Temperature and length-dependent modulation of the MH class IIβ gene expression in brook charr (Salvelinus fontinalis) by a cis-acting minisatellite

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1. Introduction

More than three decades ago, King and Wilson (1975) proposed that the phenotypic divergence between human and chimpanzees was mostly attributable to changes in gene regulation rather than changes in the protein-coding sequences of the gene. It is now recognized that the variation in gene regulation is an important factor from which evolutionary changes in diverse aspects of phenotype can be observed in all organisms. Distinctive elements with functional roles in gene regulation have been identified within the non-coding part of the genome, including repeated elements. Major histocompatibility complex (MHC) genes have been the subject of an abundant literature which made them unique candidates for studies of adaptation in natural populations. Yet, the vast majority of studies on MHC genes have dealt with patterns of polymorphism in sequence variation while very few paid attention to the possible implication of differential expression in adaptive responses. In this paper, we report the identification of a polymorphic minisatellite formed of a 32 nucleotides motif (38% G+C) involved in regulation of the major histocompatibility class IIβ gene (MHLIIβ) of brook charr (Salvelinus fontinalis). Our main objectives were: to analyze the variability of this minisatellite found in the second intron of the MHLIIβ gene and to document its effect to the variation of expression level of this gene under different environmental conditions. Distinctive number of the minisatellite repeats were associated with each different MHLIIβ alleles identified from exon 2 sequences. Relative expression levels of specific alleles in heterozygous individuals were determined from fish lymphocytes in different genotypes. We found that alleles carrying the longest minisatellite showed a significant 1.67–2.56-fold reduction in the transcript expression relatively to the shortest one. Results obtained in three different genotypes also indicated that the repressive activity associated to the longest minisatellite was more effective at 18 °C compared to 6 °C. In contrast, no significant difference was observed in transcript levels between alleles with comparable minisatellite length at both temperatures. We also depicted a significant up-regulation of the total MHLIIβ transcript at 6 °C relative to 18 °C. These results reveal for the first time that a temperature-sensitive minisatellite could potentially play an important role in the gene regulation of the adaptive immune response in fishes.

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expression have been identified within the non-coding part of the genome. These non-coding functional elements include introns, satellite DNAs and transposons, as well as pseudogenes and non-coding RNA genes (see Eddy, 2001; Balakirev and Ayala, 2003; Beauregard et al., 2008; Usdin, 2008 for reviews).

The different roles associated to introns include those in alternative splicing, transcriptional regulation, nuclear export, translation and chromatin structure. Moreover, the evolution of intron size in eukaryotes can be imputable to different models. The “selection for economy” model argues that natural selection favours short introns in highly expressed genes to minimize the cost of transcription and other molecular processes (Castillo-Davis et al., 2002; Eisenberg and Letovan, 2003). On the other hand, the “genomic design” model hypothesizes that the greater length of introns found in tissue-specific genes, possibly resulting from the accumulation of multispecies conserved sequences (MCSS) (Pozzoli et al., 2007), is an indication of their functional and regulatory complexity (Vinogradov, 2004, 2006). Furthermore, repeated sequences are frequently found in introns of higher eukaryotes. These repeated sequences are classified in two large families, called “tandem repeats” and “dispersed repeats” (Richard et al., 2008). The first family includes satellite DNAs, where the subfamilies of microsatellites and minisatellites are respectively tandemly repeated sequences of short (<10 bp) or longer motives (>10 bp and >100 bp) (Charlesworth et al., 1994). The second family contains transposons from which three subdivisions of retrotransposons can be identified: long interspersed nuclear elements (LINEs), short interspersed nuclear elements (SINEs) and long terminal repeats (LTRs) (Wicker et al., 2007). Both these families of repeated sequences have been shown to be implicated in regulation of gene expression (Ugarkovic, 2005; Feschotte, 2008; Tomilin, 2008).

Located in coding or non-coding regions of genomes, microsatellites (simple sequence repeats, SSRs) expansion or contraction can produce various effects on chromatin organization, regulation of gene activity, recombination, DNA replication, cell cycle, mismatch repair system, etc. (Li et al., 2002, 2004). SSRs variation is also well known for being responsible of human diseases such as neurologic disorders and cancers (Atkin, 2001; Orr and Zoghbi, 2007), and also for contributing to normal variation in brain and behavioral traits (Fondon et al., 2008). These expanded repeats have the potential to adopt altered DNA secondary structures that confer genetic instability, leading eventually to phenotypic changes (Wells et al., 2005; Wang and Vasquez, 2006). On the other hand, functions associated to minisatellites (variable number of tandem repeats, VNTRs) are far less well characterized with the exception of their implication in recombination events and gene expression modulation (Bois and Jeffrey, 1999; Richard and Pâques, 2000). Probably the best characterized minisatellite regulating gene expression is the one found at the insulin (INS) gene locus (Bennett et al., 1995), for which the implication in susceptibility to human type 1 diabetes has been known for more than two decades (Bell et al., 1984). Other regulatory VNTRs were identified in additional genes as well: cystatin B (CSTB) (Laloi et al., 1997), serotonin transporter (5-HT) (MacKenzie and Quinn, 1999), dopamine transporter (DAT1) (Mill et al., 2002) and type 2 bradykinin receptor (B2) (Zamorano et al., 2006).

Classical major histocompatibility complex class II (MHCII) molecules are cell-surface glycoproteins specialized in the presentation of peptides, mainly derived from extracellular proteins, to the antigen receptor of CD4 T cells in the adaptive immune system. With the MHC class I molecules, they both represent the most polymorphic genes known to date, generally with multiple loci and high allelic diversity at each of these loci (Trowsdale and Parham, 2004). Classical major histocompatibility genes have been the subject of an abundant literature over the last 20 years or so. In particular, these genes offer several assets that made them unique candidats for studies of adaptation in natural populations (Bernatchez and Landry, 2003). Yet, the vast majority of studies on MHC genes deal with patterns of polymorphism in sequence variation while very few paid attention to the possible implication of differential expression in adaptive responses. This is surprising since it has been known for many years that the modulation of the immune response is not only determined by the polymorphism of MHCII genes, but also by the cell surface expression correlation with the transcriptional level of the gene (Glimcher and Kar, 1992).

