Immunogenetics (2004) 56: 462–466
DOI 10.1007/s00251-004-0713-y

BRIEF COMMUNICATION

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Alternative splicing of major histocompatibility complex class II *DXB* transcripts in *Xiphophorus* fishes

Received: 1 December 2003 / Revised: 16 July 2004 / Published online: 3 September 2004

Abstract Classical MHC class II glycoproteins present peptides to T cells. In *Xiphophorus* fishes and in the guppy, *Poecilia reticulata*, a classical MHC class II *B*-like transcript has been identified, *DAB*, as well as a divergent MHC class II *B*-like transcript, *DXB*. In the two species of *Xiphophorus* fishes studied here, *X. multilineatus* and *X. pygmaeus*, alternative splicing of the *DXB* transcript was observed, but not of the classical type *DAB* transcripts. Two alternative splice patterns were found: a 16-codon deletion and a five-nucleotide deletion that leads to an extension of the transcript. A single *DXB* transcript that terminates before the transmembrane region was also observed. The alternative splice pattern and the divergence of *DXB* from *DAB* suggest that in fish, DXB may have an alternate function. Alternative splicing transcripts of *DXB* may allow for signaling and localization of DXB within the cell.

Keywords Xiphophorus · MHC class II · Evolution · Splicing · Nonclassical

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Classical major histocompatibility complex (MHC) class II genes code for a set of proteins that present peptides via the endosomal pathway to $CD4^+$ T cells to initiate an immune response (Watts 1997). Nonclassical MHC proteins are thought to play a role in mediating and regulating this process (Alfonso and Karlsson 2000). Both types of MHC class II molecules consist of two glycoproteins, α and β chains (Klein 1986) and have a highly conserved exon–intron structure and boundaries (Radley et al. 1994). In the human, the nonclassical class II chain, HLA-DM, regulates the unloading of CLIP and the loading of peptide from classical class II MHC (Copier et al. 1996; van Lith et al. 2001; Vogt et al. 1996) and also serves as a peptide editor in early endocytic compartments (Kropshofer et al. 1997; Pathak et al. 2001). Nonclassical MHC genes are generally less polymorphic than classical genes (Hermel et al. 1995; Servenius et al. 1987; van Lith et al. 2002), and in human, *DMB*, unlike most of its classical counterparts, has been reported to be alternatively spliced (Modrek et al. 2001). MHC class II *DMB* genes have been found in human (Kelly et al. 1991), mouse (Cho et al. 1991), rat (Hermel and Monaco 1995), cow (Niimi et al. 1995), and rabbit species (Hermel et al. 1999). To date, nonclassical MHC class II genes have not been reported as such in fish.

Recently a novel transcribed MHC class II *B*-like gene has been reported in the platyfish *Xiphophorus maculatus*, the swordtail *Xiphophorus helleri*, and the guppy *P. reticulata*, designated *DXB* with the *X* indicating an uncharacterized gene family (McConnell et al. 1998a,b).

While structurally similar to *DAB*, the classical MHC class II gene of *Xiphophorus* fishes, exon 3 of *DXB* is only 61–63% identical to *DAB*, versus the 92% identity among *XiphophorusDAB* exon 3 nucleotide sequences (McConnell et al. 1998b). The *DXB* gene also appears to be encoded at a single locus, as evidenced by Southern blot data indicating a single band when hybridized with a 190-bp β2-encoding probe, as opposed to most *DAB*-like genes (McConnell et al. 1998a,b). *Xiphophorus* fishes have been used in histocompatibililty studies since the early 1950s (Kallman 1958, 1964). This previous work, and the availability of inbred strains, makes *Xiphophorus* an ideal genus in which to study novel MHC genes. Here we identify unique characteristics of *DXB* not previously reported in fishes. We examined transcripts of *DXB* genes from wild-caught populations of *X. multilineatus* (*Ximu-DXB*) and *X. pygmaeus* (*Xipy-DXB*).

