Fish & Shellfish Immunology (2003) **14,** 177–185
doi:10.1006/fsim.2002.0431
Available online at http://www.elsevier.com/locate/fsi

SHORT COMMUNICATION

The promoter of the classical MHC class I locus in rainbow trout (*Oncorhynchus mykiss*)

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(*Received 13 November 2001, accepted 2 April 2002*)

In rainbow trout only a single classical major histocompatibility complex (MHC) class I locus has been identified. In previous studies it was shown that this locus, *Onmy-UBA*, is characterised by extensive polymorphism and ubiquitous expression. In the present study the sequence of the promoter region of *Onmy-UBA* was analysed. Within this region several fragments for transcription regulation could be detected, including interferon-sensitive enhancer elements. The promoter region is very similar to that of classical MHC class I in higher vertebrates. In agreement with the interferon sensitive elements identified in its promoter, *Onmy-UBA* expression in rainbow trout gonad cells (RTG-2 cell line) was up-regulated after infection with infectious haematopoietic necrosis virus (IHNV).

Key words: MHC class I, promoter, β2 microglobulin, IHN virus, RTG-2 cells, rainbow trout.

Classical MHC class I molecules are involved in presenting peptides from intracellular antigens at the cell surface for recognition by cytotoxic T cells [1]. This screening mechanism allows the vertebrate immune system to detect intracellular pathogens and to destroy the infected cells. All jawed vertebrates investigated thus far express one to three classical MHC class I loci at high levels in most tissues. In rainbow trout there seems to be a single classical MHC class I locus [2–5], which is now, designated *Onmy-UBA* by most research groups. The various *Onmy-UBA* alleles show unexpectedly low homology [2–5], but they all appear to be expressed at high levels [2, 4, 6].

Using an established monoclonal antibody, Onmy-UBA expression was shown to be high in endothelia, epithelia and lymphoid tissues [7], consistent with findings by

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Northern blot analysis for different tissues [2, 4, 6]. This expression pattern is reminiscent of classical MHC class I expression in higher vertebrates.

In higher vertebrates, expression of classical MHC class I genes is stimulated by a number of processes, including virus infection and treatment with recombinant interferons [8, 9]. Classical MHC class I expression in a macrophage-like cell line of Atlantic salmon was up-regulated after infection with infectious salmon anaemia (ISA) virus or infectious pancreatic necrosis virus (IPNV) [10]. Classical MHC class I expression in higher vertebrates and fish therefore seem to be regulated in a similar manner.

In channel catfish two MHC class I loci were compared, both encoding molecules with the peptide termini binding residues characteristic of classical MHC class I [11]. Interestingly only one of these two loci is ubiquitously expressed, and sequence analysis of the promoter regions indicated that only this locus, *Icpu-UA*/*26*, contains regulatory motifs indicating high constitutive expression. The other locus, *Icpu-UA*/*3*, has imperfect elements for constitutive expression but contains regulatory motifs expected to be stimulated by interferons.

In the present study the promoter region of the rainbow trout *Onmy-UBA* locus was analysed. The promoter of *Onmy-UBA* shows more similarity to the promoter of classical MHC class I loci in higher vertebrates than to channel catfish *Icpu-UA*/*3*. The fragment analysed contains more extensive sequence information than that available for the *Icpu-UA*/*26* promoter.

