp *et al.*, 1996*a*); the WCI 12 monoclonal antibody, which detects surface immunoglobulin on carp leucocytes (Secombes *et al.*, 1983); WCL 9 monoclonal antibody which reacts with thymocytes (Rombout *et al.*, umpublished); and the WCL 6 monoclonal antibody specifically recognising carp thrombocytes (Rombout *et al.*, 1994). Leucocytes isolated as described above, were incubated for 30 min on ice in 0.5 ml of

appropriately diluted (usually 1:100) Cyca-UA or Cyca-B2m polyclonal antiserum. For all the incubation and washing steps, FACS medium containing cRPMI, 1% BSA and 0.1% NaN_3 was used. After washing, binding of the polyclonal antibodies was detected by incubating the cells for 15 min on ice with phycoerythrine (PE)-conjugated goat-anti-rabbit immunoglobulin (GAR-PE; Southern, Birmigham USA), diluted 1:100 in FACS medium containing 1% of pooled carp serum. For the double labelling, leucocytes were incubated with the Cyca-B2m or Cyca-UA polyclonal antisera together with WCI 12, followed by an incubation with GAR-PE and fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse immunoglobulin (Dakopatts, Glostrup, Denmark). Cells were analyzed using a FACStar (Becton Dickinson Immunocytometry Systems, Mountain View CA, USA), with an argon laser tuned at 488 nm. The Consort 30 data analysis package was used to plot the forward (FSC) and side (SSC) scatter patterns, and to determine the percentages of fluorescent cells (FL1/FL2).

Results

Cell surface expression of Cyca-B2m and Cyca-UA

Cyca-B2m and Cyca-UA expression on leucocytes from carp lymphoid tissues during ontogeny was investigated. Fish were anaesthetized and, from the earliest time possible (3 weeks), the lymphoid tissues were dissected. The tissues were collected at several time points throughout 16 weeks, at the end of which period adult levels of expression for both Cyca-B2m and Cyca-UA are present (Rodrigues *et al.*, 1996; Van Erp *et al.*, 1996*a*).

From thymus, spleen and pronephros, cells were isolated, and labelled with the polyclonal antibodies recognizing recombinant carp β_2-microglobulin (Cyca-B2m) and MHC class I (Cyca-UA) proteins. All samples were analyzed by FACS, and the numbers of Cyca-B2m$^+$ and Cyca-UA$^+$ cells were determined. In addition, the monoclonal antibody specific for carp surface immunoglobulin (WCI 12) was used to determine the expression of Cyca-B2m and Cyca-UA on Ig$^+$ lymphocytes. For this purpose double labelling of leucocytes with the carp Cyca-B2m or Cyca-UA polyconal antibodies, and WCI 12 was carried out. Double labelling was also performed using the WCL 6 and WCL 9 monoclonal antibodies (specific for thrombocytes and thymocytes, respectively) on all cell populations under investigation.

Each point depicted in the graphs represents the mean of three measurements of duplicated experiments. Since the standard deviations were low, as were the differences between the two separate experiments, these data have been omitted from the graphs.

Pronephrocytes

The kinetics of expression of Cyca-B2m and Cyca-UA on pronephrocytes was different. Cyca-UA expression increased steadily from 3 weeks until week 16, at which time adult levels of expression were reached. At 3 weeks 10.8% of cells were labeled with the Cyca-UA polclonal antiserum, and at week 16 this figure had increased to 60.7% (Fig. 1). The increase in the number of positive cells labelled with the Cyca-B2m polyclonal antibody, however, was faster than that observed for Cyca-UA positive cells. At week 3 using Cyca-B2m polyclonal already 30.1% of cells were already positively labeled, and the adult levels of expression were found after 10 weeks. In adult carp the percentages of pronephrocytes expressing Cyca-UA or Cyca-B2m was found to be around 60%. The numbers of WCI 12^+ cells rose slowly from 2.9% at week 3, to 19.1% after 10 weeks after which the numbers of Ig^+ cells stabilized. The expression of Cyca-UA or Cyca-B2m on WCL 6^+ and WCL 9^+ pronephrocytes was also investigated. However, the percentages of both WCL 6^+ and WCL 9^+ cells found in pronephros during ontogeny was very low (data not shown).

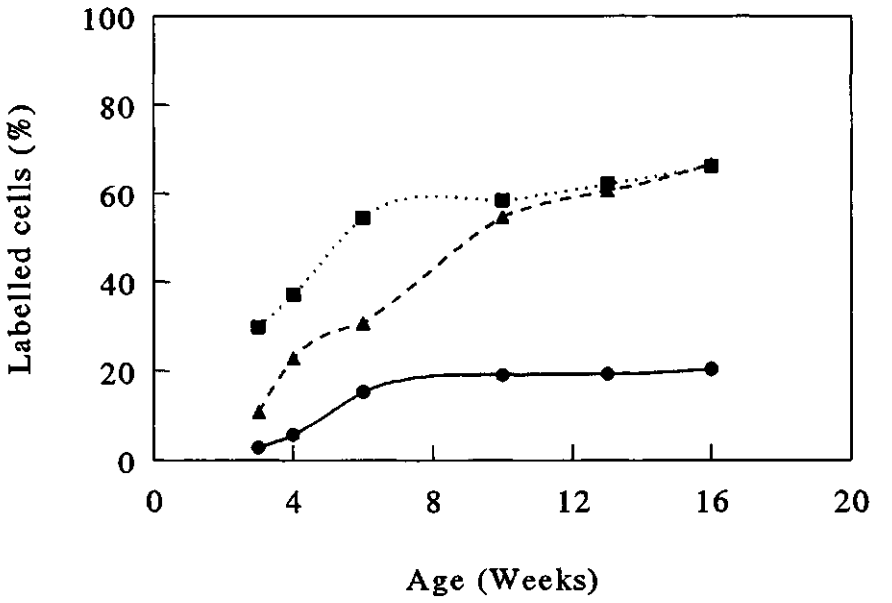

Figure 1. Leucocytes were isolated from the pronephros at different time points during development. Pronephrocytes were labelled with the Cyca-B2m and Cyca-UA polyclonal antisera. The WCI 12, a monoclonal against carp Ig was used. For the detection of the binding of the polyclonal antibodies and WCI 12, goat-anti-mouse Ig coupled to PE (GAR-PE) and goat-anti-mouse Ig conjugated to FITC were used (GAM-FITC), respectively. The percentages of positive cells labelled with antibodies recognizing Cyca-B2m (■), Cyca-UA (▲) and Cyca-IgM (●) are depicted.

Ig^+ Pronephrocytes

The expression of Cyca-UA and Cyca-B2m on WCI 12^+ pronephrocytes was investigated (Fig. 2). Generally, the percentages of WCI 12^+ cells found positive for Cyca-UA and Cyca-B2m at the several developmental stages studied were higher than the values obtained with the total leucocyte populations isolated from the pronephros. Major differences were found between the kinetics of Cyca-B2m and Cyca-UA expression on Ig^+ cells. THe proportion of Cyca-$B2m^+$/WCI 12^+ cells increased sharply from 50.1 % at week 3, to the adult-like situation at 6 weeks, at which stage almost all B cells are Cyca-$B2m^+$. The percentage of Cyca-UA^+/WCI 12^+ cells, however, was low at week 3 (9.2 %), but increased with age until week 16. At this time point, similar to the situation in adult carp almost all WCI 12^+ cells were Cyca-UA^+ and Cyca-$B2m^+$.

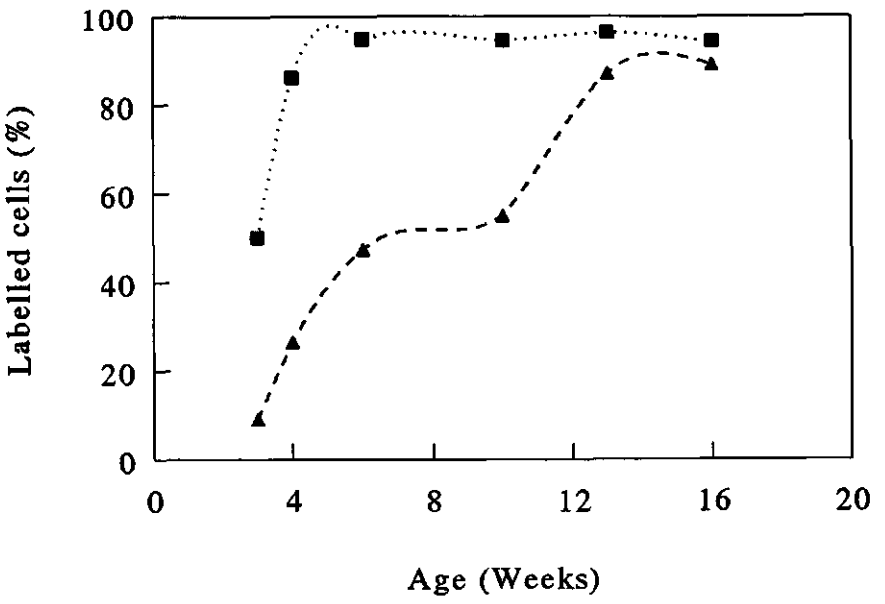

Figure 2. Leucocytes were isolated from pronephros at different time points during development. Pronephrocytes were double labelled with the Cyca-B2m or Cyca-UA polyclonal antiserum, and WCI 12, a monoclonal antibody against carp Ig. For the detection of the binding of the polyclonal antibodies and WCI 12, GAR-PE and GAM-FITC were used, respectively. The WCI 12^+ cells were gated and the percentages of positive cells labelled with Cyca-B2m (■) and Cyca-UA (▲) are depicted.

Splenocytes

On splenocytes both Cyca-UA and Cyca-B2m cellular expression increased from week 3 until week 16, the time at which adult levels of expression were reached (Fig. 3). Unlike with pronephrocytes, the numbers of Cyca-B2m$^+$ and Cyca-UA$^+$ cells in spleen did not differ very much during this period of development. At week 3, the Cyca-UA polyclonal antiserum labelled 12,5 % of the cells, and 57.1 % of positive cells were observed at week 16. With the Cyca-B2m polyclonal, 18.5 % of cells were positively labeled at week 3, and adult-like levels of expression were found after 10 weeks (56.9 %). In adult carp, both Cyca-UA nad Cyca-B2m expression have been previously determined to reach levels of 55 % of splenocytes. The number of Ig$^+$ cells rose slowly from 2.9 % to 14.9 % during the 16 weeks of the experiments. The expression of Cyca-UA and Cyca-B2m on WCL 6$^+$ and WCL 9$^+$ splenocytes was investigated. The percentages of gated WCL 6$^+$ splenocytes labelled with Cyca-UA or Cyca-B2m were similar to those of adult trombocytes, which have been shown to be class I negative, and the numbers of WCL 9$^+$ cells during ontogeny in this tissue were very low (data not shown).

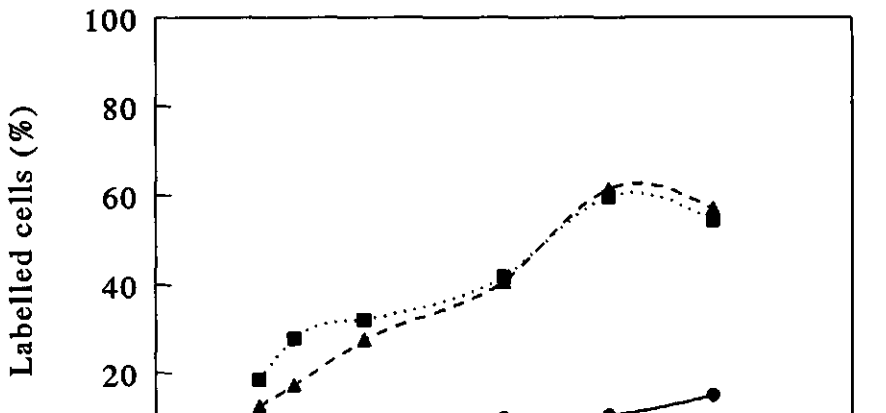

Figure 3. Leucocytes were isolated from spleen at different time points during development. Splenocytes were labelled with the Cyca-B2m or Cyca-UA polyclonal antisera. WCI 12, a monoclonal antibody against carp Ig was used. For the detection of the polyclonal antibodies and WCI 12, goat anti-mouse Ig conjugated to PE (GAR-PE) and goat anti-mouse Ig coupled to FITC (GAM-FITC) were used, respectively. The percentages of positive cells labelled with antibodies recognizing Cyca-B2m (■), Cyca-UA (▲) and Cyca-IgM (●) are depicted

Ig^+ Splenocytes

The percentages of WCI 12^+ cells in the speen expressing Cyca-UA and Cyca-B2m, during all developmental stages studied, were higher than those observed with the total splenocytes during the same larval period (Fig. 4). In addition, the numbers of Ig^+ cells found positive with the Cyca-UA or Cyca-B2m polyclonal antibodies differ significantly during ontogeny. Cyca-$B2M^+$/WCI 12^+ cells increased sharply from 52.1% at week 3 to the adult-like level already at week 6, at which stage almost all Ig^+ cells are Cyca-$B2m^+$. The percentage of Cyca-UA^+/WCI 12^+ cells, however, was low at week 3 (11.9%), but reached levels comparable to the adult situation at week 16. In previous experiments, allmost all (95%), Ig^+ cells from the spleen in adult carp were determined to be both Cyca-UA^+ and Cyca-$B2m^+$.

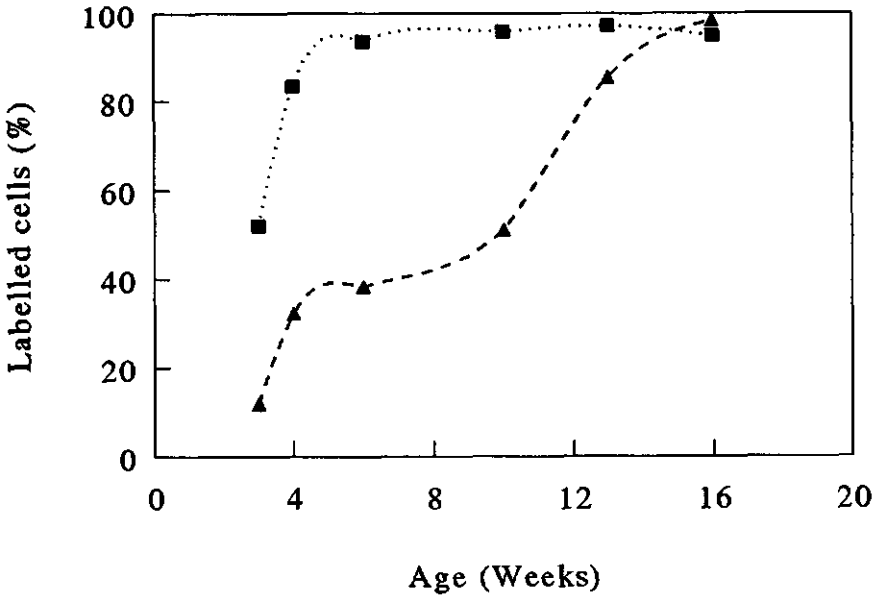

Figure 4. Leucocytes were isolated from the spleen at different time points during development. Splenocytes were double labelled with the Cyca-B2m or Cyca-UA polyclonal antiserum, and with WCI 12, a monoclonal antibody against carp Ig. For the detection of the binding of the polyclonal antibodies and WCI 12, GAR-PE and GAM-FITC were used, respectively. The WCI 12^+ cells were gated and the percentages of cells labelled with antibodies recognizing Cyca-B2m (■) and Cyca-UA (▲) are depicted.

Thymocytes

The percentages of positively labelled thymocytes with Cyca-UA or Cyca-B2m polyclonal antibodies throughout the several developmental stages studied differed to a large extent. At week 3, already 43.5% of the cells were positive for the Cyca-B2m polyclonal antiserum, but, only 11.1% of the thymocytes were Cyca-UA positive after that time. After week 6, the number of Cyca-B2m-positive cells had reached adult-like levels (93.6%), although the Cyca-UA polyclonal antibody stained only 14.6% of the cells at this age. The number of Cyca-UA positive thymocytes reached 44.9% on week 16, a value similar to that found in the adult thymus. The number of WCI 12^+ thymocytes remained very low throughout the 16 weeks of the experiment. WCL 6^+ cells were not detected during the ontogeny (data not shown). When the expression of Cyca-UA and Cyca-B2m on WCL 9^+ thymocytes (early thymocyte marker) was investigated, the percentages of gated WCL 9^+ thymocytes found positive with Cyca-UA and Cyca-B2m polyclonal antibodies were similar to those of the ungated population (data not shown).