X. multilineatus specimens were captured in the río Coy and the Arroyo Tambaque, San Luis Potosí state, Mexico. *X. pygmaeus* specimens were captured at two locations on the río Huichihuayán, San Luis Potosí state, Mexico: the towns of Huichihuayán and La Y-Griega Vieja. A total of 39 specimens were collected (25 *X. multilineatus* and 14 *X. pygmaeus*). RNA was extracted from intestinal, spleen, muscle, and liver tissue using the TRIzol reagent protocol (Life Technologies, Gaithersburg, Md.). Single-stranded cDNA was reverse transcribed from the RNA using Superscript II reverse transcriptase according to the manufacturer's protocol (Invitrogen, Carlsbad, Calif.). *DXB* PCR+1 (Borriello and Krauter 1990; Hardee et al. 1995) fragments were produced using primers TM341 (5′-ATCTCTGTTGCCAATCTAAGA-3′), TM328 (5′-ATGTGTAAAAGGCTAAATGAT-3′), and TM342 (5′-GAG*AAGCTT*ATCTCTGTTGCCAATCTAAGA-3′) for the +1 step (*Hin*dIII site is italicized). The *β-actin* was amplified with TM425 (5′-ACAGACAGAG-TATTTGCGCTCAGGTG-3′) and TM426 (5′-ACTGTCCCTGTATGCCTCTGGTCGTA-3′). For PCR +1, cDNA template was amplified using the Clontech Advantage cDNA Polymerase Mix kit following the manufacturer's specifications (BD Biosciences, Palo Alto, Calif.) along with 100 pmol primer TM328 and 10 pmol primer TM341. For RT-PCR to compare levels of *DXB* and *β-actin* transcripts, 100 pmol of each primer was used and conditions were identical to the PCR+1 except for the "+1" cycle. Thirty-five cycles of amplification were performed (94°C for 1 min, 62°C for 1 min, and 68°C for 2 min) in a MJ Research PTC-200 Peltier Thermal Cycler (Waltham, Mass.). During the last ("+1") step, 100 pmol of TM342 and 0.3 μl of Clontech Advantage cDNA polymerase mix (50×) were added to the 10-μl reaction. The mixture was then subjected to a final cycle (94°C for 5 min, 55°C for 3 min, and 68°C for 20 min), and then visualized on an 1% agarose-TBE (Tris–borate EDTA) ethidium bromide gel. PCR+1 bands of approximately 700 bp were excised and purified using the Qiagen MinElute gel extraction kit (Valencia, Calif.). Genomic DNA from an *X. multilineatus* specimen was isolated with the Qiagen DN easy Tissue kit and PCR+1 amplified under the same conditions and same primers as the cDNAs with the following exceptions: an annealing temperature of 60°C and 30 cycles of amplification were used, along with Platinum Taq HF (Invitrogen). The amplified DNA fragments were ligated into the plasmid vector pGEM-T Easy (Promega Corporation, Madison, Wis.) and then digested using the restriction enzyme *Hin*dIII as the basis to identify PCR+1 positive fragments. Plasmid clones were sequenced on both strands and identified using the NCBI BLAST program (Altschul et al. 1990). Nucleotide and amino acid sequences were aligned and analyzed using the GCG software package (Genetics Computer Group, Madison, Wis.).

In order to establish whether *DXB* exhibited a typical MHC class II expression pattern, RNA was extracted from intestine and spleen lymphoid tissues, as well as from muscle and liver nonlymphoid tissues. RT-PCR amplification of cDNA made from these RNAs showed a typical MHC class II expression pattern (Fig. 1). Bright bands are seen at the appropriate molecular weight of 700 bp for *DXB* in intestine and spleen, but are virtually absent from muscle and liver tissue cDNA. The very light band seen at 700 bp for liver may be the result of resident antigen presenting cells in this tissue. The *β-actin* is shown as a control for amount of template.

DXB cDNA fragments were successfully isolated from all 39 specimens, one per specimen. Of these sequences, 21% (8/39) were alternatively spliced, while 0% (0/78) of the *DAB* clones (2 independently RT-PCR-generated *DAB* sequences per specimen, data not shown) derived from the 39 specimens demonstrated alternative splicing. Of the *DXB* clones, three main groups of transcripts were