The homozygous gynogenetic rainbow trout strain C25 expresses the *Onmy-UBA**501 allele [4]. Genomic DNA from the liver of these fish was isolated using the proteinase K–SDS method [12]. Genome walking libraries were constructed with a Universal Genome Walker Kit (Clontech, Palo Alto, U.S.A.) according to the manufacturer's suggestions. An 826 bp fragment upstream of the *Onmy-UBA* start codon was amplified using the primer *pUBA-int1-1*, 5′-GTAGCCATAGACT TCCACTCATTGAGCTAA (which binds to the intron between the two exons coding the leader peptide and the α1 domain, intron sequence not shown) in conjunction with an adapter primer. The 25 μl reaction mixture contained 1·25 units of TaKaRa LA Taq™ DNA polymerase (Takara Shuzo, Otsu, Japan), TaKaRa LA Taq™ buffer, 400 μM of each dNTP, 2·5 mM $MgCl_2$, 5 ng adapter ligated genomic DNA and 0·2 μM of each primer. PCR conditions were: 94° C for 2 min, then 7 cycles (94° C for 25 s, 72° C for 4 min), then 32 cycles (94° C for 25 s, 67° C for 4 min), and finally 72° C for 4 min. Sequence analysis of multiple clones was performed using a 'CEQ Dye terminator Cycle Sequencing Kit' (Beckman Coulter, Fullerton, U.S.A.) and suitable primers (not shown) with an automated sequencer 'CEQ™2000 DNA Analysis System' (Beckman Coulter). The analysed sequence is shown in Fig. 1 (GenBank AF441856).

The RTG-2 cell line [13] is a commonly used fibroblast-like rainbow trout cell line previously shown to express the *Onmy-UBA**501 allele [7]. The transcriptional start point of the *Onmy-UBA**501 gene was deduced by sequence analysis of a number of 5′ RACE (rapid amplification of cDNA ends) clones obtained from RNA of RTG-2 cells using the 'SMART™ RACE cDNA Amplification Kit' (Clontech, Palo Alto, U.S.A.) according to the manufacturer's suggestions with some modifications as follows. The *Onmy-UBA**501 specific amplification was performed using 2·5 μl of the 5′-RACE-Ready cDNA product in a 50 μl reaction mixture with 1 μM of the α3 region binding primer *pa3-r*, 5′-GTTGTTCTTCCACTCCTCAGGCGTCAC, in conjunction with 1 μM of the SMART™ RACE primer UPM, plus 1·25 units Ampli Taq Gold™ polymerase (Perkin-Elmer, Tokyo, Japan), Ampli Taq Gold™ buffer, and 500 μM of each dNTP. The PCR schedule was 94° C for 10 min, then 45 cycles (65° C for 3 min, 72° C for 1 min, 94° C for 1 min), and finally 72° C for 10 min. The four longest 5′ RACE clones all ended at the same position (position 1 in Fig. 1) and included an additional upstream guanine probably derived from capping; therefore the indicated position 1 is suggested to be the transcriptional start point.

Probable transcription elements in the *Onmy-UBA* promoter are indicated in Fig. 1. They were found by comparison with literature on other MHC promoters, as well as by

Fig. 1. The promoter region of *Onmy-UBA**501. The numbering starts at the transcriptional start point. The transcribed nucleotides are indicated in italics and the start codon of the open reading frame is enlarged. Boxes indicate different transcription regulatory motifs that are shared with MHC promoters in other species (Fig. 2). Other motifs are underlined and described with a somewhat smaller font.

the DNA analysis software program TRANSFAC [14]. Those elements which are schematically compared with other promoters in Fig. 2 are boxed.

There is a perfect TATA box at position −29. The TATA element can be bound by the transcription factor TBP (TATA-binding protein; [15]). An enhancer B element, ATTGG, is found at position −59. This element can be bound by the transcription factor NF-Y [16]. The TATA box and the enhancer B element are common transcription elements that MHC class I shares with most other eukaryotic genes [15].

An X2 box, TGATGCT is found at position −84 and can be bound by the transcription factors X2BP (X2 binding protein) and ATF/CREB-like components [9]. This *Onmy-UBA* X2 box is identical to that found in the human MHC class II locus *HLA-DMB*; *DMB* has a typical MHC class II promoter similar to the *HLA-DPA*, *-DPB*, *-DQA*, *-DQB*, *-DRA* and *-DRB* loci [9]. The detection of the X2 element in the *Onmy-UBA* promoter is somewhat surprising since in mammalian MHC class I as well as channel catfish *Icpu-UA*/*26* a site *α* element is found rather than an X2 element [9, 11]. However, site *α* and X2 sequences are homologous and bind ATF/CREB family transcription factors [9]; X2 binds the X2 binding protein (X2BP) [17] but it has been hypothesised that MHC class I expression is regulated by a complex of transcription factors including X2BP as well [9]. Site *α* is a cis-acting regulatory element of the