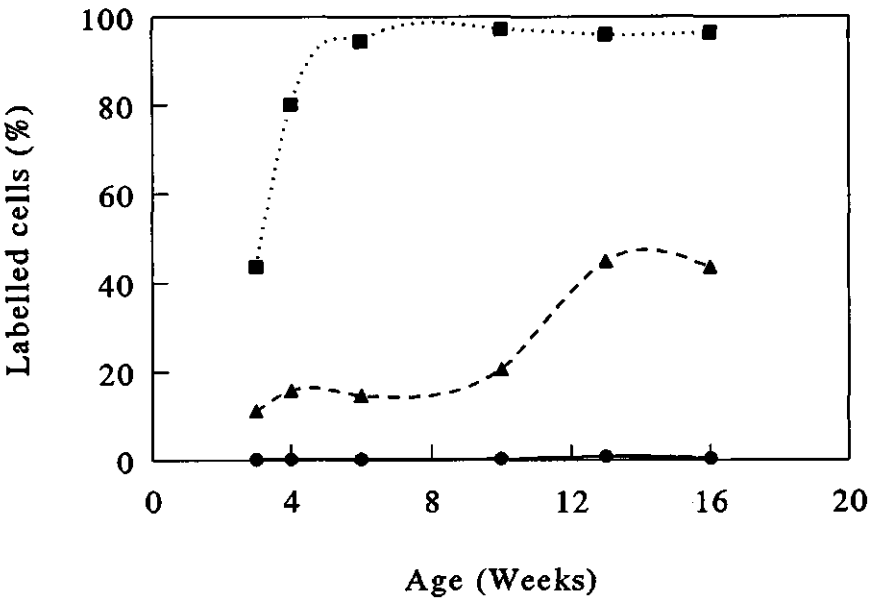

Figure 5. Leucocytes were isolated from thymus at diferent time points during development. Thymocytes were labelled with the Cyca-B2m or Cyca-UA polyclonal antisera. WCI 12, a monoclonal antibody against carp Ig was used. For the detection of the binding of the polyclonal antibodies and WCI 12, goat anti-mouse Ig conjugated to PE (GAR-PE) and goat-anti-mouse Ig to coupled FITC (GAM-FICT) were used, respectively. The percentages of positive cells labelled with the antibodies recognizing Cyca-B2m (■), Cyca-UA (▲) and Cyca-IgM (●) are depicted.

Discussion

Studies on MHC expression carried out so far in teleost fish were limited to analysis of the transcription levels of MHC genes either by northern analysis, or by semi-quantitative PCR of cDNAs. Using the latter approach it was possible to show, with some degree of detail, that carp class II *B* transcripts (*Cyca-DAB*) have a restricted tissue distribution (Rodrigues *et al.*, 1995). Although valuable, this type of studies only describes the transcription of the MHC genes, and does not fully address the question of the distribution of allegedly functional MHC molecules (Stet *et al.*, 1996). Recently, polyclonal antibodies were produced against carp β_2m (*Cyca-B2m*) and class I α chain (*Cyca-UA*), and the question of class I molecules distribution could be properly addressed (Van Erp *et al.*, 1996*a*; Rodrigues *et al.*, 1996). Studies carried out with these polyclonal antibodies revealed that generally Ig^+ as well as Ig^--lymphocytes, granulocytes were Cyca-UA- and Cyca-B2m-positive, whereas thrombocytes and erythrocytes were negative. In the present study, the ontogenetic appearance of MHC class I molecules on leukocytes from different lymphoid organs has been examined by means of indirect immunofluorescence analyses. For this purpose, cells were isolated from fish larvae, startinng at the age of 3 weeks, the earliest age from which consistent numbers of leukocytes could be isolated, and labelled with the Cyca-B2m and Cyca-UA polyclonal antibodies. The experiments were performed at several time points during the larval development, until the cellular expression had reached adult levels at week 16. Although no data are available on teleost fish MHC protein expression during ontogeny, some comparisons can made with the results from similar studies carried out in cold- and warm-blooded lower vertebrates, like *Xenopus* and chicken (Salter-Cid and Flajnik, 1995).

In carp, class I-positive cells can clearly be detected in 3 weeks old larvae. Moreover, in some experiments expression has also been seen in 2 week old larvae, although these data proved to be difficult to interpret because of the low numbers of cells analyzed (data not shown). This relatively early appearance of class I molecules in the ontogeny of the carp carp is in contrast with observations made in *Xenopus*. In the latter species, class I expression is absent on tadpole hematopoietic cells, and only appears at the time of metamorphosis climax, around 6 weeks, being the point in time where expression can be detected in all tissues (Flajnik *et al.*, 1987). However, in chicken low numbers of B-F positive cells were observed on day 10, but these values increase significantly after 3 weeks, which is around the time of hatching (Dunon *et al.*, 1990). Taking into account that carp larvae hatch within 3 days, and no metamorphosis takes place in the carp life history, it is difficult to compare these results with those of other vertebrates studied. Nevertheless, the time of detection of class I molecules

on carp larvae leukocytes points to a timing of class I expression more in line to that found in chicken than to that in *Xenopus*. The early appearance of class I molecules on carp leucocytes is most probably associated with the start of hemopoiesis and the rapid absorption of the yolk sac. Histological studies have demonstrated a precocious development of the lymphoid organs, with the first lymphocyte-like cells detectable around the first week (Botham and Manning, 1981). In addition, graft experiments in carp have showed that lymphocytes are capable of effecting allograft reaction as early as 19 days post-fertilization. This suggests that, in carp, there appears to be a rapidly maturing cytotoxic T-cell population which is able to respond to class I alloantigens.

The time of appearance of Cyca-B2m and Cyca-UA determinants at the cell surface was clearly different for all the tissues studied. In pronephros, during the first weeks (3-10) more cells were labelled with the Cyca-B2m than with the Cyca-UA polyclonal antiserum (Fig. 1). After week 10 the number of cells labelled positively with either polyclonal antibodys was similar, around 61%, which are levels consistent for adult pronephrocytes. Due to the hemopoietic function of the pronephros, a proportion of undifferentiated cells has been shown to remain Cyca-B2m- and Cyca-UA-negative (Van Erp *et al.*, 1996*a*; Rodrigues *et al.*, 1996). The analyses of gated Ig^+ pronephrocytes showed that almost all of these cells are Cyca-B2m-positive at week 4, in contrast to the Cyca-UA expression that takes 12 weeks to reach the same numbers (Fig. 2). The difference in the percentages of Cyca-B2m- and Cyca-UA-positive ungated pronephrocytes in the first weeks can largely be attributed to the differences seen in the Ig^+ cell population present in the pronephros. However, the fact that a large part of the Ig^+ pronephrocytes are Cyca-$B2m^+$/Cyca-UA^- raises the question to which molecule on these cells the carp $ß_2m$ is associated with. An explanation could be that, during the early stages of ontogeny, Ig^+ cells use not Cyca-UA but a different class I molecule, perhaps *Cyca-Z* (Okamura *et al.*, 1993) or *Cyca*-TC16 (Van Erp *et al.*, 1996*b*). However, the possibility that molecules other than those encoded by known carp class I sequences are being used can not be ruled out, such as a CD1 analogue, as has been suggested for *Xenopus* (Kaufman, 1993).

In the spleen also some differences in the timing of Cyca-B2m and Cyca-UA expression were observed (Fig. 3). Although these differences were smaller compared to those seen for pronephrocytes, they follow the same pattern of more cells positively labelled with the Cyca-B2m in the first weeks compared to the number of cells stained with the Cyca-UA antibody. The expression levels of these MHC-encoded determinants were equal at week 10, and at week 12 the percentages of positive cells for these molecules reached the adult levels of about 55%. The class I-negative population in the adult spleen most likely is accounted for by thrombocytes and several developmental stages of erythrocytes, which populations are both Cyca-B2m- and Cyca-UA-negative. The Ig^+ splenocytes, like Ig^+ pronephrocytes, were almost all Cyca-

B2m-positive at week 4, and just from week 13 on the same was seen with Cyca-UA (Fig. 4). In a similar way, the small differences found for the numbers of splenocytes expressing Cyca-B2m and Cyca-UA in the first weeks of development can be partially explained by the presence of Ig^+ cells in the ungated splenocytes. Similar to the situation discussed above in pronephros, Cyca-B2m seems to be associated with a different class I molecule in the early stages of Ig^+ splenocytes than with Cyca-UA.

In thymus, very different results from those obtained with the pronephros and spleen were observed (Fig. 5). For Cyca-B2m, adult expression levels were was reached at week 6, a time at which all thymocytes were positively labeled as in the adult fish (Rodrigues *et al.*, 1996). In contrast, the numbers of thymocytes labeled with Cyca-UA were low during ontogeny, reaching a plateau of 45 % of positively labeled cells at week 13 (Fig. 5). These results agree with data reported for chicken. During ontogeny of the latter species, the numbers of class I positive thymocytes rise until 4 weeks, resulting in a situation in which 20 % of the thymocytes are B-F-positive, which reflects the adult situation (Sgonc *et al.*, 1987). In addition, tissue sections showed that, in adult chicken, the B-F-positive thymocytes are only located in the medulla (Pink *et al.*, 1985) and, similar to carp a large subset of $ß_2$m-positive cells were class I negative (Dunon *et al.*, 1990). In carp, this observation may be explained by the assumption that not the Cyca-UA α chain is used but a different α chain such as that encoded by *Cyca*-TC16 (Van Erp *et al.*, 1996*b*). *Cyca*-TC16 expression is only found in thymus, but not in pronephros or spleen cDNAs of the R3xR8 fish, the F1 hybrids (Wiegertjes *et al.*, 1995) used in the experiment under discussion. The molecule encoded by *Cyca*-TC16 could therefore be an obvious candidate for being expressed in association with the Cyca-B2m on the cell surface of carp thymocytes. However, it can not be ruled out that other carp non-classical MHC class I molecules may be be expressed in association with Cyca-B2m such as Cyca-Z, which is assumed to be non-classical (Van Erp *et al.*, 1996*b*). Mammalian non-classical MHC-like molecules like Qa and Tla also antigens have a restricted cellular expression-pattern, some being detected only on thymocytes.

In conclusion, in the adult situation carp class I molecules use the Cyca-UA encoded α chain, in all lymphoid organs, with the exception of the thymus. During ontogeny of the lymphoid organs, clearly class I α chain genes different from Cyca-UA are being used. This situation remains present in the thymus. This suggests that class I α chain genes may play a differential role in the development of thymocytes, compared to that in pronephros or in spleen. However, in the periphery all Ig^- cells, which includes putative mature T cells, have been shown to use only the Cyca-UA α chain.

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References

Botham, J.W. and Manning, M. J. (1981) The histogeneses of the lymphoid organs in the carp (*Cyprinus carpio* L.) and ontogenic development of allograft reactivity. *J. Fish Biol.* **19**: 403-414.

Dixon, B, Stet, R.J.M., Van Erp, S.H.M. and Pohajdak, B. (1993) Characterization of ß2-microglobulin transcripts from two teleost species. *Immunogenetics* **38**: 27-34.

Dixon, B., Van Erp, S.H.M., Rodrigues, P.N.S., Egberts, E. and Stet, R.J.M. (1995) Fish major histocompatibility complex genes: an expansion. *Dev. Comp. Immunol.* **19**: 109-135.

Dunon, D., Salomonsen, J., Skjødt, Kaufman, J., and Imhof, B.A. (1990) Ontogenic appearance of MHC class I (B-F) antigens during chicken embryogenesis. *Dev. Immunol.* **1**: 127-135.

Flajnijk, M.F., Hsu, E., Kaufman, J.F. and Du Pasquier L. (1987) Changes in the immune system during metamorphosis of *Xenopus*. *Immunol. Today* **8**: 58-64.

Hashimoto, K., Nakanishi, T. and Kurosawa, Y. (1990) Isolation of carp genes encoding major histocompatibility complex antigens. *Proc. Nat. Acad. Sci. USA* **87**: 6863-6867.

Kaufman, J. (1993) Ontogeny and phylogeny of MHC-like molecules. In: *Developmental immunology.* Cooper and Nisbet-Brown, eds. Oxford: Oxford University Press, pp 53-84

Klein, J. (1986) The Natural History of the Major Histocompatibility Complex. John Wiley and sons, New York.

Okamura, K., Nakanishi, T., Kurosawa, Y. and Hashimoto, K. (1993) Expansion of genes that encode MHC class I molecules in cyprinid fishes. *J. Immunol.* **151**: 188-200.

Pink, J.R.L., Kieran, M.W., Rijnbeek, A.M. and Longenecker, B.M. (1985) A monoclonal antibody against chicken MHC Class I (B-F) antigens. *Immunogenetics* **21**: 293-297.

Rodrigues, P.N.S., Hermsen, G.T., Rombout, J.H.W.M., Egberts, E. and Stet, R.J.M. (1995) Detection of MHC Class II transcripts in lymphoid tissues of the common carp (*Cyprinus carpio* L.). *Dev. Comp. Immunol.* **19**: 483-496

Rodrigues, P.N.S., Dixon, B., Roelofs J., Rombout, J.H.W.M., Egberts, E., Pohajdak, B. and Stet, R.J.M. (1995) Expression and temperature dependent regulation of the beta$_2$-microglobulin (Cyca-B2m) gene in a cold-blooded vertebrate the common carp (*Cyprinus carpio* L.). (submited)

Rombout, J.H.W.M., Koumans-Van Diepen, J.C.E. Taverne, N. and Taverne-Thiele, J.J. (1994) Analysis of blood leucocytes and thrombopoiesis with monoclonal antibodies specific for carp thrombocytes. *Dev. Comp. Immunol.* **18** Suppl. 1, S61.

Salter-Cid L. and Flajnik M.F. (1995) Evolution and developmental regulation of the major histocompatibility complex. *Crit. Rev. Immunol.* **15**: 31-75

Secombes, C.J., Van Groningen, J.J.M. and Egberts E. (1983) Separation of lymphocyte subpopulations in carp by monoclonal antibodies: immunohistochemical studies. *Immunology* **48**: 165-175.

Sgonc, R., Hala, K. and Wick, G. (1987) Relationship between the expression of class I antigen and reactivity of chicken thymocytes. *Immunogenetics* **26**: 150-157

Stet, R.J.M., Dixon, B., Van Erp, S.H.M., Van Lierop, M.J.C., Rodrigues, P.N.S. and Egberts, E. (1996) Inference of structure and function of fish Major histocompatibility complex (MHC) molecules from expressed genes. *Fish Shellfish Immunol.* (in press)

Van Erp, S.H.M., Dixon B., Figueroa F., Egberts E. and Stet, R.J.M. (1996*a*) Identification and characterisation of a novel MHC class I gene from carp (*Cyprinus carpio* L.). *Immunogenetics* (in press)

Van Erp, S.H.M., Egberts, E. and Stet, R.J.M. (1996*b*) Evidence for multiple distinct major histocompatibility complex class I lineages in teleostean fish. *Eur. J. Immunogenetics* (in press).

Wiegertjes, G.F., Stet, R.J.M. and Van Muiswinkel, W.B. (1994) Divergent selection for antibody production in common carp (*Cyprinus carpio* L.) using gynogenesis. *Anim. Genet.* **25:** 251-257.

Chapter 5

Analyses of *MhcCyca* class I and class II transcription during ontogeny of the common carp (*Cyprinus carpio* L.).