In a previous study, we identified a 32 bp motif minisatellite located in the second intron of the MH class IIβ (MHIIB) gene in brook char (Salvelinus fontinalis) (Croisetière et al., 2008). Here, our main objective is to investigate the possible implication of this minisatellite in the MHIIB gene expression. More specifically, we (i) analyzed the variability of this minisatellite found in the second intron of the MHIIB gene in brook char and (ii) documented its effect to the variation of the expression level of this gene under different environmental conditions. Precise expression levels were determined directly from fish lymphocytes. In addition to the identification of TNF-α and LPS as regulator of the MHIIB expression in rainbow trout macrophages (Knight et al., 1998), the promoter characterisation of the MHIIB genes in Atlantic salmon (Syed et al., 2003) and the modulation of the transcriptional activity of immune system genes by low temperature (Rodrigues et al., 1998; Nath et al., 2006), results presented here improve our understanding of the factors contributing to the modulation of the expression of the MHIIB genes in teleosts.

2. Materials and methods

2.1. Fish

Individuals of approximately 50 g from a domestic strain of brook char from a local hatchery (Chesterville, Québec, Canada) were used to evaluate the total expression level (cumulative of both alleles) of the MHIIB gene in a natural environment. Our previous work confirmed the existence of a single copy of this gene in the brook char (Croisetière et al., 2008). A sample of 96 individuals from this population was previously genotyped at MHIIB gene which revealed eight different alleles (exon 2 coding sequence) with a distinct minisatellite size associated to each one (data not shown). Directly at the hatchery, head kidney tissue samples were taken for total RNA isolation from 23 random individuals in August when average water temperature was 18 °C and from 24 other individuals in November when average temperature was 6 °C.

A population of 93 brook char originating from the Rupert River (Rupert strain) in northwestern Québec, Canada, and associated waterways were maintained in a controlled environment at the LARSA (Laboratoire de Recherches des Sciences Aquatiques; Université Laval). These fish of approximately 1 kg were tagged and genotyped at the MHIIB locus (DAB) by SSCP (Single Strand Conformational Polymorphism) as previously described (Croisetière et al., 2008). Knowing their respective genotype, fish could be specifically selected for the length of the minisatellites they were carrying at the MHIIB gene and used in real-time RT-PCR with specific primers to determine allelic expression. Before proceeding to blood sampling, fish were acclimatized 5–7 days at specified temperature (6 °C and 18 °C) to attenuate the effect of stress and assure a steady state expression of the MHIIB gene.

2.2. RNA isolation from peripheral blood leukocytes (PBL) and head kidneys

Head kidneys from domestic fish (Chesterville) of each group (6 °C and 18 °C) were isolated to evaluate the total expression of
MHIIβ genes. Fish were sacrificed with an overdose of tricaine methanesulfonate (MS-222) (Sigma) before removing the head kidney that was mashed into a disposable dish using a syringe. An equivalent volume of Trizol (Invitrogen) was added before transferring the tissue sample to an Eppendorf for total RNA extraction according to the manufacturer’s protocol. For each fish, a fin sample was taken for genomic DNA isolation.

RNA from PBL of brook charr (Rupert strain) was also extracted to evaluate the relative expression levels of MHIIβ alleles at 6°C and 18°C. Since it is non-letal, RNA extraction from PBL compared to head kidneys allowed us to test gene expression of the same fish population at both experimental temperatures. Furthermore, this allowed the evaluation of the expression level of the MHIIβ gene for different alleles in a second strain and type of tissue. Fish were first anesthetised with 0.1% of tricaine methanesulfonate (MS-222) (Sigma). The blood was collected from the caudal vein using a heparinised 3 ml syringe with a 21G1 needle (Becton Dickinson) and added to 1 volume of ice-cold PBS containing 10U/ml heparin sodium salt (Sigma). A fraction of this cellular suspension (1 ml) was then spun at 500 × g for 5 min at 4°C and the buffy coat and plasma transferred to a new Eppendorf. The PBL were then pelleted and resuspended in Trizol (Invitrogen) for total RNA extraction according to the manufacturer’s protocol. Dimethyl sulfoxide (DMSO; 7% final concentration) was added to the remaining blood sample in PBS and frozen for subsequent genomic DNA isolation.

2.3. Polymerase chain reaction (PCR), cloning and sequencing

PCR was performed on genomic DNA from domestic strain fish to evaluate the size(s) of the minisatellite present in the second intron of the MHIIβ alleles. The PCR fragment containing the minisatellite was obtained with primers located in conserved regions of the second intron, after analysis of thirteen alleles in brook charr (data not shown). The sequence of the sense primer P399, located 37 or 81 bp after the last motif, was 5′-TTCTTGAATGGTTTATAGCTGCAAC-3′ and the anti-sense primer P400, located 37 to 81 bp after the last motif, was 5′-CATCGCTGTASTTTATAAATTACACTTC-3′. PCR was carried out in a total volume of 50 μl containing 20–40 ng of genomic DNA, 0.2 mM dNTPs, 7 μl of 10× PCR buffer (200 mM Tris–HCl, pH 8.4, 500 mM KCl), 2 mM MgCl₂, 5 pmol each of P399 and P400 primers, 1 unit of Taq DNA polymerase. The following PCR conditions were used: denaturation for 3 min at 94°C followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 94°C, annealing for 30 s at 54°C and extension for 2 min at 72°C. The final extension was for 10 min at 72°C.