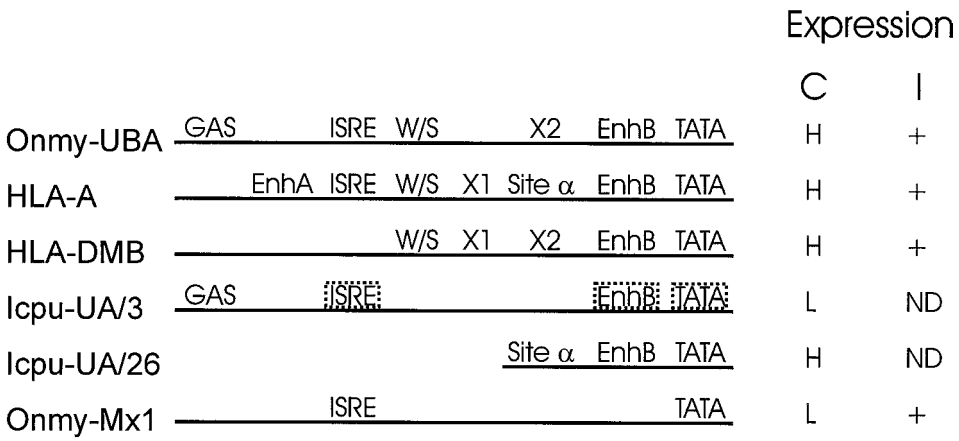

Fig. 2. Schematic comparison of the *Onmy-UBA**501 promoter with other promoters. The promoters compared were the MHC class I promoters of HLA-A (human; 9), *Icpu-UA/3* and *–UA/26* (channel catfish; 11), the MHC class II promoter of HLA-DMB (human; 9) and the promoter of the *Mx1* locus in rainbow trout (32). Compared were GAS, enhancer A, ISRE, X1, X2/site *a*, enhancer B and TATA elements. The ISRE, enhancer B and TATA boxes of *Icpu-UA/3* do not match the consensus sequences and are boxed with dotted lines. These *Icpu-UA/3* elements are: ISRE=GTTCACTTCT, consensus is (C/G)TTTCN$_{1-2}$TTTC; enhancer B=ACTGG, consensus is ATTGG; TATA=TAAAAA, consensus is TATAAA. C, constitutive expression (in characteristic cell types); I, expression induced by interferons or virus infection; H, high; L, low; +, inducible; ND, not determined.

mammalian MHC class I promoter and is essential for the IFN gamma-induced transactivation through the ISRE (interferon stimulated response element) [18]. Other elements in the *Onmy-UBA* promoter besides X2 that may bind ATF/CREB-like components are found in Fig. 1 at position −128 (TTTCGTAA) [19] and −13 (AAAAAACGTCACCAC) [19–21] (both sites were indicated by TRANSFAC).

No X1 box, which typically borders the X2 box in mammalian MHC promoters, could be detected in the *Onmy-UBA* promoter. The X1 and X2 boxes in mammalian MHC class II promoters seem to interact in binding transcription factors stimulated by interferons [22, 23].

At position −118 a probable W/S element, GAACCTT, is found at a similar distance from the X2 or site α elements as in the human MHC class II and I promoters respectively [9]. The W/S consensus sequence is GGACCT(T/C) [24]. The W/S element can bind several different transcription factors and has, like the other promoter elements described in this study, a role in activation of transcription [24]. In the channel catfish sequence *Icpu-UA/3*, no W/S element at a similar position can be found; the reported fragment of the *Icpu-UA/26* promoter is too short for analysis of this element [11].