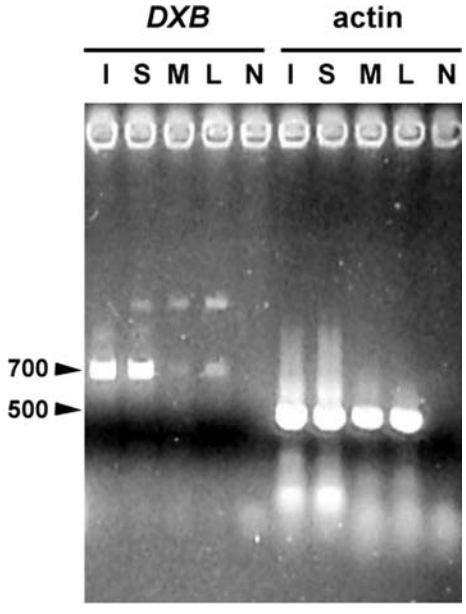

Fig. 1 RT-PCR amplification of RNA isolated from *Xiphophorus multilinatus*. The RNA was isolated from intestine (*I*), spleen (*S*), muscle (*M*), liver (*L*), and negative control (no template, *N*), then converted to cDNA for amplification using primers specific for *DXB* or for *β-actin* sequences. Approximate sizes of bands in bp are indicated on the *left*

observed; normally spliced, 48-nucleotide (16 codon) deletion spliced, and five-nucleotide deletion spliced (Fig. 2). In addition, a truncated form of *DXB* was observed in a single transcript. All splice patterns were found in both species of fish except the truncated transcript, which was found in a single *Xipy-DXB* transcript. *DXB* cDNAs following the normal splice pattern were also detected in one specimen that had alternative splicing form 1 and in one specimen with alternative splice form 2 (Fig. 3) of *DXB*, as well as having *DAB* alleles.

To determine whether the splice patterns seen could be accounted for and were consistent with the encoding genomic sequence, a *DXB* genomic clone from a *X. multilineatus* specimen was PCR-amplified, cloned, and sequenced. All alternative transcripts discussed in the manuscript were found to be compatible and consistent with this newly derived genomic sequence. In fact, the genomic sequence was isolated from a specimen from which we had isolated one of the cDNA clones with the 16-codon deletion. These two sequences (genomic and cDNA) match up base-for-base in the coding regions, likely indicating that the corresponding alternatively spliced RNA was derived from the same chromosome as the genomic clone. Potential splice signals for all normal and alternative transcripts were also conserved in all four genomic *DXB* clones that have now been isolated, each one from the four different species: *X. helleri*, *X. maculatus*, *X. multilineatus*, and *P. reticulata*. There is significant sequence conservation among all four of these genomic sequences.

The normal splice pattern in *Ximu-DXB* and *Xipy-DXB* contains a total of six exons and follows the splice pattern previously reported in other species of *Xiphophorus* as well as *P. reticulata* (Fig. 3). The 16-codon deletion occurs in exon 4, and results in the complete removal of the connecting peptide and 27% of the transmembrane region. The five-nucleotide deletion occurs at the beginning of exon 5, and results in a frame-shift which changes the amino acid sequence for all of exons 5 and 6, and extends exon 6 by 20 amino acids, resulting in a cytoplasmic tail that is nearly double the length of that found in the normal splice transcript. In both alternative splice patterns, AG is the alternative splice acceptor site.

Ximu-DXB and *Xipy-DXB* genes have many differences from the classical class II MHC *B* genes in fishes, *DAB*. Although alternative splicing has also been reported in a few other human classical MHC cII *B* gene transcripts