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Summary

The expression of MHC class I and class II genes during carp (*Cyprinus carpio* L.) ontogeny was investigated. In this study, transcription of the MHC class I α chain (*Cyca-UA*), β_2-microglobulin (*Cyca-B2m*), the MHC class II α chain (*Cyca-DXA*), and the MHC class II ß chain (*Cyca-DAB*) was investigated during the early stages of embryo development by semi-quantitative PCR. For this purpose total RNA was isolated from whole embryos, cDNA prepared, and PCR amplification carried out with specific primers for each gene. No transcripts of the genes under investigation were detected in the unfertilized egg. *Cyca-UA* was first detected on day 1 post fertilization, and the levels of transcription rose until day 3, which is the time of hatching. In contrast, Cyca-B2m was only detected on day 7. *Cyca-DXA* and *Cyca-DAB* expression was seen as early as day 1, and the transcription levels steadily increased reaching a plateau at day 3. The expression of the genes encoding the MHC class II molecules revealed to be synchronized, unlike those coding for the class I molecules. Transcripts of *Cyca-UA*, *Cyca-B2m*, *Cyca-DXA* and *Cyca-DAB* were detected on day 21 in all lymphoid tissues studied but the spleen. A week later, also in the spleen the MHC class I and class II transcripts were seen, although at lower levels than in thymus, gut and pronephros. This observation may be explained by the later appearance and slower development of the spleen during carp ontogeny. The asynchronous transcription of the *Cyca-B2m* and *Cyca-UA* points to a regulation of MHC class I expression by the β_2m gene, a situation similar to that when adult carp are subjected to low environmental temperatures. The very early transcription of class II genes shows that carp larvae are characterized by a fast development of the immune system.

Key Words: Carp ontogeny, embryo, larval lymphoid organs, transcription, *Cyca-UA*, *Cyca-B2m*, *Cyca-DXA* and *Cyca-DAB*.

Introduction

The expression of MHC class I and class II genes is differentially regulated during development (Klein, 1986). In mammals, class I transcripts are hardly detectable until the midsomite stage of embryonogenesis, and the levels remain low throughout the gestation. Mammalian MHC class II molecules are expressed later in ontogeny then class I molecules, and, therefore do not appear simultaneously throughout the embryonic life. Another difference between the two classes of MHC molecules is that the tissue distribution of class II reached during fetal life is maintained in adults, whereas class I are expressed on most somatic cells of the adult (Salter-Cid and Flajnik, 1995).

In carp, genes encoding representatives of both MHC class I and II molecules have been identified. These include the characterization of sequences encoding for β_2m (Cyca-B2m; Dixon

et al.,1993), folowed by those encoding MHC class II ß chain (Cyca-DAB; Ono *et al.*, 1993), and, most recently those sequences coding MHC class I α (Cyca-UA; Van Erp *et al.*,1996*a*) and class II α chains (Cyca-DXA; Van Erp *et al.*, 1996*b*). These genes are actively transcribed and spliced into proper mRNAs, as can be deduced from the fact that all of them have been characterized from cDNA. In addition, due to the features of these sequences it has been predicted that they encode functional MHC molecules (Stet *et al.*, 1996).

In the present study the transcription of *Cyca-UA*, *Cyca-B2m*, *Cyca-DXA* and *Cyca-DAB* during the early stages of ontogeny was investigated by semi-quantitative PCR, as has been previously reported for *Cyca-DAB* alone in the adult carp (Rodrigues *et al.*, 1995). For this purpose RNA was isolated from whole embryos, cDNA prepared, and PCR amplification carried out with specific primers for each gene.

Although some protection can be provided by immunoglobulins found in the yolk sack (Castilho *et al.*, 1993), soon after absorption of this structure the free swiming larvae is at peril of becoming infected and therefore in need of a functional integrated immune system. The ability of fish to mount a specific immune response during the development is critically dependent on the presence of the key molecules such as MHC, T-cell receptor (TCR) and B-cell receptor (BCR). Information on the timing of expression of MHC class I and class II molecules will give an idea about the onset of positive and negative selection of T cell clones in the fish larvae, and their possibilities to evoke a specific immune response. The only other ectothermic animal for which these aspects have been investigated is the clawed toad *Xenopus* (Flajnik *et al.*, 1986; Du Pasquier and Flajnik, 1990). However, this amphibian undergoes metamorphosis making it difficult to draw comparisons with carp. Therefore, a clear understanding of the timing of MHC class I and II transcription, and the cellular distribution of the gene products in carp, will provide us with a better some insight into the development of the immune system during the ontogeny of this teleost species.

Materials and methods

Animals

Common carp (*Cyprinus carpio* L.), were grown at 25°C in recirculating, filtered UV-treated water. After initial feeding on *Artemia nauplii* for a period of 3 weeks following yolk-sac absorption, the feeding regime was switched to pelleted dry food (Provimi, Rotterdam, The Netherlands) at an age-dependent daily ration. The carp were all reared in the same system starting post-hatch, and held in the same tank throughout the experiment. The experimental populations consisted of two offsprings. These were the result of two separate crosses between individuals from the R3 and R8 parental fish stocks. The R3 and R8 stocks are partly inbred strains of common carp originating from Poland and Hungary, respectively (Wiegertjes *et al.*, 1994).

Embryos and larvae

At different time points, from unfertilized eggs until larvae 2 weeks post-fertilization, samples were collected. For each time point, 20 to 30 embryos or larvae were collected, pooled, and the whole fish were snap frozen in liquid nitrogen and kept at -80°C for future use. In addition, 3 and 4 weeks-old larvae were collected for lymphoid tissue dissection.

Dissection of the larvae lymphoid tissues

Fish were anaesthetized in tricaine methane sulphonate (TMS; Crescent Research Chemicals, Phoenix AZ, USA) at 3g/10 L. In order to remove the blood of the 3- and 4-weeks-old larvae, the tail was cut off, and the larvae placed in heparinized cRPMI (RPMI 1640 adjusted to 270 mOsm). Subsequently, with the help of a binocular dissecting microscope the lymphoid tissues were removed, and placed in cRPMI on ice. From 10 to 20 larvae, the pronephros, spleen, and thymus were collected, pooled, and snap frozen in liquid nitrogen and kept at -80°C for future use.

RNA and cDNA preparation

RNA and cDNA preparations for use in semi-quantitative PCR analyses were performed as described elsewhere (Rodrigues *et al.*, 1995). Briefly, whole larvae or the dissected lymphoid tissues were thawed out in lysis buffer (4 M guanidium thiocyanide, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M 2ß-Mercaptoethanol), followed by phenol/chloroform extractions. Total RNA was precipitated in ethanol, washed and dissolved in water, and stored at -80°C.

Samples containing 10μg of total RNA were converted into cDNA using the Riboclone cDNA Synthesis System (Promega, Madison WI, USA). Efficiency of cDNA synthesis was traced by determining the incorporation of fluorescein-dUTP (Fl-dUTP) in a parallel reaction. The labelled cDNA samples were serially diluted, and blotted onto a nylon filter (Hybond N+; Amersham, Amersham, UK). Detection was carried out by an enzyme-linked immunoassay using an anti-fluorescein alkaline phosphatase conjugate with subsequent addition of chemiluminescent detection reagent (Amersham), followed by exposure to XAR5 film (Kodak, Rochester NY, USA) for 16 hours at room temperature.

Polymerase chain reaction (PCR)

For all gene transcription studies the same PCR conditions were used. The amplifications were performed in *Taq* buffer (Eurogentec S.A., Seraing, Belgium; 10X Taq buffer: 500 mM KCl, 100 mM Tris-HCl pH 9.0, 1 % triton X-100), using 1 unit of *Taq* polymerase (Eurogentec) supplemented with 1.5 mM $MgCl_2$, 0.2 μM of each primer and 200 μM of each dNTP in a final volume of 100 μl. Template concentrations were balanced according to the levels of Fl-dUTP incorporated into cDNA in a parallel reaction (see under RNA and cDNA preparation). The mixtures were subjected to a thermal cycle profile (1 min 94°C, 2 min 55°C, 1 min 72°C) for a different number of cycles, and analyzed by agarose gel electrophoresis. All PCRs were carried out on a Techne PH-3 (Techne, Cambridge, UK) thermocycler.

Primers

The primers used for the *MHCCyca* cDNAs specific PCR amplifications were as follows:

Cyca-B2m

In order to detect *Cyca-B2m* transcripts by PCR, two oligonucleotides, 5'-ATG AGA GCA ATC ATC ACT TTT GC-3' starting at codon 1, and 5'-TTA CAT GTT GGG CTC CCA AA-3' ending at codon 98 were produced based on the *Cyca-B2m* sequences previously discribed (Dixon *et al.*, 1993).

Cyca-UA

Similarly, for the detection of *Cyca-UA* transcripts, two oligonucleotides 5'-GGT GTT CAC TCA GTC CAG-3' starting at codon 1 of the α_2 domain, and 5'-GCG CCT GCA GTT TTG ATC TTG TCC-3' ending at codon 96 of the α_3 domain, were produced based on data from our laboratory with respect to *Cyca-UA* cDNA sequences (Van Erp *et al.*, 1996*a*).

Cyca-DAB

For the detection of *Cyca-DAB* transcripts, two oligonucleotides 5'-CTG ATG CTG TCT GCT TTC ACT GGA GCA-3', starting at codon -6 and 5'-GAG TCA GCG ATC CGT GAT AAA ACA G-3' ending at codon 95, were produced based on known *Cyca-DAB* cDNA sequences (Ono *et al.*, 1993).

Cyca-DXA

In addition, two sets of primers specific for the amplification of *Cyca-DXA*, with each set positioned at the end of the exons encoding for the leader peptide 5'-GGT GTC-CTG-CTT-ATG-CTC-GCT-CTT-ATT-GTC and α_1 5'-TAG TTG CTC TGT GGG CTT GTA AAC C-3' domains were used (Van Erp *et al.*, 1996*b*).

Results

Detection of *MHCCyca* transcription during carp early ontogeny

To investigate the level of *MHCCyca* expression at different stages of developmment, RNA was extracted from whole embryos and larvae according to the standard protocol. From each age group, equal concentrations of total RNA were analyzed on agarose gel, and converted into cDNA. To assess the efficiency of reverse transcriptase (RT) activity, a trace reaction was carried out. Semi-quantitative PCR relies critically on similar amounts of template being present in each individual reaction.

In order to fullfil this requirement, equal amounts of cDNA, based on the incorporation of fluorescein-dUTP from the trace reactions, were used as template for the PCR amplification (Rodrigues *et al.*, 1995). A PCR using specific primers for *Cyca-B2m*, *Cyca-UA*, *Cyca-DAB* and *Cyca-DXA* genes was carried out. The levels of the *MHCCyca* transcription in larvae from different ages were compared by evaluating the yield obtained at different cycle numbers.

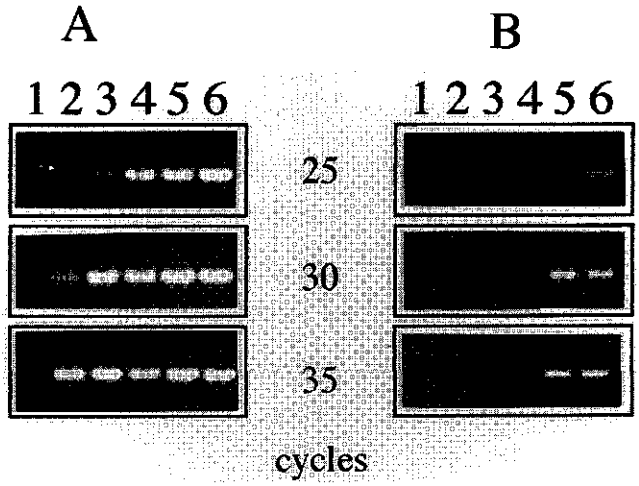

Figure 1. During two weeks after fertilization, carp larvae were collected at several time points. RNA was extracted, and converted into cDNA. A PCR with specific primers for *Cyca-UA* and the *Cyca-B2m* was carried out, and aliquots of these reactions visualized by agarose gel electrophoresis. PCR products for the *Cyca-UA* (**A**) and *Cyca-B2m* (**B**) amplification after different cycle numbers. Lanes 1 to 6 depict PCR yields from pooled carp larvae from different ages: unfertilized egg (1); 1 day embryo (2); 2 days embryo (3); 3 days embryo (4); one week larvae (5); and two weeks larvae (6).

Major differences in transcription were detected between the β_2m and class I genes (Fig. 1 and 2). For the *Cyca-UA* sequences no PCR yields were observed with the egg cDNA after 25, 30 or 35 cycles. On day 1 of embryonic live, however, such PCR products were not detected with 25 cycles, but, low yields were seen with 30, and a plateau was reached at 35 cycles. On day 2, low PCR yields were obtained with 25 cycles, and the maximum amounts of PCR products were detected with 30 and 35 cycles. After day 3 the *Cyca-UA* PCR products reached a peak for all cycle numbers used. In the case of *Cyca-B2m*, no PCR products were generated from the egg to the 3 days old embryos with either 25, 30 or 35 cycles. In contrast, after hatching, a yield was detected with the cDNA of one week old larvae using 30 and 35 cycles. With cDNAs from two weeks old larvae, *Cyca-B2m* PCR products were observed with 25 tas well as 30 and 35 cycles (Fig. 1).

The pattern of expression found for *Cyca-DAB* and *Cyca-DXA* was studied in a similar fashion as described above for the MHC class I and $ß_2$m transcription. No clear semiquantitative differences in transcription levels were found between the two class II genes. In the eggs no specific products of class II α- and ß-chain enoding cDNAs amplification could be detected. However, following fertilization, the yields of PCR products from the MHC class II transcripts increased with age until day 3, the time of hatching. At this live stage, the levels of transcription were similar to those observed for one and two weeks old carp (Fig. 2).

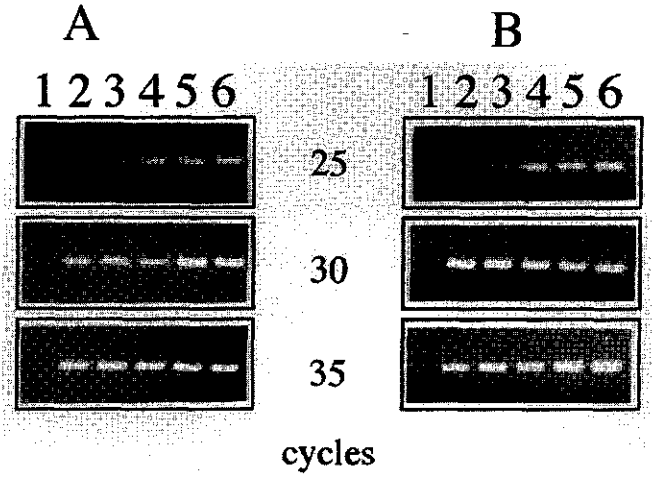

Figure 2. During two weeks after fertilization, carp larvae were collected at several time points. RNA was extracted and converted into cDNA. A PCR with specific primers for *Cyca-DAB* and the *Cyca-DXA* was carried out and aliquots of these reactions were visualized by agarose gel electrophoresis. PCR products for the *Cyca-DAB* (**A**) and *Cyca-DXA* (**B**) amplification after different cycle numbers. Lanes 1 to 6 depict PCR yields from pooled carp larvae from different ages, unfertilized egg (1), 1 day embryo (2), 2 days embryo (3), 3 days embryo (4), one week larvae (5) and two weeks larvae (6).

MHCCyca transcription in the lymphoid organs of carp larvae

In order to study the MHC expression in the carp lymphoid organs during ontogeny, 3 and 4 weeks old larvae were dissected, and thymus, spleen, pronephros and gut collected. From these organs, total RNA was isolated, and converted into cDNA. The PCR was performed under standard conditions for 30 cycles, and the yields were analysed with agarose gel electrophoresis.

For the thymus, pronephros and gut, the levels of transcription of *Cyca-B2m*, *Cyca-UA*, *Cyca-DAB* and *Cyca-DAB* did not differ significantly from each other. The expression levels observed in these lymphoid organs were also similar on day 21 and 28. In the case of the spleen, however, PCR products could not be detected on the day 21 for any of the *MHCCyca* transcripts. On the day 28, to the contrary, all of the *MHCCyca* specific PCR amplifications of spleen cDNA produced yields, almost to the same level as observed at this age in the other lymphoid organs (Fig. 3 and 4).