For each allele of the Rupert strain previously identified (Croisetière et al., 2008), a MHIIβ gene fragment containing the complete exon coding for the β1 domain (second exon) and the subsequent intron (second intron) was obtained by PCR on genomic DNA using primers in conserved regions for cloning and sequencing. The sequence of the sense primer SP4503, located at the end of the first intron of the gene, was 5′-CCTGTATTTATGTTCTCCTTTCAGATGG-3′ and the antisense primer P232, located at the N-terminal region of the β2 domain (exon 3), was 5′-CTGACCTAGCTGACATGG-3′. The reactions were carried out in a total volume of 50 μl containing 20–40 ng of genomic DNA, 0.2 mM dNTPs, 7 μl of 10× Expand high fidelity buffer (Roche), 2 mM MgCl₂, 5 pmol each of SP4503 and P232 primers, 2.6 units of Expand high fidelity enzyme mix (Roche) in a thermocycler (Biometra). The following PCR conditions were used: denaturation for 3 min at 94°C followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 52°C and extension for 3 min at 72°C. The final extension was for 10 min at 72°C.

PCR products, from 1350 to 2700 bp depending on MHIIβ Rupert alleles, were subsequently purified and extracted from agarose gel using the Illustra GFX PCR DNA and gel band purification kit (GE Healthcare) and subcloned in both orientations in pT7Blue-2 vector (Novagen) in accordance with the recommended protocol from the manufacturer.

Sequencing of both strands was performed on a 3730 DNA analyser (Applied Biosystems) using plasmid primers P235-Blue2S-Tag (5′-ACGGCTGTGCTAAATTCAAC-3′) and P247 (5′-CGGAATTCATATGTACC-3′) as well as intron 2 internal sense primer P399 (5′-TTCTTGAATGGTTTATAGCTGCAAC-3′) and anti-sense primer P400 (5′-CATCGCTGTASTTTATAAATTACACTTC-3′). Since the length of one particular allele (Safo-DAB0101) was too long to reach the nucleotide sequences from both sides (see Section 3), an Exonuclease III (New England Biolabs) digestion of the vector containing the allele was realized to allow the sequencing through the minisatellite.

2.4. Reverse transcriptase (RT)

Quantification of extracted RNA from fish of both the domestic and Rupert strain was done on a ND-1000 Spectrophotometer (NanoDrop) after treating with the DNA-free kit (Ambion) and before cDNA synthesis using SuperScript II reverse transcriptase (Invitrogen). Briefly, 500 ng of total RNA was mixed with oligo(dT)₁₂–₁₈ primer (Invitrogen), dNTPs (GE Healthcare) in a volume of 12 μl and denatured at 65°C for 5 min. This was followed by addition of 5× FS buffer, DTT, RNaseOUT (Invitrogen) and 200 units of SuperScript RT in a 20 μl final reaction volume for incubation at 42°C for 50 min, as recommended by the manufacturer.

2.5. Quantitative PCR

Quantitative PCR was performed using the FastStart SYBR Green Master (Roche Diagnostics) on a Rotor-Gene 3000 (Corbet Research). The primers used in these experiments are detailed in Table 1. All new primers were designed to flank a region containing an intron, to assure that amplification of contaminating DNA was controlled, which has not been detected in melting curve analysis. Amplification mixture (25 μl) contained template cDNA (2 μl of 1/20 dilution), 2× FastStart SYBR Green Master (12.5 μl) and 300 nM forward and reverse primers. The cycling conditions comprised 15 min polymerase activation and denaturation step at 95°C, followed by 40 cycles at 95°C for 15 s, 60°C for 45 s and 72°C for 30 s. All samples were run in triplicate with non-template controls (NTC). Relative quantification of gene expression levels was done by comparing difference in CT values by the ∆ΔCT method (Critical factors for successful real-time PCR, QiaGen), since the PCR efficiency was nearly equivalent for pairs of primers. For the domestic strain, EFAβ₂₂, β₂m, GAPDH and TCROs were evaluated as reference genes to normalize the results before calculating the relative expression levels. The stability of the reference genes was evaluated with the Bestkeeper tool (Pfaffl et al., 2004). We kept the elongation factor I alpha, paralog B (EFIA₂) as reference gene, as it showed the lowest variability in our samples and also because it has been found suitable for gene expression assay in different studies (Olsvik et al., 2005; Jorgensen et al., 2006; Ingerslev et al., 2006). Within the population of 93 brook charr (Rupert strain) kept at the LARSA, we identified four genotypes from which the allelic expression levels could be analyzed at temperatures of 18°C and 6°C: Safo-DAB*0201/*0301, Safo-DAB*0401/*0301, Safo-DAB*0101/*0301 and Safo-DAB*0101/*0201. The sizes of the minisatellites present in the MHIIβ gene from these individuals were respectively: 27 and 20, 40 and 20, 69 and 20, 69 and 27 repetitions of the minisatellite motif (Table 2). The relative allelic
expression levels were calculated by comparing differences in CT values in the same sample with allele-specific primers. Samples were included in different qPCR runs to control the reproducibility of the results. Differences for MHIIβ expression levels as a function of minisatellite length or temperature were compared using the t-test with bilateral distribution for samples with homogeneous variance.

2.6. Sequence analysis

Nucleotide sequences from the MHIIβ second intron alleles of the Rupert strain were submitted to GenBank (accession nos. GQ253476, GQ253477, GQ253478, GQ253479, GQ253480 and GQ253481). The coding sequences of the six MHIIβ alleles can also be found (GenBank accession nos. EU478851, EU478852, EU478853, EU478854, EU478855 and EU478856). Alignment of the nucleotide sequences was performed using ClustalX (Thompson et al., 1994) and MEGA 4 (Tamura et al., 2007). Identity of the intronic sequences was determined by pairwise-comparison of nucleotide sequences with the Syn-SCAN program (Gonzales et al., 2002). GeneBank published sequences from Atlantic salmon, Salmo salar (Sasa-DAB*0201; AJ439067 and Sasa-DAB*0301; AJ439069) and Oncorhynchus species (Onmy-Su2a; U34715, Onne-Wk136e; U34713, Onne-Wk136a; U34712 and Onke-An13c; U34702) (Miller and Withler, 1996) were included in the alignment.