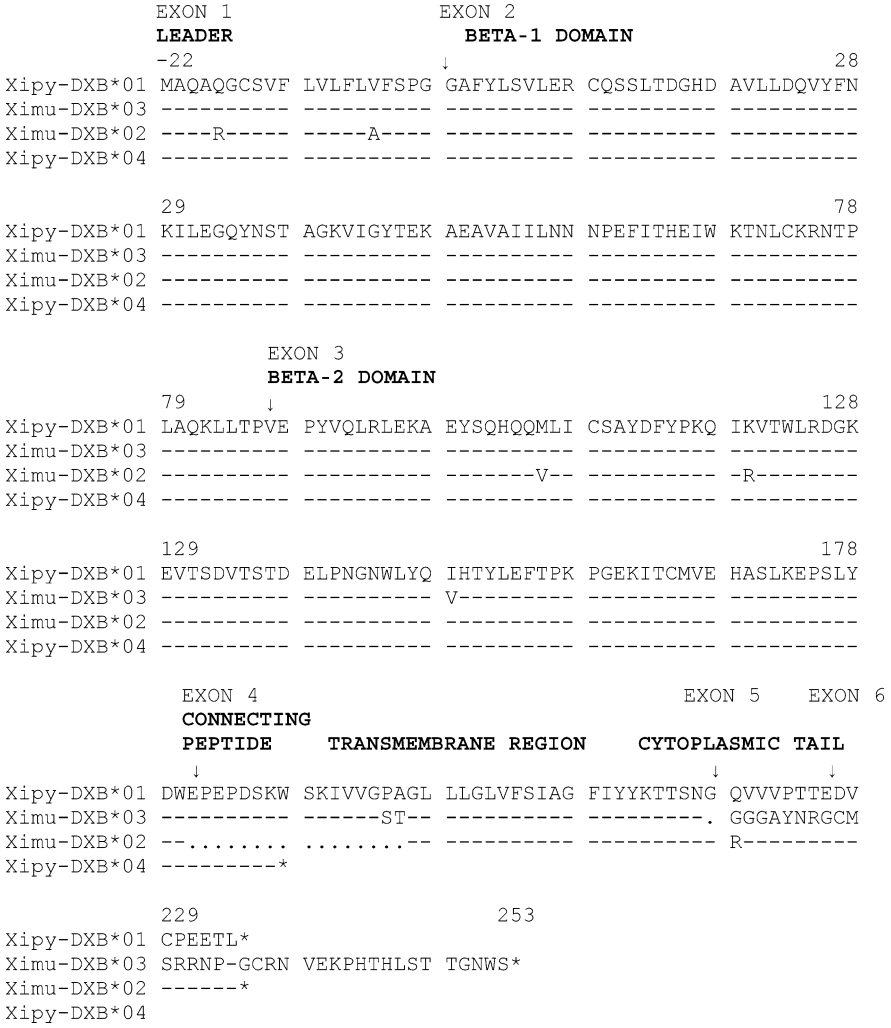

Fig. 2 Predicted amino acid sequences of *DXB* cDNA clones representative of each splicing pattern. *Down-arrow* indicates exon boundaries. Xipy-DXB*01 follows the normal splice pattern, Ximu-DXB*02 follows the 16-codon deletion splice pattern, Ximu-DXB*03 follows the five-nucleotide deletion splice pattern and Xipy-DXB*04 follows the early termination splice pattern. All splice patterns from both species studied have been deposited into GeneBank under accesion numbers AY483280–AY483286

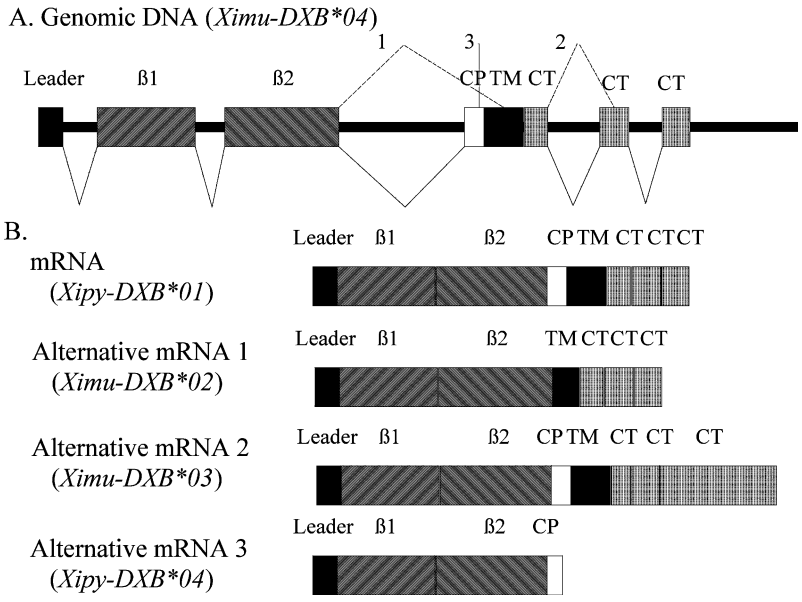

Fig. 3 **a** Exon–intron structure of an *X. multilineatusDXB* genomic clone (GenBank accession number AY671988) with inferred alternative splices for alternative mRNAs 1, 2, and 3. **b***DXB* mRNA and inferred alternative *DXB* mRNAs. Alternative splice pattern 1 was observed in at least one member of *X. multilineatus* and at least one member of *X. pygmaeus*, as was alternative splice pattern 2. The truncated pattern was found in one clone derived from a *X. pygmaeus* specimen