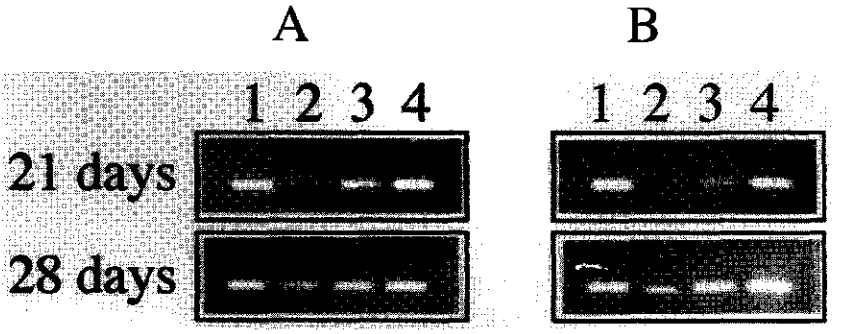

Figure 3. Several larvae of three and four weeks-old were dissected, and the lymphoid organs collected. From thymus, spleen, pronephros and gut RNA was extracted and cDNA prepared. A PCR with specific primers for *Cyca-UA* and the *Cyca-B2m* was carried out, and aliquots of these reactions visualized by agarose gel electrophoresis. PCR products for the *Cyca-UA* (**A**) and *Cyca-B2m* (**B**) amplification after 30 cycles. Lanes 1 to 4 depict PCR yields from lymphoid tissues of 21 and 28 days old carp larvae: thymus (1), spleen (2), pronephros (3) and gut (4).

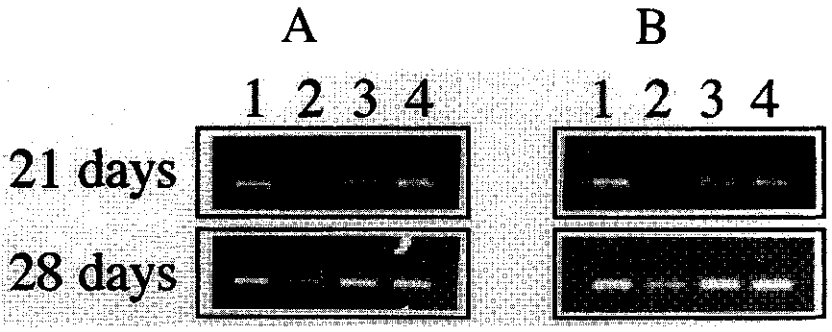

Figure 4. Several larvae of three and four weeks-old were dissected and the lymphoid organs collected. From thymus, spleen, pronephros and gut RNA was extracted and cDNA prepared. A PCR was performrd using specific primers for *Cyca-DXA* and *Cyca-DAB* and aliquots of these reactions visualized by agarose gel electrophoresis. PCR products for the *Cyca-DXA* (**A**) and *Cyca-DAB* (**B**) amplification after 30 cycles. Lanes 1 to 4 depict PCR yields from lymphoid tissues of 21 and 28 days old carp larvae: thymus (1), spleen (2), pronephros (3) and gut (4).

Discussion

The recent studies on MHC expression in teleost fish using polyclonal antibodies to prokaryotic expressed proteins, or by semi-quantitative PCR, have mainly focused on the cell and tissue distribution of the MHC transcripts or MHC-encoded molecules in adult fish lymphoid organs. In carp, the transcription of MHC class II*B* genes (*Cyca-DAB*) seems to be restricted to the lymphoid tissues, not being detected in muscle. Within these tissues, *Cyca-DAB* is highly expressed in certain cell types like thymocytes, B lymphocytes and adherent cells, but is not detected on erythrocytes (Rodrigues *et al.*, 1995). The carp MHC class I molecules (composed of Cyca-UA and Cyca-B2m) seem to have a wider distribution than the class II transcript *Cyca-DAB,* since they are found not only in lymphoid tissues but also in non-lymphoid tissues like muscle. However, Cyca-UA and Cyca-B2m molecules are present on the cell surface of Ig^+ and Ig^- lymphocytes, and granulocytes but not on erythrocytes and thrombocytes (Rodrigues *et al.*, 1996; Van Erp *et al.*, 1996*a*). In the present study, the transcription patterns of known representatives of putative functional carp MHC class I and II genes during the early ontogeny were investigated. For this purpose the levels of transcription of the *Cyca-B2m, Cyca-UA, Cyca-DAB* and *Cyca-DXA* genes in carp embryos and larvae were analyzed by semi-quantitative PCR. This approach was used on cDNA prepared from the whole embryo, due to the limitations of collecting tissues or cells at those early developmental stages.

Cyca-UA transcripts can be detected in the carp embryo as early as 1 day post fertilization, and the levels of transcription increase until day 3, which is the day of hatching. In contrast, *Cyca-B2m* transcripts are only detected at day 7, and the expression of this gene still increases from that point in time onwards. Clearly in carp MHC class I transcription precedes that of $ß_2m$, and levels of both are comparable on day 21 at least in the organs studied. The transcription of class I in carp embryos which can already be seen on day 1 post-fertilization, seems to start earlier compared to that in chicken, in which the *B-F* transcription was first detected on day 6.5 (Dunon *et al.*, 1990*a*). The expression of *Cyca-B2m* is clearly not synchronized with that of the class I α chain (*Cyca-UA*), and is shown to start at a later developmental stage. Similar observations have been reported for chicken embryos where $ß_2m$ messengers are detected in several tissues later than *B-F* mRNA, with the exception of the thymus (Dunon et al., 1990*b*; Dunon, et al, 1990*a*). In *Xenopus*, MHC class I molecules have not been observed during the early stages of development. This absence of MHC class I expression is believed to be a protective measure to prevent possible autoimmune reactivity

after metamorphosis (Flajnik *et al.*, 1987), a process which does not occur in carp. In *Xenopus*, the class I expression is first seen at the peak of metamorphosis climax, and this observation raises the question how the situation might be in other fish species that do undergo extensive morphological changes, like eels and flatfishes. In mammals, an asynchronous regulation of MHC class I and β_2-microglobulin transcription has been reported to occur during the early stages of development (Morello *et al.*, 1985). However, in mammals during early ontogeny the transcription of β_2m precedes that of MHC class I α chain (Jaffe *et al.*, 1991). Thus, in fish class I expression seems to be regulated by β_2m transcription as no MHC class I expression is possible without β_2m (Townsend *et al.*, 1990). This is in contrast to suggestions that class I molecule cell surface expression is regulated by the transcription of the TAP gene, providing the availability of proper peptides for binding the in MHC class I cleft (Bikoff *et al.*, 1991). In carp, the lack of MHC class I molecules on cells during early ontogeny, inferred from the absence of *Cyca-B2m* transcription, is difficult to reconcile with a fail safe co-regulation of these molecules, because there are no maternal-foetal interactions that would require this. The absence of class I molecules would imply that positive and negative selection of T cells in the thymus, which is dependent on MHC expression would be compromised, and so no MHC class I-restricted T cells may be generated during the early live of the carp larvae. However, it should be stressed that the thymus has been observed to became actively lymphopoietic at day 7 (Botham and Manning, 1981), which time coincides with the detection of *Cyca-B2m* transcription in the whole larvae.

Cyca-DXA and *Cyca-DAB* transcripts can be detected in carp embryos at day 1 post fertilization, and the levels of transcription increase until the day of hatching, which is day 3. The possibility that MHC class II molecules are expressed at an earlier live stage than MHC class I molecules in carp larvae is in contrast to what has been reported with mammallian embryonic development where MHC class II molecules are detected later than MHC class I. In *Xenopus*, MHC class II proteins were detected as early as day 7 in the thymus, and appear in other tissues later during development (Du Pasquier and Flajnik, 1990). The early expression of MHC class II molecules in carp larvae, suggests a fast developing immune system, but this conclusion awaits further experimental confirmation, although data on allograft rejection seem to support this notion (Botham and Manning, 1981).

The transcription levels observed for the MHC class I and class II genes (*Cyca-B2m, Cyca-UA, Cyca-DAB* and *Cyca-DXA*) in the carp larval lymphoid organs such as thymus, spleen, pronephros and gut are comparable. In mammals, also a concordant expression of MHC class I α chain and β_2m transcripts has been observed in the developing lymphoid organs (Jaffe et al., 1991). At day 21, transcription of all genes is seen in all tissues but the spleen, and at day 28 all tissues show MHC class I and class II expression, although in spleen the expression

observed was still at lower levels than in other tissues. This lower level of transcription in spleen can be explained by the large proportions in this organ of erythrocytes and thrombocytes which are class I negative (Rodrigues *et al.*, 1996; Van Erp *et al.*, 1996*a*). In addition, during the development of carp the spleen is the last organ to become lymphoid (Botham and Manning, 1981).

In conclusion, MHC class I molecules do not play a role in the ontogeny of carp until day 7. The expression of MHC class I seems to be regulated by β_2m. Hence research into MHC class I functional aspects should take into consideration regulatory elements of the β_2m gene.

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References

Bikoff, E.K., Jaffe, L., Ribaudo, R.K., Otten, G.R., Germain, R.N. and Robertson, E.J. (1991) MHC class I surface expression in embryo-derived cell lines inducible with peptide or interferon. *Nature* **334**: 235-237.

Botham, J.W. and Manning, M. J. (1981) The histogeneses of the lymphoid organs in the carp *Cyprinus carpio* L. and ontogenic development of allograft reactivity. *J. Fish Biol.* **19**: 403-414.

Castillo, A., Sánchez, C., Dominguez, J., Kaattari, S.L. and Villena, A.J. (1993) Ontogeny of IgM-bearing cells in rainbow trout. *Dev. Comp. Immunol.* **17**: 419-424.

Dixon, B., Stet, R.J.M., Van Erp, S.H.M. and Pohajdak, B. (1993) Characterization of ß2-microglobulin transcripts from two teleost species. *Immunogenetics* **38**: 27-34.

Dunon, D., Salomonsen, J., Skjødt, K., Kaufman, J. and Imhof, B.A. (1990*a*) Ontogenic appearance of MHC class I (B-F) antigens during chicken embryogenesis. *Dev. Immunol.* **1**: 127-135.

Dunon, D., Kaufman, J., Salomonsen, J., Skjødt, K., Vainio, O., Thiery, J-P. and Imhof, B.A. (1990*b*) T cell precursor migration towards β_2-microglobulin is involved in thymus colonization of chicken embryos. *EMBO J.* **9**: 3315-3322.

Du Pasquier, L. and Flajnik, M.F. (1990) Expression of MHC class II antigens during *Xenopus* development. *Dev.Immunol.* **1**: 85-95.

Flajnik, M.F., Hsu, E., Kaufman, J.F. and Du Pasquier L. (1987) Changes in the immune system during metarmorphosis of *Xenopus*. *Immunol. Today* **8**: 58-64.

Flajnik, M.F., Kaufman, J.F., Hsu, E., Manes, M., Parisot, R. and Du Pasquier, L. (1986) Major histocompatibility complex-encoded class I molecules are absent in immunologically competent *Xenopus* before metamorphosis. *J.Immunol.* **137**: 3891-3899.

Jaffe, L., Robertson, J. and Bikoff, E.K. (1991) Distinct patterns of expression of MHC class I and $ß_2$-microglobulin transcripts at early stages of mouse development. *J.Immunol.* **147**: 2740-2750.

Klein, J. (1986) *The Natural History of the Major Histocompatibility Complex.* John Wiley and sons, New York.

Ono, H., O'hUigin, C., Vincek, V., Stet, R. J. M., Figueroa, F. and Klein, J. (1993) New ß chain-encoding MHC class II genes in the carp. *Immmunogenetics* **38**: 146-149.

Morello, D., Duprey, P. and Babinet, C. (1985) Asynchronous regulation of mouse H-2D and beta-2 microglobulin RNA transcripts. *Immmunogenetics* **22**: 441-452.

Rodrigues, P.N.S., Hermsen, G.T., Rombout, J.H.W.M., Egberts, E. and Stet, R.J.M. (1995) Detection of MHC class II transcripts in lymphoid tissues of the common carp (*Cyprinus carpio* L.). *Dev. Comp.Immunol.* **19**: 483-496.

Rodrigues, P.N.S., Dixon, B., Roelofs, J., Rombout, J.H.W.M., Pohajdak, B., Egberts, E. and Stet, R.J.M. (1996) Expression and temperature dependent regulation of the beta2-microglobulin (*Cyca-B2m*) gene in a cold-blooded vertebrate the common carp (*Cyprinus carpio* L.). (submitted)

Salter-Cid, L. and Flajnik, M.F. (1995) Evolution and developmental ragulation of the Major Histocompatability Complex. *Critical Rewiews in Immunology* **15**: 31-75.

Stet, R.J.M., Dixon, B., Van Erp, S.H.M., Van Lierop, M.J.C., Rodrigues, P.N.S. and Egberts, E. (1996) Inference of structure and function of fish Major Histocompatibility Complex (MHC) molecules from expressed genes. *Fish and Shellfish Immunol.* (in press)

Townsend, A., Elliott, T., Cerundolo, V., Foster, L., Barber, B. and Tse, A. (1990) Assembly of MHC class I molecules analysed in vitro. *Cell* **62**: 285-295.

Van Erp, S.H.M., Dixon B., Figueroa F., Egberts E. and Stet, R.J.M. (1996*a*) Identification and characterisation of a novel MHC class I gene from carp (*Cyprinus carpio* L.). *Immunogenetics* (in press)

Van Erp, S.H.M., Egberts,E. and Stet, R.J.M. (1996*b*) Characterization of major histocompatibility complex class II A and B genes in a gynogenetic carp clone. *Immunogenetics* (in press)

Wiegertjes, G.F., Stet, R.J.M. and Van Muiswinkel, W.B. (1994) Divergent selection for antibody production in common carp (*Cyprinus carpio* L.) using gynogenesis. *Anim. Genet.* **25**: 251-257.

Chapter 6

General discussion.

The structure and function of Major Histocompatibility Complex (MHC) encoded molecules is well documented, and has been extensively studied especially in humans and those mammals generally used as experimental animals (Klein, 1986). The importance of MHC molecules is their involvement in antigen presentation. It and is widely accepted that, in general the MHC class I molecule is involved in presentation of endogenous antigen to cytotoxic $CD8^+CD4^-$ T cells, whereas MHC class II present exogenously derived processed antigen to T helper cells of the $CD8^-CD4^+$ phenotype (Germain and Margulies, 1993). The expression of MHC molecules and their tissue distribution can not be dissociated from age, and immunological state of the animal and the species considered. In general, studies carried out so far have demonstrated that MHC class I and II molecules display a very different tissue distribution. MHC class I genes are expressed on most somatic cells, whereas class II molecules are known to have a restricted tissue distribution, being expressed predominantly on cells of the immune system.

For many years, indirect evidence such as allograft rejections, mixed lymphocyte reactions and in vitro antibody responses, suggested the existence of MHC-encoded molecules in teleost fish (Stet and Egberts, 1991). During this period attempts to isolate MHC proteins were not successful and the existence of MHC molecules fish was still uncertain. However, in 1990 Hashimoto and co-workers with the use of polymerase chain reaction (PCR) and the available information on known MHC sequences was able to describe partial genomic MHC sequences from carp (Hashimoto *et al.*, 1990). This finding suggested the presence of MHC class I and class II genes, and implied the expression of MHC-encoded molecules. Following this report, many studies using similar molecular techniques, have been on MHC genes from several teleost species (Dixon *et al.*, 1995). The growing interest in fish MHC research started to yield a considerable body of data. As the new teleost MHC class I and class II sequences were surfacing, and became better understood, also more questions were raised. One of the questions emerging was the issue of functionality of the reported MHC genes in the context of the level of knowledge of the fish immune system. One of the ways to address this question is to analyze the tissue distribution and expression levels of the MHC class I- and II-encoded products. Not only functionality deduced from biochemical characteristics of the MHC molecules (Stet *et al.*, 1996), but more importantly their presence in certain microenvironments creating possibilities for interactions with other receptors, like the T cell-receptor (TCR), will increase our understanding of the teleost immune system. The ideal model for this type of study is the common carp, since it is one of the few teleost fish for which all main representatives of MHC genes that play a role in the initiation of the immune response, are known. The research described in this thesis deals with the tissue distribution and cellular expression of MHC-encoded

products in the common carp (*Cyprinus carpio* L.). In this general discussion, all the findings presented in the previous chapters are be integrated in a comprehensive way, answering some questions and putting forward other questions, and also generating ideas for future research.