Table 2
Characteristics of the second intron in MHIIβ alleles from brook charr (Safo-DAB) and other salmonid fishes.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Intron length (nt.)</th>
<th>Minisatellite</th>
<th>Hpa I SINE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Motif*</td>
<td>No. repeats</td>
</tr>
<tr>
<td>Safo-DAB0101</td>
<td>2410</td>
<td>I</td>
<td>69b</td>
</tr>
<tr>
<td>Safo-DAB0201</td>
<td>1382</td>
<td>I</td>
<td>27</td>
</tr>
<tr>
<td>Safo-DAB0301</td>
<td>1095</td>
<td>I</td>
<td>40</td>
</tr>
<tr>
<td>Safo-DAB0401</td>
<td>1696</td>
<td>I</td>
<td>40</td>
</tr>
<tr>
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<td>I</td>
<td>19</td>
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<td>I</td>
<td>25</td>
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<tr>
<td>Sasa-DAB0201</td>
<td>1334</td>
<td>II</td>
<td>25c</td>
</tr>
<tr>
<td>Sasa-DAB0301a</td>
<td>478</td>
<td>I</td>
<td>≥7</td>
</tr>
<tr>
<td>Onmy-Su2a</td>
<td>563</td>
<td>I</td>
<td>1</td>
</tr>
<tr>
<td>Onne-Wk136e</td>
<td>547</td>
<td>I</td>
<td>1</td>
</tr>
<tr>
<td>Onne-Wk136a</td>
<td>731</td>
<td>I</td>
<td>1</td>
</tr>
<tr>
<td>Onke-An13c</td>
<td>773</td>
<td>I</td>
<td>1</td>
</tr>
</tbody>
</table>

N/A, Not available.

a Consensus sequence, type I (32 nt.): ACATTACAGTATGACTAGTCAGCTATGTAGTG; consensus sequence, type II (40 nt.): TATGATATGTAGTACACTGGAAT.

b 10 partial motifs.

c 1 partial motif.

d Incomplete intronic sequence.
3. Results

3.1. Nucleotides sequences and minisatellite from the second intron of MHIIβ gene

The first objective of this study was to document the variability of a minisatellite found in the second intron of the MHIIβ gene in brook charr. As a first step, we sequenced the second intron of the six brook charr MHIIβ alleles of the Rupert strain identified in a previous study (Croisetière et al., 2008). Characteristics of the second intron of these alleles and of other salmonids MHIIβ gene are given in Table 2. The intron length differed among all six exon alleles identified in brook charr of the Rupert strain and ranged from 1023 to 2410 nucleotides. The alignments of the second intron nucleotide sequences flanking a minisatellite in 5′ (Fig. 1) and 3′ (Fig. 2) are presented for selected salmonid species to illustrate the diversity found at this locus. A schematic representation of this diversity in the second intron of the MHIIβ gene of salmonids is also shown in Fig. 3.

Four regions of the intron indicated as Box I to Box IV showed variable level of sequence divergence in pairwise comparisons (Figs. 1–3). The first 261 nucleotides of the second intron (Box I) showed pairwise divergence values ranging from 1.20% to 7.02% (mean of 5.52%) among brook charr alleles to a maximum of 12.9%.
(mean of 8.46%) when compared to other salmonid species. Box II corresponds to the first minisatellite motif from brook char and Atlantic salmon, and the sole motif reported in Pacific salmon species. This 32 nucleotide sequence diverged by 0 to 4 nucleotides (mean of 1.33 nt; 4.17%) within brook char while divergence for up to 8 nucleotides were found (mean of 3.05 nt; 9.52%) between species. Box III comprises 36 nucleotides with divergence ranging from 0 to 5 nucleotides (mean of 2.27; 9.45%) among brook char alleles and up to 9 substitutions (mean of 4.22 nt; 13.4%) among species. Box IV is composed of the last 58 nucleotides of the intron with divergence values ranging from 0.00% to 5.00% (mean of 2.33%) within brook char alleles and up to 17.9% (mean of 7.07%) among species.

The minisatellite identified within the second intron has a consensus motif of 32 nucleotides (type I in Table 2): ACAT-TACATAGTACTAGTACCTATGTAGTG; which is 38% G+C rich. Search for homologue sequences did not reveal its occurrence in salmonid species other than Atlantic salmon. PCR amplification of a fragment containing this minisatellite with the primer pairs P399/P400 revealed that each allele contains a different number of the minisatellite repeats with 69, 27, 20, 40, 19 and 25 repetitions found in the Safo-DAB*0101 to Safo-DAB*0601 allele, respectively (Table 2). An example of the minisatellite found in allele Safo-DAB*0301 is shown (Fig. 4). With the exception of the Safo-DAB*0101 allele, sequencing also revealed that the purity of the repeated motif was very high, ranging from 95.6% to 99.7%. In contrast, allele Safo-DAB*0101 showed a high level of mutation over the whole minisatellite length; with 86% (59 out of 69) of the motifs being mutated in comparison with the consensus sequence. Moreover, these mutated motifs included ten additional ones that showed a deletion of about 50% of the nucleotides relative to the consensus motif (Fig. 3).

Fig. 2. Alignment of the second intron nucleotide sequences following the minisatellite found in the MHII/H9252 gene from brook char and other salmonids. The last minisatellite motif sequence of each allele is in bold. Dots (.) indicate identity with Safo-DAB*0101; dashes (–), gaps introduced to maximize the alignment. Portions of the intronic sequences showing high conservation are identified with boxes (III–IV). The motifs (type II) forming the second minisatellite in Atlantic salmon are alternatively indicated by a grey and white rectangle. The complete Hpa I SINE sequence is underlined. GeneBank accession numbers for Atlantic salmon (Salmo salar; Sasa) and Pacific salmon (Oncorhynchus species; Oomy, Onne and Onke) sequences are AJ439067, AJ439069 and U34715, U34713, U34712, U34702 respectively.
3.2. MHIIβ gene expression level is inversely correlated to minisatellite length

A quantitative real-time RT-PCR experiment was realized on head kidney RNA from the domestic strain to evaluate the possible effect of minisatellite length on gene expression level of MHIIβ at both temperatures of 18°C and 6°C. This strain was selected because the fish were exposed to natural fish farm environment and also because of availability of specimens. However, since the genotypes of the individuals at the fish farm are variable in function of new incorporated stocks, the coding sequence of the MHIIβ gene was not considered and the focus was oriented on the minisatellite length found in the second intron.