At position −135 the ISRE sequence CTTTCAGTTTC can be found. The sequence (G/C)TTTCN$_{1-2}$TTTC is found in all promoters stimulated by *α* and *β* interferons and confers interferon inducibility [25, 26]. The ISRE contains two GAAA motifs (complementary strand) and GAAA motifs can be found at positions −297, −522, −565 and −687 as well. Both the ISRE and the GAAA elements suggest stimulation of expression by interferons *α* and *β* [25].

In human and mouse MHC class I loci an enhancer A element that may be involved in specificity of tissue expression can be found directly upstream from the ISRE [8]. In the *Onmy-UBA* promoter, as with the promoter for the channel catfish *Icpu-UA/3*, no sequence with high homology to the enhancer A element was detected.

A GAS sequence (*γ* activated sequence; consensus TTNCNNNAA [27]), TTGCAGTAA, was found upstream of the ISRE in *Onmy-UBA* at position −236. No GAS elements are normally present in mammalian MHC class I promoters, but in the

channel catfish *Icpu-UA/3* this element was found at a position similar to that in *Onmy-UBA*. The GAS element indicates the capacity for up-regulation by interferon γ. Although no GAS elements have been detected in human classical MHC class I genes, interferon γ does stimulate their expression [9].

In the 5′UTR at position 65 the sequence GGAGATAACA is predicted to bind the GATA-binding factors 1, 2 and 3 [28] (prediction by TRANSFAC). At a similar position in the allele *Onmy-UBA**401 such binding site is lacking though [4], questioning the importance of this element.

In many studies retroelements and other DNA sequences that stimulate recombination are discussed as factors in the establishment of MHC diversity (e.g. [29]). Because the variation between the Onmy-UBA alleles is probably higher than for any well-studied gene locus so far [4], the sequence shown in Fig. 1 was investigated for such DNA elements.

At position −647 the sequence CCCCCCCCCCCCCCCCCACCACACACACACACA CACAC is present. Short direct repeats are sites of genetic instability [30]. AC-repeats have also been found in the 3′ untranslated regions of *Onmy-UBA* alleles [4].

The sequence from position −501 to −267 is 89% identical to a portion of the Atlantic salmon clone BHMS513 sequence (GenBank AF257001) that has not been associated with any gene. In the fragment −539 to −249 there are also high homologies with (parts of) other Atlantic salmon (GenBank AF256844, AF256948), lake trout (GenBank U27090) or rainbow trout (GenBank AF291718, Z54210) sequences, probably all of which are noncoding. It has been estimated that 84% of the DNA in the rainbow trout genome is comprised of repetitive DNA sequences [31] and several repetitive families have been distinguished in the rainbow trout genome [32–36]. Among these are the short interspersed repetitive elements (SINEs) of the *Hpa* I family [33, 35]. The *Onmy-UBA* promoter fragment −385 to −249 in Fig. 1 is >80% identical to (parts of) the coho salmon *Hpa* I SINE sequence Hpa(Oki)-51 and the kokanee salmon *Hpa* I SINE sequence Hpa(ON)-51 [33]. However, the tRNA-related region of this family of repeats appears to be deleted in the *Onmy-UBA* promoter compared to Hpa(ON)-51 [35].

Interestingly, the 66 bp sequence from position −490 to −425 would encode for a protein fragment with 75% identity and 90% homology to a fragment in the carboxy-terminal part of the putative Tes*1* transposase (related to the Tc*1* transposases) found in an intron in the pacific hagfish vasotocin gene [37]. The fragment is probably too small to be conclusive regarding evolutionary relationship. This fragment is not within an open reading frame with a start codon, although it is the only tentative translation product in the *Onmy-UBA* promoter region with significant homology to a known protein. However, there is an AATAAA sequence at position −303 and it remains to be clarified if the upstream region in Fig. 1 is a 3′ untranslated region of a yet unidentified gene.