MHC class II expression

As a matter of simplification it is accepted that MHC class II genes, under normal circumstances, are constitutively expressed on cells belonging to certain hemopoietic lineages (Klein, 1986). Therefore, the tissue distribution of class II molecules is restricted to tissues that constitute the immune system, in which among others a large number of cells of the lympho-myeloid lineages are found. In addition, it is known that MHC class II expression can be upregulated in other cell types that play an active role during an immune response, like T lymphocytes and certain types of macrophages. However, recent studies suggest that the MHC class II distribution is more complex than was originally thought, and that MHC class II molecules are also expressed by non-lymphoid tisues and cells such as microglia and keratinocytes. Conflicting results reported by different research groups dealing with MHC class II expression levels and distribution are often found in the literature. Most likely the affinity of the reagents like antibodies and the methods used, such as indirect immunofluorenscence to detect the MHC-expression levels of a given cell, can account for the discrepancies (Klein, 1986).

Studies on the presence of MHC molecules in other than mammalian species rely on the cross-reactivity of the antibodies available. This type of cross-reactivity has been successfully employed in the chicken and *Xenopus* (Kaufman *et al.*, 1991), mainly to demonstrate the presence of MHC class II molecules. Similar attempts to use such xenoantibodies in fish did not yield any positive results, and although immunizations to obtain alloantibodies to MHC products in carp pointed to the of presence histocompatibility genes (Kaastrup *et al.*, 1989), it proved difficult to produce convincing reagents identifying *bona fide* teleost MHC molecules. The difficulties in finding the molecules drove the researchers to look for the MHC gene sequences proper. The first teleost MHC class II sequence to be reported was that of *TLAIIß-1* (Hashimoto *et al.*, 1990), which was later renamed to *Cyca-DYB* according to a proposed standard nomenclature (Klein *et al.*, 1990). However, to date no full-length cDNA data of these partial genomic sequences have been published. A few years later a functional MHC class II beta gene, based on full-length sequence characteristics, designated *Cyca-DAB*, was reported (Ono *et al.*, 1993). Soon after this, a functional MHC class II α (*Cyca-DXA*) gene, based on the same criteria, was also identified from cDNA (Van Erp *et al.*, 1996*a*).

Without the availability of antibodies to class II molecules, the only possibility to study the expression of these genes is at the transcription level. The strategies we used for this purpose were, the direct estimation of mRNA using semi-quantative Northern hybridizations, or PCR amplification using specific primers of cDNA obtained from mRNA. The choice between the two strategies depended on the availability of the amount tissue from which the mRNA had to be extracted.

During ontogeny, the expression of the MHC class II α and β genes starts simultaneously, at day 1 after fertilization, and increases proportionally in time (Fig 1). The onset of MHC class II expression is relatively early compared to that in other vertebrates, such as chicken and *Xenopus* (Salter-Cid and Flajnik, 1995). This is probably linked to the relative short time to hatching, at which time point a certain degree of immunocompetence should have been acquired, irrespective of the presence of immunoglobulin presumed to be contained in the yolksac (Castillo *et al.*, 1993). In the studies under discussion the expression data were obtained using pooled material consisting of whole larvae, and are thus not informative of the tissues

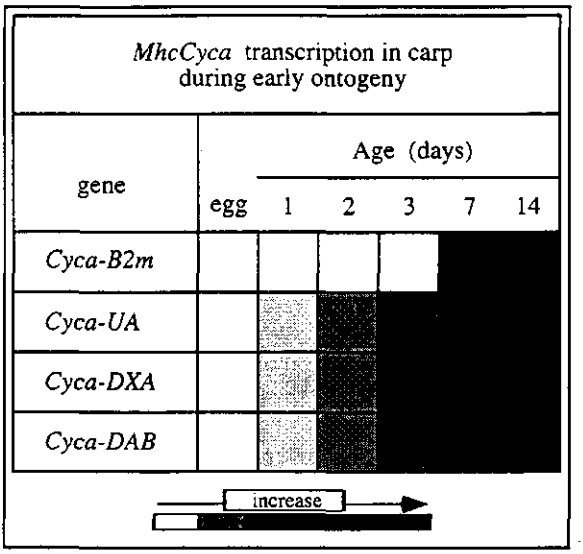

Figure 1. During two weeks after fertilization carp larvae were collected at several time points. RNA was extracted and cDNA prepared. PCR was carried out with specific primers for *Cyca-B2m*, *Cyca-UA*, *Cyca-DXA* and *Cyca-DAB* and yields of these reactions were visualized by agarose gel electrophoresis. Depicted is a graphic representation of the results.

in which the expression takes place. These type of studies are only possible after three weeks, at which time lymphoid organs can be dissected with some confidence. At this point MHC class II expression was detected mainly in the thymus, pronephros and intestine, but very low levels were observed in the spleen (Fig. 2). The choice of the organs studied was determined by observations in the adult situation as depicted in figure 3. From the expression pattern observed on day 21, it can be inferred that the detection of class II transcripts in the earlier stages is mainly accounted for by the expression in the thymus and intestine. These organs are present early in ontogeny, and the thymus is the first organ to become lymphopoietic, followed by the pronephros and in a later stage by the spleen (Botham and Manning, 1981). This observation can explain the early detection of MHC class II transcription. In the thymus it is unclear whether the thymocytes proper, as in the adult situation, or the non-lymphoid cells, such as epithelial cells, are responsible for the class II expression early in ontogeny. This question can only be answered by in situ hybridizations, or using antibodies to class II molecules.

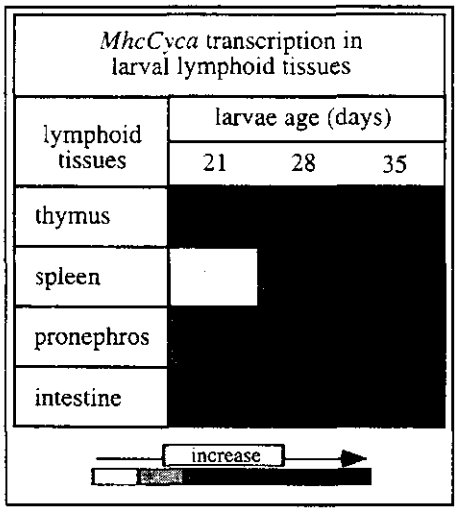

Figure 2. The expression patern of MHC genes in larval lymphoid tissues during development. Larvae were dissected, and the lymphoid organs collected. From thymus, spleen, pronephros and intestine, RNA was extracted and cDNA prepared. PCR was carried out with specific primers for *Cyca-B2m*, *Cyca-UA*, *Cyca-DXA* and *Cyca-DAB*, and the yields from these reaction were visualized by agarose gel electrophoresis. Depicted is a graphic representation of the results.

Twenty-one days after fertilization an increase in MHC class II gene transcription is observed mainly in the spleen. The expression reaches adult levels at day 35, after which no change have been observed in those organs studied. In the adult situation,e expression studies can be expanded to other tissues, including blood and non-lymphoid tissues. Such studies have shown that, basically, MHC class II expression is restricted to the lymphoid organs (Fig. 3). In the non-lymphoid organs, with the possible exception of the liver, MHC transcripts seem to be absent, as can be concluded from the results of Northern hybridizations. However, it should be realized that Northern hybridizations have limitations as to the level of expression that can be detected. Therefore, the presence in non-lymphoid organs of a low number of class II positive cells can not be excluded. In *Xenopus*, MHC class II positive cells, mainly identified as dendritic cells and macrophages, have indeed been detected by antibodies in non-lymphoid tissues, such as skin and brain (Flajnik *et al.*, 1990).

The thymus has been shown in the studies presented here to express the highest level of class II mRNA, followed by the other lymphoid organs including blood. The thymus consists of thymocytes, embedded in a reticulum of other cell types. These thymocytes can be isolated and fractionated by density centrifugation and assayed for the expression of MHC class II genes. The two fractions of thymocytes obtained with this procedure were morphologically identical, but differed in their MHC class II expression levels, suggesting they might represent different subpopulations. This difference in MHC class II expression may reflect the fact that these fractions contain cells from different thymic compartments, such as medulla and cortex. This is supported by observations in *Xenopus* where a differential expression of MHC class II molecules between the cortical and medullary thymocytes has been reported (Du Pasquier and Flajnik, 1990).

In the other organs under investigation the level of MHC class II transcription seems to correlate with the presence of MHC class II-positive B lymphocytes. Under this assumption we can also conclude that the neutrophilic granulocytes, contained in fraction three of the density-fractionated pronephrocytes, are most likely to be MHC class II negative. Fraction two contains, apart from B cells, also a high proportion of adherent cells. Studies on the MHC class II expression of isolated adherent cells demonstrated that such cells constitutively express these molecules. It could be argued that adherence, the method used for the isolation, itself is responsible for this observation. However, in fraction two the number of B cells alone could not account for the class II expression observed. Therefore, it seems likely that these adherent cells representing macrophages and do indeed constitutively express class II molecules, and can therefore be considered professional antigen presenting cells.

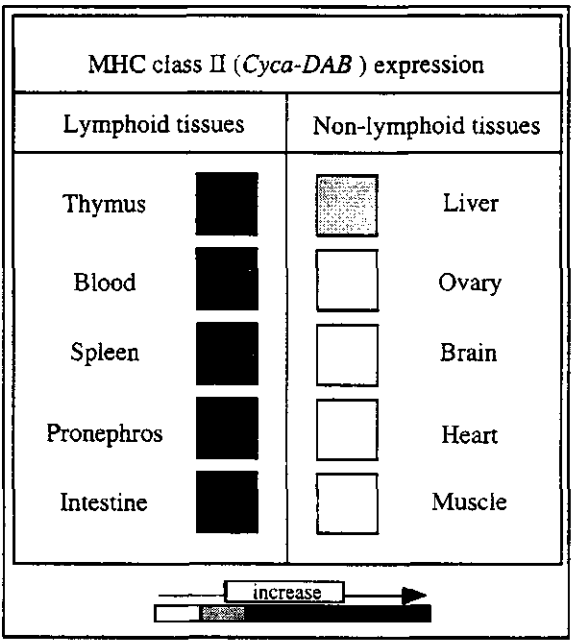

Figure 3. Tissue-dependent Cyca-DAB expression in adult carp. The levels of *Cyca-DAB* transcription in different organs was analyzed by Northern dot blot. From the organs total RNA was extracted, serially diluted and blotted onto nitrocellulose. The blot was hybridized with a carp MHC class II probe. Depicted is a graphic representation of the results. Data on the non-lymphoid tissues, with exception of the muscle, are from Ono and co-workers (1993).

Peripheral blood can easily be separated into erythrocytes and leucocytes by density centrifugation (Fig. 4). Studies on MHC class II expression of erythrocytes revealed that these cells are negative. The isolated leucocytes can further be separated into adherent and non-adherent cells, and the latter can be divided into Ig^+ and Ig^- leucocytes. The MHC class II mRNA expression observed in blood is almost all accounted for by the expression in adherent cells and B lymphocytes. This implies that circulating T cells do not express class II molecules, unlike the thymocytes. Thus, in general the situation in fish resembles that of most other vertebrates, including the uncertainty concerning the status of MHC class II expression in T cells (Klein, 1986).

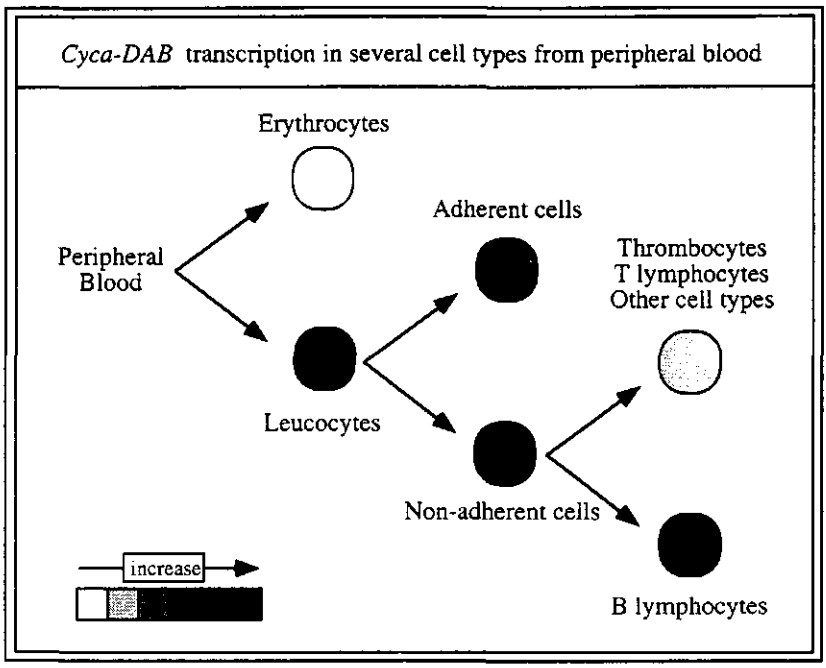

Figure 4. *Cyca-DAB* expression in peripheral blood. The levels of *Cyca-DAB* transcription in different cell types from peripheral blood were analyzed by semiquatitative PCR followed by Southern dot blot. Cells were isolated, cDNA prepared and PCR amplification with specific primers for *Cyca-DAB* performed. PCR products were serially diluted, and blotted onto nitrocellulose. The blots were hybridized with a carp MHC class II probe. Depicted is a graphic representation of the results.

MHC class I expression

As a matter of generalization it is accepted that, by virtue of their function, MHC class I molecules are expressed on every cell of any given organism with an integrated immune system. However, clear differenceS in both the level expression, and the usage of different classical and non-classical class I genes have been reported (Klein and O'hUigin, 1994). Studies on the nature of MHC class I expression are greatly facilitated by detection of β_2-microglobulin on the assumption that all class I molecules on the cell surface consist of a class I α chain, encoded by any given class I gene, and β_2-microglobulin. The identification and characterization

of the *Cyca-B2m* cDNA (Dixon *et al.*, 1993) made it possible produce a carp β_2-microglobulin recombinant protein using a prokaryotic expression system, in order to obtain a polyclonal antiserum. Concurrently, a polyclonal antiserum was raised against a classical class I gene product (Cyca-UA) using a similar approach (Van Erp *et al.*, 1996*b*), which was used in conjuction with the β_2m antiserum in studies of class I molecule expression. The use of the Cyca-B2m antiserum allows the indirect detection of all class I α chains encoded by the different class I genes.

In addition to the use of these antibodies for the detection of class I molecules on the cell surface, mRNA studies similar to those described for the class II expression have been performed. Such studies were performed in those cases where the availability of cells for immunofluorescence experiments is a problem, and therefore, one has to resort to the detection of mRNA of the different genes. This is the case in studies on the MHC class I expression during ontogeny up to the moment when enough cells can be isolated from the different organs, *i.e.*, 3 weeks after fertilization.