To estimate the size of the minisatellite present in both MHIIβ genes, a PCR amplification with the P399/P400 conserved primer pair was realized on genomic DNA from 23 individuals for which the head kidney was sampled at 18°C and 24 individuals at sampled at 6°C directly at the fish farm. Depending on MHIIβ allele, PCR products of approximately 750, 925, 975, 1600 and 2000 bp, corresponding to approximately 19, 25, 27, 46 and 59 motif repetitions respectively, were generated (Fig. 5A). We identified 23 homozygote and 24 heterozygote individuals in the 47 fish sampled at both temperatures, with the former ones clearly showing increased intensity of the PCR product (e.g. #4, #8 at 18°C). Fish carrying both short minisatellites, heterozygote (#3 and #15; 19 and 25 repeats) or homozygote (#4 and #14; 25 repeats), and long minisatellites, homozygote (#1 and #7; 46 repeats), were selected at 18°C, while at 6°C fish were also selected for short minisatellites, heterozygote (#10 and #22; 19 and 25 repeats) or homozygote (#8 and #15; 25 repeats), and long minisatellites (#12; 46 and 59 repeats) for the quantitative RT-PCR experiment.

Fig. 5B shows the relative total MHIIβ gene expression values (generic P456/P457 primer pair) in head kidney for short or long minisatellites present in the gene, at temperatures of 18°C and 6°C with EFlAB as reference genes. The results are the cumulative of two RT-PCR experiments where each sample is tested each time in triplicate. At 18°C, the MHIIβ genes having a short minisatellite (19 and 26 repeats) showed a relative expression of 1.12 ± 0.12 while it was 0.75 ± 0.17 for the genes with a long minisatellite (46 repeats). This real-time PCR results demonstrated a significant 1.49-fold down-regulation (t-test, p < 0.01, n = 8 and 4) of the MHIIβ expression for gene carrying a long minisatellite. At 6°C, the relative expression was 2.31 ± 0.27 for the genes with a short minisatellite and

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**Fig. 3.** Properties of the second intron of the MHIIβ gene in salmonids. (A) Schematic representation of the allelic diversity found in the MHIIβ gene of various salmonid species. Identity of the motifs and elements correspond to the letters and color shapes indicated in (B), (C), (D) and (E). The sequence of 22 motifs repeated twice in Saff-DAB*0101 is marked by an asterisks (*). (B) Complete motif sequences with corresponding identity indicated below by a letter and color boxes. Motifs with one or two mutations relative to the original are represented by boxes with few or many shading respectively. Dots (.) indicate identity with the consensus motif. (C) Partial motif sequences with the element identity indicated below. Asterisks (*) indicate gaps introduced to maximize the alignment; dashes (–), sequence not related to the motif. (D) Motif sequence of the second minisatellite (type II) present in the second intron of MHIIβ gene from Atlantic salmon. (E) Identity of the partial and complete Hpa I SINEs.
expression revealed no significant difference in the transcript levels between these alleles at both temperatures.

We also analyzed individuals with the Safo-DAB*0401/*0301 genotype (40 and 20 repeats), for which the difference in minisatellite length is more important. In those fish, we detected an allelic imbalance in the expression profile between both alleles. The relative expression level of the Safo-DAB*0401 allele in function of Safo-DAB*0301 allele, as studied in five individuals, was $0.51 \pm 0.06$ and $0.59 \pm 0.06$ at 18°C and 6°C, respectively (primers P452–P478/P457) (Fig. 6). This represents a 1.96-fold and 1.69-fold reduction in the expression for the allele carrying the longer minisatellite. Moreover, these values indicate a significant difference in the expression levels between 18°C and 6°C (t-test: $p < 0.05$, $n = 9$).

The last two genotypes for which we analyzed the relative expression levels were Safo-DAB*0101/*0301 (69 and 20 repeats) and Safo-DAB*0101/*0201 (69 and 27 repeats), with the Safo-DAB*0101 allele carrying the longest minisatellite found in this population present in both ones. In the former genotype, found in three individuals (2 analyzed at 6°C because of one mortality between sampling), the relative allelic expression was 0.45 $\pm$ 0.05 at 18°C and 0.60 $\pm$ 0.07 at 6°C (primers P451–P452/P457). This corresponded to a significant 2.22-fold and 1.67-fold reduction in the expression of the Safo-DAB*0101 allele compared to Safo-DAB*0301 at both temperatures (t-test: $p < 0.001$, $n = 6$ (6°C)) (Fig. 6). This result was confirmed with the pair of primer P539–P540/P232 (data not shown). Found in 11 individuals, the second genotype in which Safo-DAB*0101 was present also showed a reduction in the transcript expression of the allele carrying the longest minisatellite and a significant difference in the relative expression levels between temperatures (t-test: $p < 0.05$, $n = 10$). A relative allelic expression of 0.39 $\pm$ 0.06 (2.56-fold reduction) and 0.51 $\pm$ 0.03 (1.96-fold reduction) at 18°C and 6°C respectively was found for the Safo-DAB*0101/*0201 genotype (Fig. 6). The results presented for the last three genotypes (Safo-DAB*0401/*0301, Safo-DAB*0101/*0301 and Safo-DAB*0101/*0201) further indicates that the repressive activity associated to the longest minisatellite was more effective at 18°C compared to 6°C and therefore highlighted a functional cis-acting polymorphism for the minisatellite present in the second intron of the MHIβ gene.