Figure 2 shows a schematic comparison of the *Onmy-UBA* promoter with human classical MHC class I and MHC class II promoters, channel catfish MHC class I promoters and the rainbow trout Mx1 promoter. Mx1 is a protein involved in the immune system and its promoter was recently analysed by Collet & Secombes [38]; they found that Mx1 expression is strongly enhanced after immune stimulation. Of the possible regulatory elements only the GAS, enhancer A, ISRE, W/S, X1, X2/site *a*, enhancer B and TATA box are compared in Fig. 2. It is difficult to predict differences in transcription regulation of the compared loci since transcription regulation depends on many different factors and elements, including ones not compared in Fig. 2 such as (unidentified) silencers and Sp1 boxes. Moreover, extrapolation of findings from mammals to fish would be imperfect. For example we do not know how to interpret the lack of enhancer A and X1 elements or the presence of an X2 element instead of a site *a* sequence in trout compared to mammalian classical MHC class I promoters. Yet it is interesting that the *Onmy-UBA* promoter shows the fundamental characteristics of mammalian classical MHC class I promoters, namely interferon inducible elements as well as perfect elements for constitutive expression at similar locations. This in contrast to the channel catfish *Icpu-UA3* promoter that lacks perfect enhancer B and

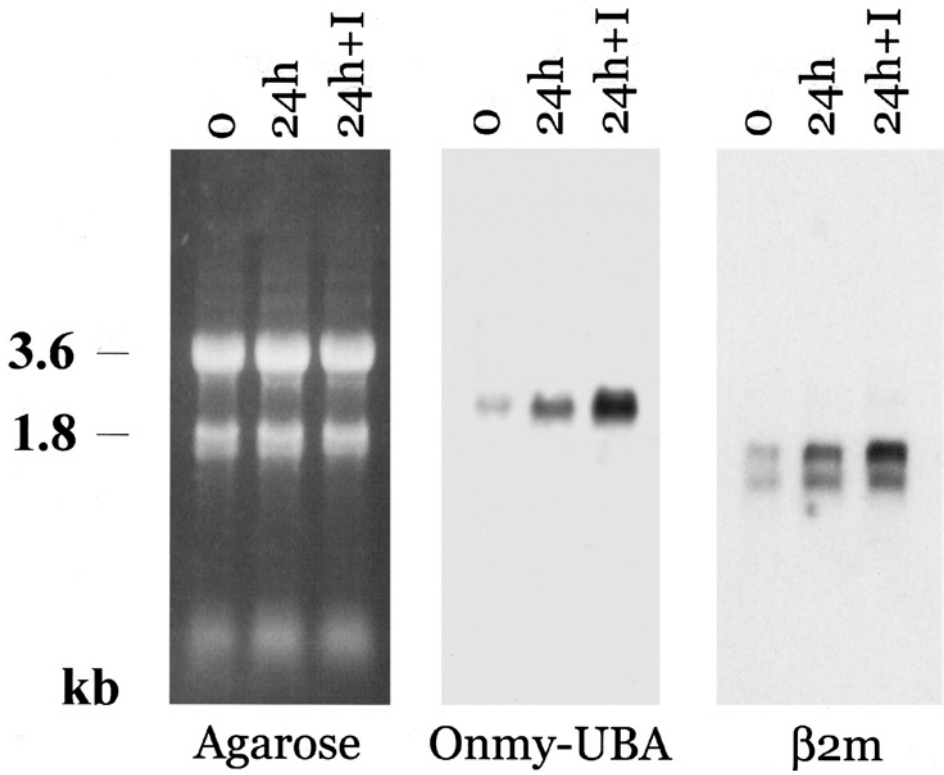

Fig. 3. Up-regulation of *Onmy-UBA* and *β*2m expression in IHNV-infected RTG-2 cells. RTG-2 cells grown in MEM/10% FBS medium at 20° C that had just become confluent were mock infected or infected with IHNV strain G4 [44] at a multiplicity of infection (M.O.I.) of 5. At the time of infection (T=0) 5 ml of the 20 ml of growth medium was replaced with fresh medium in infected and uninfected cell cultures. Total RNA was isolated after 24 h from both uninfected (24 h) and infected (24 h+I) cells. 7·5 *μ*g of total RNA per lane was separated in 1% agarose (Agarose) and blotted to a nylon membrane for Northern blot analysis with a 170 bp probe *pr5-a2* [4] derived from the *a*2 region of *Onmy-UBA**501. After stripping, the same blot was used for analysis with a probe consisting of a complete rainbow trout *β*2m open reading frame (sequence identical to *Jb-9* [40]) (*β*2m). Northern blot analyses were performed using the 'PCR DIG probe synthesis kit' (Boehringer, Mannheim, Germany) according to the manufacturer's recommendations and hybridisation signals were monitored by X-ray sensitive film. The washing step for both Northern blots consisted of two 15 min washes in 2 × SSC/0·1% SDS at 68° C. The pixel intensities of the rectangle band areas were calculated by use of the program 'Quantity One' of BIO-RAD Quantitation Software, setting the gel background as 0 and the intensity at the time of infection as 1. The pixel intensities for the lanes T=0, 24 h and 24 h+I in the agarose pictograph (adding the 3·6 and 1·8 kb ribosomal bands) were 1, 1·14, and 0·98, in the *Onmy-UBA* hybridisation pictograph 1, 1·67, and 2·59, and in the *β*2m hybridisation pictograph (both signals were added) 1, 3·10, and 4·52 respectively.