During early ontogeny the transcription of *Cyca-UA* preceeds that of *Cyca-B2m* (Fig 1.). Expression of the latter starts after hatching, whereas the former is already detected at day 1. The expression of any given MHC class I constituent on the cell surface is critically dependent on the trimolecular configuration of the class I α chain, peptide and the β_2m molecule (Townsend *et al.*, 1990). Therefore, it is unlikely that the carp larvae are expressing MHC class I molecules of any type on the surface of cells prior to day 3. It can, however, be conceived that MHC class I α chain molecules are present inside the cell, but can not yet be used as restriction elements for self and non-self. Experiments using the β_2m antiserum would have been able to confirm the absence of MHC class I molecules on the cell surface at these stages of development. However, such studies have not been performed, as isolation of cells from 3 day old larvae is technically very difficult. With respect to other species than fish, it has been suggested that class I molecule expression is not essential in early ontogeny in mice and in chicken (Salter-Cid and Flajnik, 1995). In *Xenopus*, the situation concerning the expression of MHC class I alpha chain is complicated, since so far an MHC has not been identified at the surface of the cells in the tadpole stage, which relates to the fact that this species undergoes metamorphosis (Flajnik *et al.*, 1987). These studies on MHC class I expression during *Xenopus* development, however, have been severely hampered by the lack of reagents detecting β_2m. In carp, β_2m transcription is detected from day 7 onwards, and reaches a level similar to that of *Cyca-UA* transcription at day 21 (Fig. 2). At this time the expression level in the spleen is low compared to that in other lymphoid tissues studied, an observation which can be attributed to the relatively late influx of lymphoid cells in this organ (Botham and Manning, 1981). However, at day 35 the expression levels of all MHC genes studied have

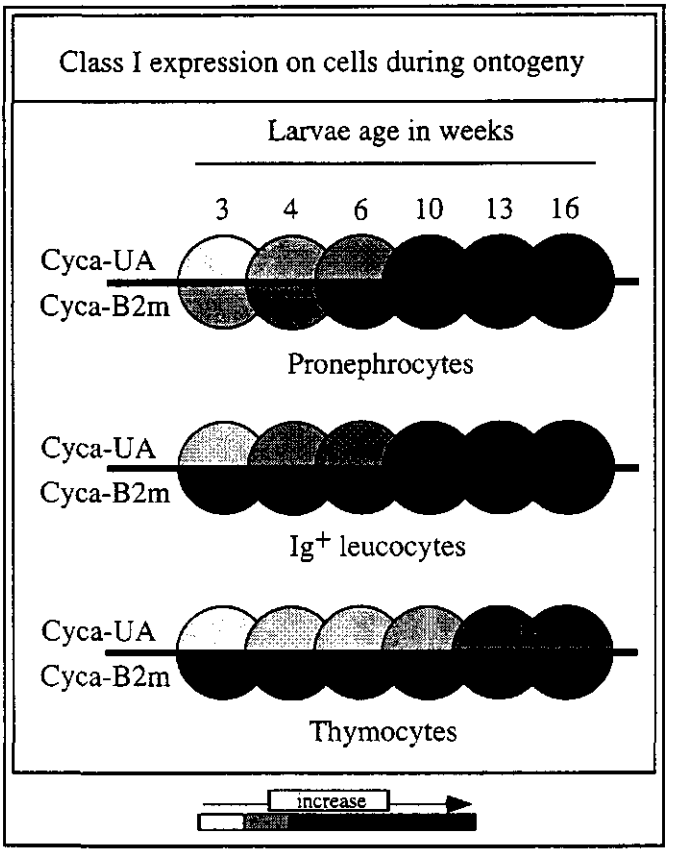

Figure 5. MHC class I cell surface expression during carp ontogeny. The appearance of Cyca-UA and Cyca-B2m on the cell surface during ontogeny was investigated. Leucocytes were isolated at different time points. Cells were isolated, and labelled with the Cyca-UA and Cyca-B2m polyclonal antisera. The number of positive cells labelled was determined by FACS analysis. Depicted is a graphic representation of the results.

reached their maximum, as determined from the adult situation.

From three weeks onwards organs can be isolated from the larvae, and the cell surface expression of the MHC genes on positively identifyable cell types be studied using the available antisera to both Cyca-UA and Cyca-B2m. In all cell populations obtained from the different organs, a clear difference in time of appearance of Cyca-UA and Cyca-B2m was observed (Fig. 5). However, with the exception of the thymus, the discrepancies early in development between the expression of the two proteins were seen to disappear after 13 weeks. Thus, it

seems that leucocytes from the organs studied, use a different class I gene product in the earlier stages of ontogeny, and this is clearly evident in the Ig^+ leucocyte population. This conclusion is based on the fact that since β_2m is not a transmembrane protein, its detection on the cell surface implies that it is associated with a class I α chain. In the thymus, to the contrary, a situation in which a difference in expression of the class I α chain and the β_2m molecule is maintained, persists even in the adult life stage of carp. Although several lineages of class I genes, such as *Cyca-Z*, *Cyca*-TC16, *Cyca*-C4, and *Cyca-UA* have been described in carp (Van Erp *et al.*, 1996c), only of the latter category the mRNAs have been fully characterized, and were shown to incorporate all elements of a functional transcripts. The differential expression of the two genes could be accounted for by the expression of a class I α gene from one of the lineages other than the *Cyca-UA* family. However, we have been unable to detect transcription of *Cyca-Z*, although the genes are present in the fish used. The possibility that genes of the other remaining lineages are used can not be excluded. Certainly in the thymus the difference persists and, therefore seems to have a functional significance, an observation which deserves further attention with respect to the identification of the class I genes expressed. In mammals, non-classical class I genes such as CD1, *Tla*, and *Qa* have been shown to be preferentially expressed in the thymus, and especially on cortical thymocytes. If this is also the case in carp, it would suggest that the useage of genes from different classical and non-classical lineages continues to play a role, in the thymus microenvironment because the discrepancy between the expression of the classical class I and β_2m genes remains present. The idea that non-classical class I genes could play an important role in carp thymocyte differentation would support the notion that these type of molecules perform essential functions, as has been suggested by Shawar and co-workers (1994).

In adult carp, the expression of MHC class I molecules was studied on the different cell populations of the blood. These studies showed essentially that two cell populations were negative for class I, namely erythrocytes and thrombocytes, whereas and the remaining myeloid and lymphoid cells were positive (Fig. 6). The high levels of expression of class I molecules on the latter cell types is consistent with observations in other species (Klein, 1986). The lack of class I molecules on erythrocytes, however, seems to contradict previous suggestions based on alloantiserum reactivities, that class I-like molecules are present on carp erythrocytes based on alloantiserum reactivities (Kaastrup *et al.*, 1989). These studies have demonstrated that a correlation exists between the presence of an alloantigen and skin transplant rejection times, from which the presence of a class I-like alloantigen on erythrocytes was inferred. Based on the present studies it is likely that this alloantigen represents a different MHC-linked alloantigenic system, perhaps analogous to the B-G encoded molecules reported in chicken, which are found mainly on erythrocytes (Kaufman, 1993).

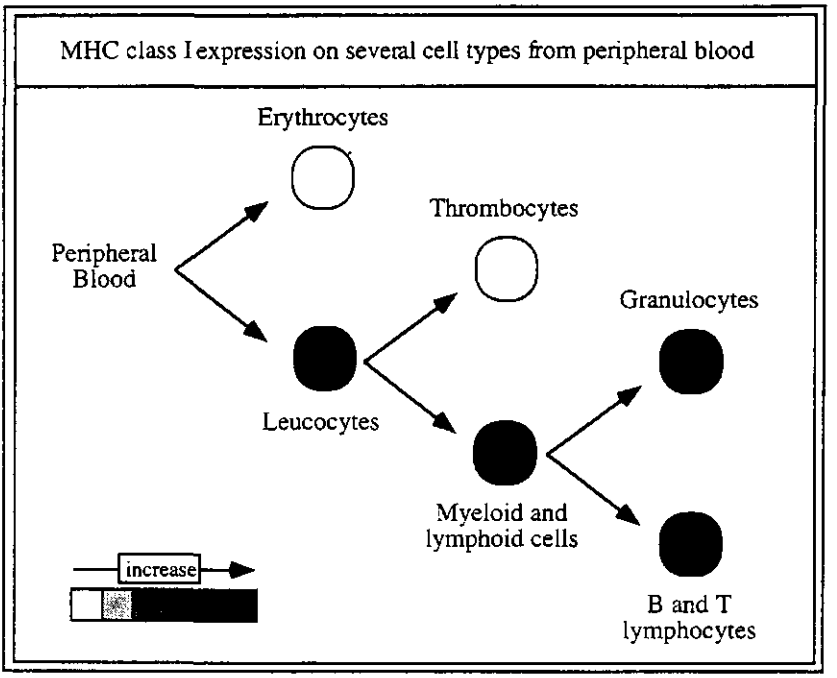

Figure 6. MHC class I expression in blood cells.The expression of class I molecules on different cell types from peripheral blood was studied. Cells were isolated, and labelled with the class I polyclonal antibodies raised against Cyca-UA and Cyca-B2m, in combination with other cell surface markers. The cells were analyzed on a FACS, and the positive cells for class I molecules determined. Depicted is a graphic representation of the results.

The most important finding in the studies under discussion is the regulation of MHC class I molecule expression by the transcription of the β_2m gene. In experiments in which carp were subjected to rapid temperature changes, involving transfer to lower temperatures, it was observed, from the changes in the reactivities of the peripheral blood leucocytes with polyclonal antisera to both Cyca-UA and Cyca-B2m, that class I molecule expression in these cells is down-regulated. A similar down regulation was not seen in the case of the expression of Ig molecules on the B cells. The MHC class I expression did return to normal levels, except in those cases in which the carp were kept at 6°C for a prolonged period. These observations suggests that, at low temperatures, cell surface trafficking of macromolecules like Ig is still possible, but that for the MHC class I proteins other mechanisms which play an important role in determining the level of expression are operative. In general, results from studies with

mammalian cells at the level of mRNA of class I and $ß_2$m genes clearly demonstrated that a trancriptional regulation of $ß_2$m is responsible for the expression of cell-surface class I molecules. However, it can not be excluded that the MHC class I expression is regulated in part at a post-transcriptional level where proteins of the class I α chain are present in the cytoplasm, but are unable to come out onto the cell-surface due to the lack of $ß_2$m (Vitiello *et al.*, 1990). In *Xenopus* prior to metamorphosis no class I molecules can be detected on the cell-surface, and it was hypothesized that this absence may be due to the lack of $ß_2$m expression in the tadpole life (Kaufman *et al.*, 1991). However, formal proof could not be provided as no reagents for the detection of $ß_2$m are available in *Xenopus* . In later studies, however, it has been revealed that class I α chains are present in the cytoplasm of tadpole cells (M.J.Flajnik, pers. comm.). These data, therefore, seem to point also to a transcriptional regulation of the $ß_2$m gene determining the class I membrane molecule expression in anuran amphibia. Although we were unable to perform studies on MHC expression using antibodies in the early stages of carp ontogeny, the results of the temperature shift experiments support the notion that MHC class I molecules are not being expressed in early embryonic stages, due to a lack of $ß_2$m transcription. This brings us to the conclusion that also in teleostean fish the cell surface expression of MHC class I molecules is regulated through the transcription of the $ß_2$m gene. This transcription seems to be influenced by factors such as developmental stage and environmental conditions, unlike the transcription of the MHC class I gene.

Concluding remarks

Based on the detection of MHC class II transcription, the studies described in this thesis reveal that, presentation of exogenous antigen in carp can be performed by a number of different cell types with a restricted tissue distribution. MHC class II expression was found on B cells, adherent cells, and thymocytes. The immunological significance of the latter observation is remains to be determined, but this phenomenon has also been observed in other species. Additional experiments into the nature of the regulation of MHC class II expression by, *e.g.*, cytokines might reveal other cell types capable of presenting antigens. Also, the availability of antibodies recognizing MHC class II molecules are expected to facilitate peptide binding studies, which in turn help to identify B- and T-cell epitopes on antigens derived from pathogenic organisms.

MHC class I molecules play an essential role in the positive and negative selection of T lymphocytes. The main function of MHC class I molecules is the binding and presentation of endogenously derived peptides. The expression of MHC class I molecules has been shown to be critically dependent upon regulated by the presence of $ß_2$m molecules. Therefore, investigations into the transcription regulation of the $ß_2$m gene are expected to contribute to an understanding of how to manipulate class I function. In this respect the temperature sensitive nature of this regulation mechanism is of great significance. Sudden ambient temperature changes which can occur under aquacultural conditions can have a temporal effect on the immunocompetence of the fish due to the down regulation of class I expression. Depending on the severity of the temperature change, the fish for some time will be at risk due to the inability of presenting non-self peptides.

In conclusion, the experiments on expression of MHC class I and class II molecules described in this thesis have provided some clues into the nature of the research that can be performed in relation to presentation of both endogenous and exogenous antigens in teleost fish.

References

Botham, J.W. and Manning, M. J. (1981) The histogeneses of the lymphoid organs in the carp *Cyprinus carpio* L. and ontogenic development of allograft reactivity. *J. Fish Biol.* **19**: 403-414.

Castillo, A., Sánchez, C., Dominguez, J., Kaattari, S.L. and Villena, A.J. (1993) Ontogeny of IgM-bearing cells in rainbow trout. *Dev. Comp. Immunol.* **17**: 419-424.

Dixon, B., Stet, R.J.M., Van Erp, S.H.M. and Pohajdak, B. (1993) Characterization of ß2-microglobulin transcripts from two teleost species. *Immunogenetics* **38**: 27-34.

Dixon, B., Van Erp, S.H.M., Rodrigues, P.N.S., Egberts, E. and Stet, R.J.M. (1995) Fish major histocompatibility complex genes: an expansion. *Dev. Comp. Immunol.* **19**: 109-135.

Du Pasquier, L. and Flajnik, M.F. (1990) Expression of MHC class II antigens during *Xenopus* development. *Dev.Immunol.* **1**: 85-95.

Flajnik, M.F., Hsu, E., Kaufman, J.F. and Du Pasquier L. (1987) Changes in the immune system during metarmorphosis of *Xenopus*. *Immunol. Today* **8**: 58-64.

Flajnik, M.F., Ferrone, S., Cohen, N. and Du Pasquier, L. (1990) Evolution of the MHC: antigenicity and unusual tissue distribution of *Xenopus* (frog) class II molecules. *Molec. Immunol.* **27**: 451-462.

Germain, R.N. and Margulies, D.H. (1993). The biochemistry and cell biology of antigen processing and presentation. *Ann. Rev. Immunol.* **11**: 403-450.

Hashimoto, K., Nakanishi, T. and Kurosawa, Y. (1990). Isolation of carp genes encoding major histocompatibility complex antigens. *Proc. Nat. Acad. Sci. USA.* **87**: 6863-6867.

Kaastrup, P., Stet, R.J.M., Tigchelaar, A.J., Egberts, E. and Van Muiswinkel, W.B. (1989) A major histocompatibility locus in fish: Serological identification and segregation of transplantation antigens in the common carp (Cyprinus carpio L.). *Imunnogenetics* **30**: 284-290

Kaufman, J. (1993) Ontogeny and phylogeny of MHC-like molecules. In: *Developmental Immunology*. Cooper and Nisbet-Brown, eds. Oxford University Press, Oxford, pp 53-84.

Kaufman, J., Flajnijk, M..and Du Pasquier L. (1993) The MHC molecules of ectothermic vertebrates. In: *Phylogenesis of immune functions*. Warr and Cohen, eds. CRC press, Boca Raton, Florida, pp 125-149.

Klein, J. (1986). *Natural History of the Major Histocompatibility Complex*. John Wiley and Sons, New York.

Klein, J., Bontrop, R.E., Dawkins, R.L., Erlich, H.A., Gyllensten, U.B., Heise, E.R., Jones, P.P., Parham, P., Wakeland, E.K. and Watkins, D.I. (1990). Nomenclature for the major histocompatibility complexes of different species: a proposal. *Immunogenetics* **31**: 217-219.

Klein, J. and O'hUigin, C. (1994) The conundrum of non-classical major histocompatibility complexe genes. *Proc. Nat. Academy Sci. USA*. **91**: 6251-6252.

Ono, H., O'hUigin, C., Vincek, V., Stet, R.J.M., Figueroa, F. and Klein, J. (1993). New β chain encoding MHC class II genes in the carp. *Immunogenetics* **38**: 146-149.

Salter-Cid, L. and Flajnik, M.F. (1995) Evolution and developmental regulation of the Major Histocompatability Complex. *Crit. Rev.Immunol.* **15**: 31-75.

Shawar, S., Vyas, J.M., Rogers, J.R. & Rich, R.R. (1994). Antigen presentation by major histocompatibility complex class I-b molecules. *Ann. Rev. Immunol.* **12**: 839-880.

Stet, R.J.M. and Egberts E. (1991). The histocompatibility complex in teleostean fishes: from multiple histocompatibility loci to a major histocompatibility complex. *Fish Shellfish Immunol.* **1**: 1-16.

Stet, R.J.M., Dixon, B., Van Erp, S.H.M., Van Lierop, M.C., Rodrigues, P.N.S. and Egberts, E. (1996) Inference of structure and function of fish Major Histocompatibility Complex (MHC) molecules from expressed genes. *Fish Shellfish Immunol.* (in press)

Townsend, A., Elliott, T., Cerundolo, V., Foster, L., Barber, B. and Tse A. (1990). Assembly of MHC class I molecules analyzed in vitro. *Cell* **62**, 285-295.