4. Discussion

4.1. Instability of the minisatellite found in the second intron of MHIβ gene

In this study, a minisatellite composed of a 32 nucleotides motif was identified in the second intron of the MHIβ gene in brook char. This minisatellite has already been revealed in the same gene in Atlantic salmon (Stet et al., 2002) and as a single 32 nucleotides motif in Pacific salmon (Oncorhynchus species) (Miller and Withler, 1996). Search for homologue sequences of the minisatellite did not reveal its occurrence in other species. The length of the intron found in brook char is comparable to that found in Atlantic salmon, but longer than for Pacific salmon. The presence of the motif as a single or as a minisatellite sequence in all salmonids species, combined with the low sequence divergence of Box I and Box IV support the hypothesis of a single and same ancestral expressed MHIβ gene carrying a long minisatellite. These results further revealed an allelic imbalance in the expression profile between both alleles. The relative expression level of the Safo-DAB*0401 allele in function of Safo-DAB*0301 allele, as studied in five individuals, was $0.51 \pm 0.06$ and $0.59 \pm 0.06$ at 18°C and 6°C, respectively (primers P452–P478/P457) (Fig. 6). This represents a 1.96-fold and 1.69-fold reduction in the expression for the allele carrying the longer minisatellite. Moreover, these values indicate a significant difference in the expression levels between 18°C and 6°C (t-test: $p < 0.05$, $n = 9$).

The last two genotypes for which we analyzed the relative expression levels were Safo-DAB*0101/*0301 (69 and 20 repeats) and Safo-DAB*0101/*0201 (69 and 27 repeats), with the Safo-DAB*0101 allele carrying the longest minisatellite found in this population present in both ones. In the former genotype, found in three individuals (2 analyzed at 6°C because of one mortality between sampling), the relative allelic expression was 0.45 $\pm$ 0.05 at 18°C and 0.60 $\pm$ 0.07 at 6°C (primers P451–P452/P457). This corresponded to a significant 2.22-fold and 1.67-fold reduction in the expression of the Safo-DAB*0101 allele compared to Safo-DAB*0301 at both temperatures (t-test: $p < 0.001$, $n = 6$ (6°C)) (Fig. 6). This result was confirmed with the pair of primer P539–P540/P232 (data not shown). Found in 11 individuals, the second genotype in which Safo-DAB*0101 was present also showed a reduction in the transcript expression of the allele carrying the longest minisatellite and a significant difference in the relative expression levels between temperatures (t-test: $p < 0.05$, $n = 10$). A relative allelic expression of 0.39 $\pm$ 0.06 (2.56-fold reduction) and 0.51 $\pm$ 0.03 (1.96-fold reduction) at 18°C and 6°C respectively was found for the Safo-DAB*0101/*0201 genotype (Fig. 6). The results presented for the last three genotypes (Safo-DAB*0401/*0301, Safo-DAB*0101/*0301 and Safo-DAB*0101/*0201) further indicates that the repressive activity associated to the longest minisatellite was more effective at 18°C compared to 6°C and therefore highlighted a functional cis-acting polymorphism for the minisatellite present in the second intron of the MHIβ gene.

4. Discussion

4.1. Instability of the minisatellite found in the second intron of MHIβ gene

In this study, a minisatellite composed of a 32 nucleotides motif was identified in the second intron of the MHIβ gene in brook char. This minisatellite has already been revealed in the same gene in Atlantic salmon (Stet et al., 2002) and as a single 32 nucleotides motif in Pacific salmon (Oncorhynchus species) (Miller and Withler, 1996). Search for homologue sequences of the minisatellite did not reveal its occurrence in other species. The length of the intron found in brook char is comparable to that found in Atlantic salmon, but longer than for Pacific salmon. The presence of the motif as a single or as a minisatellite sequence in all salmonids species, combined with the low sequence divergence of Box I and Box IV support the hypothesis of a single and same ancestral expressed MHIβ locus in salmonids (Glammann, 1995; Langefors et al., 2000).

Atlantic salmon minisatellites (type I) are showing an increase accumulation of mutations with purity of 92.0% and 95.4% over the entire length. For microsatellites, it has been demonstrated that expansion and contraction mutations on the tandem repeat tend to remove the imperfections found in the motifs by the mechanism of polymerase slippage, while in the absence of this purifying activ-
Fig. 5. MHIIβ expression level in brook charr head kidney as a function of minisatellite length and temperature. (A) Genomic PCR showing the length of the minisatellite present in the second intron of MHIIβ alleles (domestic strain) amplified with the P399/P400 primers. A total of 23 fish and 24 fish were sampled at 18°C and 6°C, respectively. Short and long minisatellites have 19 or 25 and 46 or 59 repeats of the 32 nucleotide motif. (B) Box plots giving the relative expression distributions, evaluated by real-time RT-PCR, of total MHIIβ mRNA from fish with variable minisatellite length. Symbol legend: diamond, short minisatellite; circle, long minisatellite. Results from samples at 18°C and 6°C are normalized for EFAβ. The top and bottom of each rectangle represent the 75th and 25th percentiles, respectively; the bar and symbol in the rectangle indicate the median and average expression value respectively; the top and bottom whiskers show the maximal and minimal allelic expression value. Results are the cumulative data of two independent real-time RT-PCR experiments where samples are analyzed in triplicate. Comparisons of the distributions using the t-test returned significant results: *p < 0.01 and **p < 0.001.

Fig. 6. Expression level of MHIIβ alleles as a function of both minisatellite number of repeats and temperature. Box plots showing the relative allelic expression distributions of MHIIβ mRNA in brook charr lymphocytes (Rupert strain) at 18°C and 6°C. The allelic expression levels are expressed in function of the other one in parenthesis. The top and bottom of each rectangle represent the 75th and 25th percentiles, respectively; the bar and symbol in the rectangle indicate the median and average allelic expression value respectively; the top and bottom whiskers show the maximal and minimal allelic expression value. Symbol legend: circle, value at 18°C; diamond, value at 6°C. Values presented are from a single (Safo-DAB*0201/*0301 and Safo-DAB*0101/*0201), two (Safo-DAB*0401/*0301) or three (Safo-DAB*0101/*0301) independent real time RT-PCR experiment, where all samples are analyzed in triplicate. Comparisons of the distributions using the t-test returned significant results: *p < 0.05 and **p < 0.001.
identified by asterisks in Fig. 3. Since the minisatellite found in this allele is composed of motifs showing a complex and high level of polymorphism, this repetition seems to reflect a recombinative event that occurred previously and support this mechanism to explain the expansion of this minisatellite sequence as previously suggested (Debrauwere et al., 1997).