TATA box elements. Further analysis is needed to clarify whether expression of the *IcpuUA/26* locus involves interferon sensitive transcription elements.

In mammals *α*, *β* and *γ* interferons are all capable of inducing increased expression of class I MHC surface antigens [8]. *α*- and *β*-interferons differ from *γ*-interferon in that they are produced by fibroblasts and other cells during viral infection, whereas *γ* interferon is produced by subsets of leucocytes only. Although interferons have not been isolated from rainbow trout, their presence after virus infection is suggested by functional analysis [39].

Stimulation of *Onmy-UBA* expression after infectious haematopoietic necrosis virus (IHNV) infection of RTG-2 cells is shown by the Northern blot analysis in Fig. 3. The

experimental procedure is described in the figure legend. Although the *Onmy-UBA* expression was up-regulated after the cell monolayer achieved confluency even without infection, infection with IHNV induced an additional increase in expression (Fig. 3). The β2 microglobulin (β2m) expression appeared regulated in the same manner (Fig. 3); β2m molecules form heterodimers with MHC class I molecules and co-regulation of the *Onmy-UBA* and β2m transcripts was expected. Two different transcripts for rainbow trout β2m (Fig. 3) were described previously by Shum *et al*. [40]. The results shown in Fig. 3 were repeated in an independent experiment (not shown). The up-regulation of *Onmy-UBA* expression after virus infection is in agreement with the interferon-sensitive elements in its promoter. The reason for the stimulation of *Onmy-UBA* expression after cell confluency without infection is unclear, but for mammalian classical MHC class I dependence of expression on culture conditions has been described as well [41, 42].

Comparison of the promoter sequences suggested that *Onmy-UBA* expression is similar to that of classical MHC class I in higher vertebrates. This was confirmed by analysis of transcripts and proteins. The overall expression profile of *Onmy-UBA* is very similar to that of classical MHC class I in mammals, with strongest expression in endothelia, epithelia and lymphoid tissues [7] and up-regulation after virus infection (this study). Why *Onmy-UBA* expression is very low in muscle and nervous tissues [4, 7] is unclear but presumably yet unidentified silencer sequences are present as found in mammalian MHC class I promoters [43].

This work was supported by 'the promotion of basic research activities for innovative biosciences' funded by Bio-oriented Technology Research Advancement Institution (BRAIN), Japan. We thank Dr James D. Moore, Bodega Marine Laboratory, USA for his help editing this manuscript.

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