Van Erp, S.H.M., Egberts, E. and Stet, R.J.M. (1996*a*) Characterization of major histocompatibility complex class II A and B genes in a gynogenetic carp clone. *Immunogenetics* (in press)

Van Erp, S.H.M., Dixon B., Figueroa F., Egberts E. and Stet, R.J.M. (1996*b*) Identification and characterisation of a novel MHC class I gene from carp (*Cyprinus carpio* L.). *Immunogenetics* (in press)

Van Erp, S.H.M., Egberts, E. and Stet, R.J.M. (1996*c*) Evidence for multiple distinct major histocompatibility complex class I lineages in teleost fish. *Eur. J. Immunogenetics* (in press)

Vitiello, A., Potter, T.A. and Sherman, L.A. (1990) The role of β_2-microglobulin in peptide binding by class I molecules. *Science* **250**: 1423-1426.

The common carp (*Cyprinus carpio* L.) has been the experimental animal of choice because many features of the immune system of this Cyprinid fish have been well characterized. The immune system consists of an integrated set of organs containing cells such as Ig^+ B lymphocytes, Ig^- leucocytes, and macrophages, capable of performing a specific immune response. The prerequisites for such a response upon an antigenic challenge are cell-surface molecules, like T- and B-cell receptors, and major histocompatibility complex (MHC)-encoded molecules. T cells are only capable of recognizing processed antigen when it is presented in the context of an MHC molecules. In mammals it has been firmly established that peptides derived from the antigenic proteins are bound to MHC-encoded molecules, and that the peptide/MHC configuration is recognized by the T-cell receptor (TCR). Thus, for a better understanding of the initiation of a specific immune response more knowledge is needed about the presence and function of the molecules involved in antigen presentation.

In **Chapter 1**, a description is given of the current knowledge on the MHC in fish, and in particular in the common carp. The first MHC genes were described for carp, however, these were only partial genomic sequences, and it proved difficult to establish that these were functional genes. This first report was soon followed by a wealth of other sequences in a variety of other teleost species. Overall, the MHC gene structure and that of the $beta_2$-microglobulin ($ß_2m$) do not seem to differ from those described for mammalian species. The MHC genes show an exon and intron structure remarkably similar to their mammalian counterparts, including the fact that the introns are all phase 1. The only major difference may lie in the fact that teleost fish have more than one MHC, similar to the situation described for the chicken and *Xenopus* MHC.

Most sequences reported are, however, partial sequences obtained by Polymerase Chain Reactions (PCR) on genomic DNA, and do not provide information on the function of the encoded molecules. For a limited number of species, including the carp, full-length cDNA sequences have been reported, and can be used to infer the functionality of the encoded proteins. Analyses of these cDNAs have indicated that invariably the main functional characteristics, such as the presence of conserved peptide-binding residues and cysteines forming disulphide bridges, are present. Thus, although abundant theoretical evidence seems to suggest the presence of MHC molecules, formal proof has yet to be presented.

In **Chapter 2**, studies are described which aim at providing evidence for the the presence of MHC class II molecules in lymphoid organs. To this end RNA was isolated from several organs, some with known immunological functions. The cDNA prepared from it was used as a template in the PCR amplification of *MHCCyca-DAB* transcripts. The presence of these transcripts appeared to be confined to tissues such as thymus, spleen, pronephros and intestine,

which have been demonstrated to perform immunological functions. Further analyses carried out on isolated leucocyte subpopulations indicated that a direct correlation exists between the levels of *Cyca-DAB* expression and the number of Ig^+ cells present. In addition, adherent cells were shown to abundantly express class II transcripts. The most important finding was the fact that thymocytes were the cell population with the highest expression of *Cyca-DAB* mRNA. Although we were unable to detect the MHC class II molecules themselves due to the lack of proper reagents like antibodies, these studies reinforced the notion that class II expression is restricted to those microenvironments where antigen presentation takes place.

To overcome the problem of detection of the molecules proper, a different strategy was adopted, *i.e.*, prokaryotic expression of cDNA sequences for the production of recombinant proteins that can be used to immunize rabbits. In **Chapter 3**, experiments are described with a polyclonal antibody raised against carp $ß_2$-microglobulin (Cyca-B2m). This antiserum was used to assess the expression of class I molecules on the cell surface of different cell populations. The results of these experiment show that erythrocytes and thrombocytes are negative, whereas leucocytes of lympho-myeloid lineages are class I positive. In addition, a brightly class I positive population of Ig^- lymphocytes was identified, which may constitute putative circulating T lymphocytes.

Subsequently, experiments were designed to study the effect of temperature on the expression of class I molecules on peripheral blood leucocytes (PBL). These experiments revealed a long lasting absence of class I molecules at low permissive (6°C) temperatures, which could be restored by increasing the temperature. These results were confirmed by using an antiserum raised against the carp class I α chain (Cyca-UA). However, the presence of Ig on the cell surface of B cells remained unchanged in the course of the experiments. The transcription of the genes involved was also studied, using PCR amplification on cDNA prepared from RNA. Normal transcription of *Cyca-UA* was observed, which contrasts the low levels of transcription found for *Cyca-B2m*. Therefore, the absence of class I molecules is considered to be the result of a lack of sufficient *Cyca-B2m* transcription, prompting the conclusion that class I cell surface expression is regulated by a temperature sensitive transcription-mechanism of the $ß_2$m gene.

In **Chapters 4** and **5**, the MHC class I and class II molecules were studied during carp ontogeny using different approaches. In earlier stages of development, studies on the expression of the MHC class I and class II molecules have been restricted to the detection of transcripts using PCR amplification of cDNA. In later developmental stages, where it was possible to obtain cell suspensions from immunological organs of the larvae, the expression of MHC class I molecules was studied by using polyclonal antibodies to $ß_2$m and the MHC class I α chain. In unfertilized eggs no transcription of any of the genes was detected. Transcription of *Cyca-UA*, *Cyca-DAB*, and *Cyca-DXA* starts as early as day 1, and increases steadily reaching a plateau

at day 3. In contrast, transcription of *Cyca-B2m* was shown to start at day 7. After 14 days, the levels of expression of the genes under investigation have reached a plateau. At this point in time, organs can be dissected and used for the detection of transcription. These experiments demonstrated that from the lymphoid organs investigated, the spleen is the only one where a significant lower level of transcription of the MHC and β_2m genes was found. This observation correlats with the late development of this organ and subsequent late influx of lymphoid cells. The absence of *Cyca-B2m* transcripts suggests a lack of class I cell surface expression, similar to the situation in the temperature experiments, and corroborates the conclusion that MHC class I molecules do not play a major role during early ontogeny.

At three weeks after fertilization it is possible to obtain enough cells from the immunologically important organs to perform FACS analyses with antibodies identifying MHC class I molecules. These studies revealed that, in the pronephros and spleen, cells are present, up to week 13, which are positive for Cyca-B2m, but do not express the Cyca-UA class I α chain. This cell population seems to consist mainly of Ig^+ cells. No difference in the percentage of Cyca-B2m- and Cyca-UA-positive cells is observed after week 13, reflecting the adult situation. In the adult thymus, to the contrary, there remains a population of thymocytes which is Cyca-B2m-positive, but Cyca-UA-negative. The identity of the class I α chain, that is associated with the Cyca-B2m during ontogeny and in the adult thymus, is yet to be revealed. The suggestion is that a non-classical class I-like molecule may play a role in thymocyte differentation. In the adult carp, peripheral blood consists of Cyca-UA- and Cyca-B2m-negative erythrocytes and thrombocytes, whereas the other leucocytes are positive for these molecules.

In **Chapter 6**, the data presented in this thesis are discussed in connection with what is known from other vertebrates, mainly *Xenopus* and chicken. The MHC class I and class II molecules are dealt with separately, as their distribution patterns differ to a large extent. Basically, the expression pattern of class II molecules follows that of other species studied. The only exception is the early onset, in carp, of MHC class II transcription during ontogeny. As for the expression of class I molecules on cells of the immune system it is discussed that different class I α chain-encoding genes are being used in carp. This conclusion is based on the observations from experiments with the antiserum to the Cyca-B2m molecule. However, the most interesting finding, with respect to the MHC class I expression, is the temperature-dependent regulation of β_2m transcription. This mechanism is thought to be responsible for the lack of MHC class I cell surface expression that has been observed at low ambient temperatures in this ectothermic vertebrate species. This is the first report in a cold-blooded vertebrate in which this mechanism has been firmly established.

O sistema imunológico da carpa (*Cyprinus carpio* L.) tem vindo a ser progressivamente estudado e actualmente encontra-se relativamente bem caracterizado. Embora com certas diferenças importantes, os componentes básicos do sistema imunológico presentes em outros grupos de animais são também parte integrante do sistema imune em peixes. Mais particularmente em carpa, o sistema consiste de um grupo integrado de orgãos contendo células como os limfócitos B Ig^+, leucócitos Ig^-, e macrófagos, que são capazes de efectuar uma resposta imuno específica contra um determinado antigénio. Os pré-requesitos necessários para esta resposta específica presença de moléculas na superfície celular, como os receptores das células T e B, e as moléculas codificadas pelo receptor de major histocompatibilidade (MHC). As células T só são capazes de reconhecer o antigénio quando este é processado e apresentado no contexto das moléculas de MHC. Em mamíferos é geralmente aceite que péptidos derivados do processamento do antigénio ligam-se às moleculas codificadas pelo MHC, e é esta configuração péptido/MHC que é reconhecida pelo receptor da célula T. Desta forma, para um melhor entendimento da iniciação da resposta imune específica, são necessários mais estudos sobre a presença e a função das moléculas envolvidas na apresentação de antigénio.

No **Capítulo 1**, é feita uma revisão acerca do conhecimento actual sobre MHC em peixes, e mais particularmente em carpa. Os primeiros genes do MHC a serem descritos em peixes foram encontrados em carpa, no entanto estas sequências genómicas eram somente parciais, e revelou-se mais tarde difícil provar que estes eram de facto genes funcionais. Após o referido estudo, uma grande quantidade de sequências obtidas em diferentes Teleósteos foram publicadas. Em geral, estes trabalhos mostram que a estrutura dos genes do MHC e da beta$_2$-microglobulina (β_2m) não parecem ser muito diferente daqueles descritos para mamíferos. Os genes situados no MHC apresentam uma estrutura exon e intron muito semelhante aos equivalentes em mamíferos, incluindo o facto que os introns serem todos fase 1. A única diferença de vulto talvez seja a possibilidade de os peixes teleósteos possuirem mais de um MHC, uma situaçao semelhante ao que está descrito para o MHC da galinha e do sapo (*Xenopus*).

A maior parte das sequências publicadas são no entanto sequências parciais obtidas através do uso da técnica "Polymerase Chain Reactions" (PCR) em DNA genómico, não sendo muito informativas sobre a função das moléculas codificadas. Para um número limitado de espécies, incluindo a carpa, têm sido publicadas sequências completas de cDNA, que podem ser usadas para inferir sobre a funcionalidade das proteínas por elas codificadas. A análise destes cDNAs revelaram que invariavelmente as principais características funcionais, como a existência de resíduos de ligações peptídicas conservados, e cistinas formando pontes disulfídicas, estão presentes. No entanto apesar da abundante evidência teórica sugerir a presença de moléculas codificadas pelo MHC, uma prova formal tem ainda que ser apresentada.

No **Capítulo 2**, são apresentados estudos cujo o principal objectivo é de mostrar evidência para a presença de moléculas do MHC classe II nos orgãos limfoides. Para este efeito RNA foi isolado de vários orgãos com conhecida função imunológica. cDNA foi preparado a partir deste RNA e usado como cadeia molde na amplificação especifíca dos transcritos *MhcCyca-DAB*. A presença desses transcritos parece estar confinada a tecidos como o timo, baço, pronefros e intestino, tecidos que se sabe desempenharem funções imunológicas em carpa. Análises levadas a cabo com sub-populações de leucócitos indicam que existe uma correlação directa entre o nivel de expressão de *Cyca-DAB* e o número de células Ig^+ presentes. As celúlas aderentes evidenciaram uma abundante expressão de transcritos para class II. A descoberta mais importante foi o facto de os timócitos serem a população celular com a mais elevada expressão de mRNA *Cyca-DAB*. Embora nós não fossemos capazes de detectar as moléculas de MHC classe II dado a inexistência de reagentes adequados, tais como anticorpos, estes estudos reforçaram o ponto de vista segundo o qual a expressão de classe II é restricta aqueles micro-ambientes onde a apresentação antigénio tem lugar.

Para ultrapassar o problema da detecção das moléculas de MHC, uma estratégia diferente foi adoptada, *i.e.*, a expressão procariótica das sequencias de cDNA para a produção de proteína recombinante que pode ser usada posteriormente para imunizar coelhos por forma a produzir anticorpos policlonais. No **Capítulo 3**, são descritas experiências levadas a cabo com o anticorpo policlonal produzido contra a $ß_2$-microglobulina (Cyca-B2m) recombinante de carpa. Este anticorpo foi usado para estudar a expressão das moléculas de classe I na superficie das células das diferentes populações celulares. Os resultados desses experimentos mostram que os eritrócitos e trombócitos são negativos para classe I, enquanto os leucócitos das linhagens linfo-mieloide são positivos. Por outro lado, uma população de linfócitos Ig^- intensamente positiva para classe I foi identificada, sendo possivelmente constituida por linfócitos T circulantes.

Subsequentemente, foram planeadas experiências com o objectivo de estudar o efeito da temperatura na expressão das moléculas de classe I em leucócitos do sangue periférico (PBL). Estes estudos revelaram uma ausência prolongada de moléculas de class I a baixas temperaturas (6°C) na superficie celular, ausência que podia ser revertida pelo aumento da temperatura. Estes resultados foram confirmados pelo uso do anticorpo policlonal produzido contra a cadeia α do MHC classe I (Cyca-UA) da carpa. No entanto, a presença de Ig na superfície das células B permaneceu inalterada durante o curso das experiências. A transcrição dos genes involvidos foi também estudada, usando PCR em cDNA preparado a partir de RNA isolado dos diferentes grupos experimentais. Para foi observada *Cyca-UA* uma transcrição normal, o que contrasta com os baixos niveis de transcrição encontrados para *Cyca-B2m*. Desta forma, a ausência de moléculas de classe I parece ser o resultado da insuficiente transcrição de *Cyca-B2m*, levando concluir que a expressão da classe I na superfície celular é regulada por um mecanismo de

transcrição do gene que codifica a $ß_2$m que é sensivel á temperatura.

Nos **Capítulos 4** e **5**, a expresão das moléculas de MHC classe I e classe II foram estudadas durante a ontogenia da carpa usando diferentes métodos. Nos estadios iniciais de desenvolvimento, os estudos sobre expressão das moléculas de MHC classe I e classe II foram limitados á detecção de transcritos usando PCR em cDNA. Nos estadios de desenvolvimento mais tardios, em que era possivel obter suspenções celulares dos orgãos do sistema imunológico da larva, a expressão das moléculas de MHC class I foi estudada com o uso de anticorpos policlonais contra a $ß_2$m e a cadeia α de MHC class I. Nos ovos não fertilizados não foi detectada transcrição para os genes estudados. A transcrição de *Cyca-UA*, *Cyca-DAB*, e *Cyca-DXA* começa cedo, no dia 1 após a fertilização, e aumenta continuamente atingindo um nivel constante no dia 3. No entanto, a transcrição de *Cyca-B2m* inicia-se sómente no dia 7. Passados 14 dias, os niveis de expressão dos genes sob investigação alcançaram um nivel constante. Neste altura, os orgãos podem ser dissecados e usados para a detecção da transcrição. Estas experiências demonstraram que dos orgãos linfoides investigados, o baço é o único onde o nivel de transcrição dos genes de MHC e $ß_2$m foi significativamente mais baixo. Esta observação está correlacionada com o tardio desenvolvimento deste orgão e o subsequente tardio influxo de células linfoides. A ausência de transcritos de *Cyca-B2m* sugere a inexistência de class I na superficie celular, uma situação semelhante á observada nas experiências com a temperatura, e apoiando o ponto de vista segundo o qual as moléculas de MHC class I não desempenham um papel crucial durante as fases iniciais da ontogenia.