4.2. Minisatellite modulation of the MHIIB gene expression level

We also described a functional effect associated to the presence of the polymorphic minisatellite on the transcriptional activity of the MHIIB gene. Regulation of the transcriptional activity by minisatellites has already been demonstrated in few studies (e.g. Pogliese et al., 1997; MacKenzie and Quinn, 1999; Zamarano et al., 2006). This genetic element is added to a different minisatellite (type II 40 nucleotides motif, Figs. 2, 3 and Table 2), a partial and a complete Hpa I SINE that were identified previously at the same location in salmonids (Figs. 1–3 and Table 2) (Miller and Withler, 1996; Stet et al., 2002). The possibility of MHIIB gene expression modulation offered by these types of genetic elements could constitute a fine tuning mechanism to ensure an appropriate immune response to pathogens, while limiting deleterious effects to host tissues.

In fact, two strains of brook char showed a reduction in MHIIB gene expression for different alleles possessing the longest minisatellite. The effect was also seen at the total RNA expression level in head kidney tissue (Fig. 5) or by comparison of the allele’s relative expression level in lymphocytes of heterozygous fish (Fig. 6). This analysis of the transcriptional allelic variation allows the detection of small differences observed between alleles and also eliminates the inter-individual variation that could come from different genetic background as well as environmental or physiological conditions rather than direct genetic factors. Also, the real-time RT-PCR results on heterozygous fish (Fig. 6) were subsequently confirmed with different probes for the same allele (Table 1). With the exception of the few last 3’ nucleotides that were allele specific, the primers for the quantification between these alleles were localized at the same position at the end of the second exon and produced same size amplicons with minimal variation in nucleotide sequence. The selection of housekeeping gene is also known to be a critical factor in gene expression experiments. We used EF1α that was reported with the most stable transcription level in a selection of genes in Atlantic salmon (Olsvik et al., 2005). This reference gene also showed a stable level of expression at both temperatures in the tissues tested for the quantification experiments (Croisetière et al., in preparation). Moreover, we observed this effect in multiple heterozygous individuals presenting three different genotypes where the MHIIB alleles carrying the longest minisatellite (Safe-DAB0101 and Safe-DAB0401) showed reduced transcript expression when compared to the allele with the shortest one (Fig. 6). It is noteworthy that the difference between the expression level of the allele Safe-DAB0101 relatively to Safe-DAB0301 was not greater than Safe-DAB0401 relatively to Safe-DAB0301, even if the number of motif found in Safe-DAB0101 is superior (69 versus 40). One possible explanation for this could be the presence of multiple mutations in this allele that decrease its impact.

Finally, our results also confirmed that reduced expression level associated to alleles carrying a long minisatellite is modulated by the temperature. The temperature effect on teleost MHC gene expression has been addressed for more than a decade now (Rodrigues et al., 1998). Here, results clearly showed a more pronounced difference of MHIIB gene expression level at 18°C between alleles of different minisatellite length (Fig. 6). This could possibly reflect a greater thermodynamical energy for the adoption of a DNA secondary structure as functional mechanism.

4.3. Reduced immune gene expression at high temperature in brook char?

When we compared at both temperatures the total expression level of the MHIIB genes carrying short minisatelites, we found that the expression was 2.08-fold higher at 6°C than at 18°C (Fig. 5). The difference was approximately of the same order for long minisatellites, however with fewer samples tested. This increased expression at low temperature is in accordance with the data of Raida and Buchmann (2007) for constitutive expression of MHIIB gene in rainbow trouts’ head kidney. In their paper, Table 4 indicates a difference of 0.8 raw Ct between MHCI expression at 5°C and 15°C, while the expression of the reference gene EF1α was stable. Since MHCI and EF1α show almost the same PCR efficiency (101.1% vs 100.0%), this represents a 1.74-fold higher expression of MHIIB at 5°C, a result in accordance with ours. On the other hand, Nath et al. (2006) observed a downregulation of MHIIB gene expression at mRNA and protein levels in rainbow trout at 2°C. However, this conflicting result seems to be the consequence of the exposition to temperature below the immunologically nonpermissive temperature of −4°C (Fryer et al., 1976; Bly and Clem, 1991) and near the lower lethal temperature of −0.7°C for salmonids (Saunders, 1995). Furthermore, since the gene coding for the ribosomal protein S11 also shows a decrease in transcript at 2°C, the effect reported by Nath et al. (2006) seems to be the result of a more general and severe repression of the transcription.

From the evaluation of the four candidate housekeeping genes that was realized, the β2m gene was surprisingly ranked as the worst gene in the samples tested. A close examination of the Ct obtained with the β2m gene revealed an interesting fact. The mean of the Ct at 18°C for the β2m expression was 18.18 ± S.E. 0.44 while it was of 16.89 ± S.E. 0.40 at 6°C. This difference was significant (t-test; p < 0.001, n = 6) and represented a 2.78-fold higher expression of β2m at 6°C once corrected with the reference gene EF1α (data not shown). This result concord with studies performed in Atlantic salmon and rainbow trout that show no decrease of the β2m transcript after 10 days at temperature of 2°C (Kales et al., 2006), unlike to a reduction of the expression at 6°C reported in common carp (Rodrigues et al., 1998). The reasons why Kales et al. (2006) did not detect a significant variation in the β2m gene expression at low temperature could be explained by the low sensitivity of their technique or the differences in the temperatures to which the fish have been exposed. The conflictual data described in this report regarding the expression level of the MHIIB and the β2m as a function of temperature clearly highlights the need of further investigations on immune gene expression level at boundary or extreme habitat temperatures.