(Briata et al. 1989; Kappes et al. 1984; Tsukamoto et al. 1987), the amount of nucleotide divergence from *DAB* and the alternative splicing of *DXB* suggest that it may serve in a nonclassical role similar to that of HLA-DM. In DM, the lysosomal targeting is controlled by a tyrosine-based motif located in the DM β chain (van Lith et al. 2001). Modrek and co-workers (2001) recently observed four alternative splice patterns in *DMB*, with exon 4 (transmembrane) and exon 5 (part of the cytoplasmic tail) as the exons that can be included or removed. They hypothesized that this serves as a method of control of DM as exon 5 encodes the targeting motif YTPL (Copier et al. 1996) and exon 4 encodes the membrane anchoring sequence. In *Ximu-DXB* and *Xipy-DXB*, the splice patterns are different in that exon 5 is never completely removed, although the five-nucleotide deletion and frame-shift does occur in this exon. A tyrosine-based sorting motif in the form of YXX∅ (where *X* indicates any amino acid and ∅ indicates any amino acid with a large hydrophobic side chain), a common transmembrane targeting signal (Bonifacino and Dell'Angelica 1999), is not readily apparent in any of the forms of DXB, possibly due to the evolutionary divergence between fish and higher vertebrates. Tyrosine residues are present in the cytoplasmic tail of both alternatively spliced forms of *DXB* that are absent in the normal splice pattern (Fig. 1).

Interestingly, when the 16-codon deletion transcript is modeled with the TransMem program of GCG, it does not show a removal of the transmembrane domain—it shows a reduction in the total size of the transmembrane from 22 to 19 amino acids, with a shift of the last four amino acids of the β2 domain into the transmembrane and the last tyrosine of the normal transmembrane into the cytoplasmic tail. Using chimeric molecules Potter and co-workers (1999) have recently demonstrated that correct spacing of the tyrosine-based motif from the transmembrane domain is necessary for localization of DM to the lysosomal compartments. They have also shown that the motif, when moved, results in DM being distributed to the cell surface. It is possible that in Ximu-DXB and Xipy-DXB, a putative signaling motif present in the cytoplasmic tail is shifted towards the transmembrane by alternative splicing, and hence a form of control of DXB is not by removal of a signal but movement of the location of the signal. It is also possible that the reduction in size of the transmembrane and change in the amino acid composition in the transmembrane contribute to localization of the protein within the cell (Cosson and Bonifacino 1992; Yang et al. 1997).

The five-nucleotide deletion splice pattern leads to an amino acid change in the last two-thirds of the cytoplasmic tail as well as an extension. The cytoplasmic tail of the normally splicing *DXB* contains six potentially charged amino acids, and the extended cytoplasmic tail of the five-nucleotide deletion splice pattern contains seven potentially charged amino acids, some of which are in nearly identical locations and are of the opposite charge. These potentially charged amino acids, as well as the extended length of the cytoplasmic tail, could function in signaling and trafficking of DXB.

The truncated splice form of *DXB* ends before the transmembrane encoding region of the normal transcript, and when modeled does not show a transmembrane domain. This particular cDNA could encode a soluble form of DXB. However, since this pattern was only found in one individual, further studies to detect this cDNA transcript in additional fish should be performed as well as assays to ascertain the presence of a soluble protein form of DXB. All three alternate forms of the *DXB* transcripts were isolated from intestine cDNA. We cannot exclude the possibility that different splice forms occur in different cell types, different stages of differentiation, or only in select (MHC allele-dependent) individual fish.

The *DXB* gene is very different from its classical counterpart in its nucleotide composition. Here we show for the first time in fish alternative splicing of an MHC

class II *B*-like gene. The combination of nucleotide divergence of *DXB* compared with *DAB* (McConnell et al. 1998a), along with the alternative splicing of *DXB*, suggests that in *Xiphophorus* species, *DXB* may function in a different role in the processing and presentation of peptides than the classical type *DAB* gene. Further studies of the polymorphism of *DXB* in comparison to *DAB* in these two species of *Xiphophorus* are in progress.

Acknowledgments We thank Adam W. Boles for technical assistance. This work was supported by National Science Foundation Grant MCB-0110541.

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