Três semanas após a fertilização é possivel obter um número suficiente de células dos orgãos imunologicamente importantes, de forma a poder levar a cabo estudos usando anticorpos que identificam as moléculas do MHC class I. Estes estudos mostraram que, em pronefros e no baço, até a semana 13, existem células que são positivas para Cyca-B2m mas não expressam a cadeia α da classe I (Cyca-UA). Esta população parece consistir maioritáriamente de células Ig^+. Depois da semana 13 não foram observadas diferenças entre as percentagens de células positivas marcadas com anticorpos contra Cyca-B2m ou Cyca-UA, reflectindo a situação no adulto. No entanto, no timo do adulto, permanece uma população de timócitos que são Cyca-B2m-positivos mas Cyca-UA-negativos. A natureza da cadeia α do MHC class I que está associada com a Cyca-B2m durante a ontogenia e no timo do adulto, tem ainda que ser identificada. É aqui sugerido que uma molécula do tipo classe I, não clássica, possa desempenhar um papel na differenciação do timócito. Na carpa adulta, o sangue periférico consiste de eritrócitos e trombócitos que são Cyca-UA e Cyca-B2m negativos, enquanto os outros leucócitos são positivos para estes anticorpos.

No **Capítulo 6**, os dados apresentados nesta tese são discutidos e integrandos com conhecimentos de outros vertebrados, especialmente *Xenopus* (sapo) e galinha. As moléculas

de MHC classe I e class II são discutidas separadamente, dado que os seus padrões de distribuição são muito diferentes. Basicamente, o padrão de expressão das moléculas de MHC class II é semelhante ao das outras espécies estudadas. A única excepção, é o rápido começo da transcrição dos genes que codificam para MHC class II durante a ontogenia da carpa. É discutida a possibilidade de diferentes genes codificando a cadeia α do MHC class I estarem a ser usados para expressão de moléculas de classe I na superficie das células do sistema imune em carpa. Esta conclusão é baseada em observações das experiências com o anticorpo contra a molécula Cyca-B2m. No entanto, a descoberta mais interessante, com relação à expressão de MHC class I, é o facto de a regulação da transcrição da $ß_2m$ estar dependente da temperatura. Este mecanismo é provavelmente o reponsável pela ausência de MHC class I na superfície celular, que foi observado na carpa quando colocada a uma temperatura ambiente baixa. Este é a primeira vez em que este mecanismo fica provado para um vertebrado poiquilotérmico como a carpa.

Samenvatting

Voor het werk beschreven in dit proefschrift is gebruik gemaakt van de karper als proefdier omdat het immuunsysteem van deze cyprinide in veel opzichten goed is onderzocht. Het immuunsysteem is opgebouwd uit een aantal organen met daarin cellen zoals Ig^+ B lymfocyten, Ig^- leucocyten, en macrofagen; hiermee is het in staat een specifieke immuunrespons uit te voeren. Voor zo'n respons die volgt op een contact met antigeen zijn celoppervlakte moleculen noodzakelijk, zoals T- en B-cel receptoren, en moleculen die gecodeerd worden door het major histocompatibility complex (MHC). Na bewerking ervan kunnen T cellen antigeen alleen herkennen als het aangeboden wordt in samenhang met MHC moleculen. Uit onderzoek bij zoogdieren is komen vast te staan dat peptiden afkomstig van antigene eiwitten gebonden worden aan moleculen die door het MHC worden gecodeerd, en dat het deze peptide/MHC configuratie is die door de T-cel receptor wordt herkend. Voor een beter begrip van hoe een specifieke immuunrespons wordt opgestart is het daarom noodzakelijk dat wij onze kennis vergroten met betrekking tot het voorkomen en het functioneren van de moleculen die betrokken zijn bij de presentatie van antigeen.

In **Hoofdstuk 1** wordt een beschrijving gegeven van de huidige staat van onze kennis met betrekking tot het MHC bij vissen, met name bij de karper. De eerste MHC genen die in dit verband zijn beschreven waren afkomstig van de karper, maar dit waren slechts onvolledige genomische sequenties, en het bleek lastig vast te stellen of deze genen ook daadwerkelijk functioneel zijn. Deze eerste publikatie werd al spoedig gevolgd door het bekend worden van een groot aantal andere sequenties bij verscheidene andere soorten beenvissen. In het algemeen lijkt de structuur van de MHC genen, en die van het gen voor beta$_2$-microglobuline (β_2m), niet veel te verschillen van de overeenkomstige elementen bij zoogdieren, met inbegrip van het feit dat de intronen alle fase 1 zijn. Het enige belangrijke verschil zou wel eens het feit kunnen zijn dat, evenals beschreven bij de kip en *Xenopus*, ook bij beenvissen meer dan één MHC aanwezig is.

De meeste sequenties waarover nu gepubliceerd is bevatten slechts een deel van de informatie waaruit het gen bestaat, en ze geven daarom geen informatie over de functie van de moleculen die erdoor gecodeerd worden. Voor maar een beperkt aantal soorten, waaronder de karper, zijn ook volledige cDNA sequenties beschreven, en deze kunnen wel gebruikt worden om daaruit af te leiden hoe de gecodeerde eiwitten functioneren. Analyses van zulke cDNAs laten zien dat ze, zonder uitzondering, beschikken over de meest belangrijke functionele kenmerken zoals het voorkomen van geconserveerde aminozuren die betrokken zijn bij de binding van peptiden, en van cysteïnes die zwavelbruggen kunnen vormen. Alhoewel er dus een overmaat aan theoretische bewijskracht is voor het bestaan van MHC moleculen bij de vis, moet toch het formele bewijs ervan nog steeds geleverd worden.

In **Hoofdstuk 2** wordt onderzoek beschreven dat tot doel heeft om aanwijzingen te verkrijgen over het voorkomen van MHC klasse II moleculen in lymfoïde organen. Hiervoor is RNA geïsoleerd uit verschillende organen. Van dit RNA is cDNA gemaakt, en dit laatste is vervolgens gebruikt als matrijs voor een PCR-afhankelijke vermeerdering van *MhcCyca-DAB* transcripten. Het voorkomen van deze transcripten bleek zich te beperken tot organen zoals de thymus, de milt, de kopnier en de darm, waarvan bekend is dat ze een rol spelen in het functioneren van het immuunsysteem. Verdergaande analyses op geïsoleerde subpopulaties van leucocyten leverden aanwijzingen op dat er een direct verband bestaat tussen de niveaus van expressie van *Cyca-DAB*, en de hoeveelheid Ig^+ cellen. Bovendien bleken adherente cellen over een hoge expressie van klasse II transcripten te beschikken. De belangrijkste waarneming was echter het feit dat de thymocyten de celpopulatie is met het hoogste niveau aan expressie van *Cyca-DAB* mRNA. Ofschoon wij dus niet in staat zijn geweest om de MHC klasse II moleculen zelf aan te tonen vanwege het ontbreken van goede reagentia zoals antilichamen, heeft dit deel van het onderzoek de gedachte versterkt dat de expressie van klasse II zich beperkt tot die micromilieus waarin zich de presentatie van antigeen afspeelt.

Om het probleem te omzeilen dat wij de MHC moleculen zelf niet kunnen identificeren, is gebruik gemaakt van een andere aanpak, nl. expressie van cDNA sequenties in een prokaryoot organisme voor de produktie van recombinante eiwitten, welke op hun beurt weer gebruikt kunnen worden voor de immunisatie van konijnen. In **Hoofdstuk 3** worden experimenten beschreven die zijn uitgevoerd met een polyclonaal antiserum dat is opgewekt tegen het karper β_2-microglobuline (Cyca-B2m). Dit antiserum is gebruikt om bij verschillende celpopulaties vast te stellen of klasse I moleculen op de celmembraan tot expressie komen. De resultaten van deze experimenten laten zien dat erythrocyten en thrombocyten in dit opzicht negatief zijn, terwijl leucocyten die behoren tot de lymfo-myeloïde differentiatieroute daarentegen positief zijn voor klasse I. Daarnaast werd een populatie van Ig^- lymfocyten geïdentificeerd die sterk positief is voor klasse I, en waarschijnlijk bestaat uit mogelijke T cellen in circulatie.

Vervolgens zijn experimenten opgezet om het effect van de temperatuur te onderzoeken op de expressie van klasse I moleculen van leucocyten in het perifere bloed (PBL). Uit deze experimenten kwam naar voren dat bij lage temperatuur (6°C) voor langere tijd geen klasse I moleculen op zulke cellen kunnen worden gevonden, maar dat deze moleculen weer verschijnen wanneer de temperatuur verhoogd wordt. Deze resultaten konden bevestigd worden met behulp van een antiserum dat gemaakt was tegen de karper klasse I α keten (Cyca-UA). Met betrekking tot het Ig, dat voorkomt op het oppervlak van de B cellen, bleek zich tijdens deze experimenten daarentegen geen veranderingen voor te doen. Er is ook gekeken naar de transcriptie van de betreffende genen, en wel met behulp van PCR-afhankelijke amplificatie van cDNA dat verkregen was uit mRNA. De transcriptie van *Cyca-UA* bleek bij lage temperatuur normaal

te verlopen, in tegenstelling tot de transcriptie van *Cyca-B2m* welke duidelijk verlaagd was. Het ontbreken van klasse I moleculen bij lage temperatuur kan daarom opgevat worden als zijnde het gevolg van een gebrek aan voldoende transcriptie van *Cyca-B2m*, en leidt dus tot de conclusie dat de expressie van klasse I op het celoppervlak gereguleerd wordt door een temperatuur-gevoelig transcriptiemechanisme van het β_2m gen.

In **Hoofdstuk 4** en **Hoofdstuk 5** is op verschillende manieren onderzoek gedaan naar de klasse I en klasse II MHC moleculen tijdens diverse ontwikkelingsstadia van de karper. De analyses van de expressie van de klasse I en klasse II MHC moleculen hebben zich tijdens de vroege ontwikkeling beperkt tot de detectie van transcripten met behulp van PCR-afhankelijke amplificatie van cDNA. Voor de latere ontwikkelingsstadia was het mogelijk om celsuspensies te maken van die larvale organen welke in immunologisch opzicht een belangrijke rol spelen. De expressie van de klasse I MHC moleculen is bestudeerd met gebruik van polyclonale antisera gericht tegen β_2m en de MHC klasse I α keten. Van geen enkel van de bovengenoemde genen kon transcriptie worden aangetoond in onbevruchte eieren. Transcriptie van *Cyca-UA*, *Cyca-DAB*, en *Cyca-DXA* wordt voor het eerst gezien worden op dag 1 na de bevruchting, en neemt vanaf dan gestadig toe totdat op dag 3 een plateau wordt bereikt. De transcriptie van *Cyca-B2m* bleek daarentegen te beginnen op dag 7. Uiteindelijk bereiken de expressie niveaus van alle genen die in dit verband zijn onderzocht een plateau op 14 dagen na bevruchting. Dat is ook het tijdstip waarop het mogelijk wordt om organen te verwijderen, die dan vervolgens gebruikt kunnen worden om daarin gentranscriptie aan te tonen. Zulke experimenten lieten zien dat, van de lymfoïde organen die op deze manier zijn onderzocht, de milt het enige orgaan is waar een significant lager niveau van transcriptie wordt aangetroffen met betrekking tot de MHC genen en het β_2m gen. Deze waarneming komt overeen met de late ontwikkeling van dit orgaan, en het daarmee samenhangende late tijdstip van instroom van lymfoïde cellen. De afwezigheid van *Cyca-B2m* transcripten doet de veronderstelling rijzen dat ook hier, vergelijkbaar met de situatie in de experimenten waarbij de omgevingstemperatuur is gevariëerd, sprake is van het ontbreken van klasse I expressie op het celoppervlak, hetgeen aanleiding geeft tot de conclusie dat de MHC klasse I moleculen in de vroege ontwikkeling geen belangrijke rol spelen.

Drie weken na bevruchting kunnen voldoende cellen verkregen worden van de in immunologisch opzicht belangrijke organen om daarmee FACS analyses uit te voeren, na reacties op die cellen met antilichamen die de MHC klasse I moleculen kunnen aantonen. Dergelijk onderzoek bracht naar voren dat, in de kopnier en de milt, tot en met week 13 na de bevruchting cellen voorkomen die positief zijn voor Cyca-B2m, maar niet de Cyca-UA klasse I α keten tot expressie brengen. Deze celpopulatie lijkt vooral te bestaan uit Ig^+ cellen. Na week 13 zijn er geen verschillen meer te zien tussen de percentages aan cellen positief

voor Cyca-B2m en positief voor Cyca-UA, analoog aan de situatie zoals die in het volgroeide individu wordt aangetroffen. In de thymus van volwassen dieren blijft daarentegen een populatie thymocyten aanwezig die positief is voor Cyca-B2m, maar negatief voor Cyca-UA. Dit doet vermoeden dat een klasse I α keten verschillend van Cyca-UA geassociëerd met Cyca-B2m voorkomt op het oppervlak van cellen tijdens de embryonale en larvale ontwikkeling van de karper, en op cellen in de thymus van volwassen individuen. De identiteit van deze klasse I α keten moet nog worden vastgesteld. Het ligt voor de hand te veronderstellen dat een op MHC klasse I gelijkend eiwitmolecuul een rol speelt in de differentiatie van thymocyten. Bij karpers in volwassen toestand blijken in het perifere bloed de erythrocyten en thrombocyten negatief te zijn voor zowel Cyca-UA en Cyca-B2m, in tegenstelling tot de overige leucocyten die voor beide moleculen positief zijn.

In **Hoofdstuk 6** worden de gegevens die uit het in dit proefschrift beschreven onderzoek zijn voortgekomen besproken in samenhang met hetgeen in dit opzicht van andere gewervelde dieren, met name *Xenopus* en de kip, bekend is. Hierbij worden de MHC klasse I en klasse II moleculen afzonderlijk behandeld, aangezien deze een in grote mate van elkaar verschillend patroon van verdeling over de diverse celtypen laten zien. In principe is er geen verschil in het patroon van expressie van de MHC klasse II moleculen bij de vis met dat van andere diersoorten waarbij hieromtrent onderzoek is gedaan. De enige uitzondering hierop is dat bij de karper de MHC klasse II transcriptie al vroeg in de ontwikkeling van start gaat. Wat betreft de expressie van klasse I moleculen op cellen behorend tot het immuunsysteem wordt de mogelijkheid besproken dat de karper daarbij gebruik maakt van genen coderend voor verschillende klasse I α ketens. Deze conclusie is gebaseerd op waarnemingen uit experimenten waarin gebruik is gemaakt van een antiserum gericht tegen het Cyca-B2m molecuul. De meest belangwekkende bevinding met betrekking tot de MHC klasse I expressie is echter dat de transcriptie van β_2m temperatuur-afhankelijk is gereguleerd. Gedacht wordt dat dit mechanisme verantwoordelijk is voor het ontbreken van de expressie van klasse I op het celoppervlak; een verschijnsel dat bij de karper, een exotherme diersoort, wordt aangetroffen in situaties waarin de omgevingstemperatuur laag is. Dit proefschrift vormt daarmee de eerste publikatie waarin is vastgesteld dat ook bij koudbloedige gewervelde diersoorten sprake is van het voorkomen van een dergelijk regulatiemechanisme van de MHC klasse I expressie.

Curriculum vitae

Pedro Nuno Simões Rodrigues was born on February 17, 1966 in Porto, Portugal. In 1984 he started a four years university degree in Biological Sciences at the "Abel Salazar" Institute of Biomedical Sciences, University of Porto, Portugal. During 1988 he carried out a 6 months research project in biochemistry at the Plymouth Marine Laboratory, NERC, England (UK). In September 1989 he finished his degree named "Licenciatura", after which he worked as a research assistant at the department of Cell Biology, University of Porto, Portugal. In October 1990 he started a two years postgraduate degree in Immunology at the Institute of Biomedical Sciences, University of Porto. During 1991 he carried out a 8 months research project on immunobiology at the Medical Faculty, Free University, Amsterdam, The Netherlands. In February 1992 he finished his post graduate degree named "Mestrado" at the Porto University, Portugal. From May 1992 he has been working on a PhD research project on immunology, studying the immune system of fish at the Department Experimental Animal Morphology and Cell Biology at the Wageningen Agricultural University, The Netherlands. The results of those 4 years of research are presented in this thesis.