One can question what could be the advantages for fish to reduce their MHIIB expression level at higher temperature? By reducing the MHIIB gene expression level at higher temperature (and possibly other immune relevant genes), the advantage could also be a diminution of the energy cost to mount an efficient immune response. This hypothesis is based on the fact that temperature is important in governing the rate at which body processes are carried out. This might be the consequences of an increased cellular metabolism and/or functional activity. It has been suggested that membrane fluidity could be involved in the activation of T cells in function of temperature (Vallejo et al., 1992). As previously mentioned, other studies also demonstrated that some fish MHC genes have a temperature-dependent expression level (Rodrigues et al., 1998; Kales et al., 2006; Nath et al., 2006). Another possible advantage on the field of energy economy implicates a fine tuning by the minisatellite of the MHIIB gene expression level. The closely linkage of the minisatellite with the exon 2 coding for the protein β1 domain and responsible of antigen presenta-
that specific alleles of MHIIβ gene correlates with increase resistance to pathogen (Langefors et al., 2001; Grimholt et al., 2003; Croisetière et al., 2008), the selection for an allele with a particular minisatellite length from another one coding for an identical protein, but with a different minisatellite length, is clearly possible. The basis for this selection would be an optimal expression level of the allele, involving a certain energy cost, in function of the efficiency to present antigens and induce an appropriate immune response. Here, it would be very interesting to develop transgenic fish with the same MHIIβ-coding sequence, but differing at minisatellite length, and to test them in challenge experiment against bacteria to evaluate the contribution of gene expression level to the resistance and susceptibility to pathogens.

4.4. Other elements contributing to the polymorphism of the MHIIβ gene in salmonids

Two additional elements could be distinguished from the analysis of the second intron nucleotide sequences of the MHIIβ gene in salmonids: a second minisatellite composed of a 40 nucleotides motif (type II) found exclusively in Atlantic salmon (Figs. 2, 3 and Table 2, small white and grey rectangles) and a Hpa I SINE (Figs. 1–3 and Table 2) (Kido et al., 1991; Miller and Withler, 1996). The accumulation of genetic element in the second intron of the salmonids MHIIβ gene with its expression restricted to specialized tissues (Ting and Trowsdale, 2002) support the hypothesis of 'genomic design' model for the evolution of this intron (Pozzoli et al., 2007).

SINES contain an internal RNA polymerase III promoter that consists of an A box downstream from the transcription start site, and a B box located approximatively 50 bp downstream from the B box (Roy et al., 2000). Moreover, it contains several potential transcription factor binding sites. Wang et al. (2004) reported that SINE retroposition does not appear randomly in the direction of insertion, with all SINES within known host gene in rainbow trout inserted in the opposite orientation to the host gene transcription. As they suggested, insertion in both orientations could interfere with gene expression, the insertion in the same orientation showing the greatest possibility of effects. The fact that the complete Hpa I SINE found in brook char MHIIβ gene is in the same orientation and shows almost intact A and B box thus suggests that this element might present a regulatory role modulating the expression of the gene during stress period or pathogen infection. Furthermore, the closely insertion of two Hpa I SINES in the same gene is unexpected since SINES insertion is believed to be random (Kido et al., 1995). The dual insertion events detected in brook char MHIIβ gene is puzzling and calls for further studies on the mechanisms of SINES retroposition and regulatory functions.

The presence of multiple elements for which the implication in recombination events has been demonstrated previously probably highlights the importance of this type of mechanism in generating polymorphism of the MHIIβ gene. The high concentration and variability of elements found in this intron, just downstream of the exon 2 coding for the domain implicated in peptide binding and presentation could reflect the selective pressure to assure polymorphism of the MHIIβ gene. A higher incidence of these elements in the MHIIβ gene of salmonids may be the consequence of the fact that it is the only MHII locus identified thus far (Glammann, 1995; Langefors et al., 2000). Allele and gene conversion were identified as a mechanism to generate polymorphism of MHC in multiple organisms (Pease et al., 1983; Zangenberg et al., 1995). Also, it was demonstrated that the Eb gene of the mouse contains a recombination hotspot located within the second intron. Interestingly, a segment of the intron showed strong similarity to retroposon long terminal repeat (LTR), env, and pol genes indicating that this segment of the second intron may have evolved through retroposon insertion (Zimmerer and Passmore, 1991).

4.5. Identification of a new regulatory element submitted to natural selection?

MHIIβ gene exhibit polymorphism that is undoubtedly relevant for resistance or susceptibility toward infectious diseases (Trowsdale and Parham, 2004; Dionne et al., 2009), where different mechanisms are responsible for the evolution and maintenance of this polymorphism (Bernatchez and Landry, 2003; Aguilar and Garza, 2007). Since teleosts express a single "minimal, essential" locus of both α and β chain forming the MHII molecule (Glammann, 1995; Langefors et al., 2000) and also MHIIα and MHIIβ loci are genetically linked in Atlantic salmon (Stet et al., 2002), this animal model was used in association studies to identify resistance and susceptibility alleles, with the focus on the coding sequences (Langefors et al., 2001; Grimholt et al., 2003; Croisetière et al., 2008). Other studies demonstrated the implication of non-coding region polymorphism in the modulation of the MHCIβ transcriptional activity. The expression level of MHCII molecules was shown to be affected by mutations in the promoter, some of them revealing binding sites for transcription factors (Shewey et al., 1992; Baumgart et al., 1998). The results presented in this paper clearly demonstrate the implication of a new mechanism to fine tune the expression of the MHIIβ gene. Some reports are emerging to implicate microsatellite in morphological and behavioral evolution in vertebrates (Fondon and Garner, 2004; Hammock and Young, 2005) indicating a more important contribution of VNTR regulating phenotypic expression than previously presumed. Given the amply documented implication of the MHCIβ gene in the immune response and fitness, this report is the first, to our knowledge, to present a minisatellite that could potentially be submitted to selective forces and therefore play an important role in the immune response of fish populations in the wild.

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References


