doi: 10.1111/j.1744–313X.2005.00496.x

Polymorphisms of the upstream regulatory region of the major histocompatibility complex *DRB* genes in domestic horses

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Summary

Sequence information was obtained on the variation of the *ELA-DRB* upstream regulatory region (URR) after polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) cloning and sequencing of ≈ 220 bp upstream of the first exon of horse *DRB* genes. The sequence of the proximal URR of equine DRB is composed of highly conserved sequence motifs, showing the presence of the W, X, Y, CAAT and TATA conserved boxes of major histocompatibility complex (MHC) class II promoters. Five different polymorphic horse *DRB* promoter sequences were detected in five horse breeds. The results demonstrate the existence of polymorphism in the nucleotide sequences of the *ELA-DRB* URR, located in the functionally important conserved consensus sequences, the X2 box, the Y box and the TATA box, while conservation were observed in X1 and CAAT boxes. The nucleotide diversity among horse URRs was intermediate between that seen within human and mouse DRB promoters, suggesting the existence of another important source of variability in *ELA-DRB* genes. In addition, phylogenetic comparisons, identity analysis and sequence organization suggested that the reported sequences would correspond to an expressed *ELA-DRB* locus. However, further information about the functional significance of these promoter polymorphisms will probably be acquired through expression studies on the different sequences.

Introduction

Major histocompatibility complex (MHC) class II molecules are polymorphic heterodimeric surface glycoproteins that are involved in the presentation of antigens to T-helper lymphocytes (Kappes & Strominger, 1988). The expression of these antigens at the surface of antigen-presenting cells (APCs) is essential for the recognition of foreign antigens by the T-cell receptor, and is regulated in a cell-specific manner and controlled via *cis*-acting elements in the upstream regulatory region (URR) located in the 5′-flanking regions of *HLA* class II genes (Benoist & Mathis, 1990; Peterlin *et al*., 1990; Glimcher & Kara, 1992; Ting & Trowsdale, 2002).

The promoter elements of the class II genes are remarkably conserved. All classical and non-classical class II promoters contain elements that are necessary for optimal constitutive and cytokine-induced gene expression (Ting & Baldwin, 1993; Ting & Trowsdale, 2002; Hake *et al*., 2003). In addition to sequence conservation, the orientation and spacing of the elements are proposed to be critical for an accurate transcription (Harton & Ting, 2000). The URRs encompass approximately 200 base pairs (bp) upstream from the transcription start and consist of highly conserved sequences, i.e. TATA, CAAT and X1 (the RFX-binding site), Y [the reversed CAAT site (rCAAT)], X2 [the cAMP response element (CRE)] and W boxes (Auffray *et al*., 1987; Reith *et al*., 1995; Mitchinson & Roes, 2002). Their sequences represent sites of interaction with nuclear regulatory proteins — transcription factors such as NF-YB, NF-YC, RFX5, RFXANK and CREB-1 (Ting & Trowsdale, 2002) — which form a 'transcriptosome' by interacting with the Class II transactivator (CIITA) and mediate transcriptional control (Ting & Trowsdales, 2002). Despite the highly conserved sequences of the *cis*-acting elements, polymorphism has been found in the URRs of *HLA*-D genes, namely in *DRA* (Pinet *et al*., 1991), *DQB1* (Andersen *et al*., 1991; Reichstetter *et al*., 1994), *DQA1* (Del Pozzo *et al*., 1992), *DRB1* (Perfetto *et al*., 1993; Singal *et al*., 1993; Louis *et al*., 1993; Singal & Qiu, 1994; Louis *et al*., 1994) and *DOB* (Hake *et al*., 2003). It has also been shown that the nucleotide polymorphism of the proximal promoter region of *DRB* genes has certain functional consequences on their transcriptional activities (Louis *et al*., 1993; Louis *et al*., 1994; Singal *et al*., 1993; Singal & Qiu, 1994; Janitz *et al*., 1997; Sindwani & Singal, 2001).

The MHC of the horse, also called the equine leukocyte antigen (*ELA*) system, is located on chromosome 20 (20q14–22), as determined by *in situ* hybridization (Ansari *et al*., 1988; Måkinen *et al*., 1989). As in other species, the

The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database and have been assigned the accession numbers AF344426, AY354918, AY354919, AY3549120 and AY572852.

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Received 21 June 2004; revised 7 October 2004; accepted 28 October 2004

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number of expressed *DRB* genes appears to be variable, with two or three *DRB* genes reported in domestic horses and two in Przewalski horses (Fraser & Bailey, 1996; Hedrick *et al.*, 1999; Díaz *et al.*, 2001).

To date, no sequence information exists regarding the URRs of class II genes in horses, and relatively little is known about the genetic control of *ELA-DRB* gene expression. The purpose of the current study is to analyse the equine *DRB-URR* by means of polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and DNA sequencing. This analysis would allow us to address the following: first, whether the equine URR exhibits the common organization observed in other mammals and, second, whether polymorphism is present in their promoter region.

Materials and methods

Animals

To screen *ELA-DRB* promoter variation, a total of 90 peripheral blood samples from different horse breeds were used in order to maximize the chances of detecting polymorphism. These samples included Argentine Creole horses (AC; $n = 33$), Peruvian Paso (PP; $n = 20$), Thoroughbred (TB; $n = 15$), Spanish Pure Breed (SP; $n = 10$) and Spanish native breeds (SN; $n = 12$) horses. The Spanish native breeds group was represented by only 12 unrelated animals belonging to different, but related, breeds (Losino, $n = 3$; Asturcón, $n = 2$; Mallorquín, $n = 3$; Menorquín, $n = 2$ and Potoka, $n = 2$).

Genomic and plasmid DNA isolation

Purification of genomic DNA from blood lymphocytes was performed by using the DNAzol® method (Invitrogen, Carlsbald, CA, USA) and isolation of recombinant plasmid DNA was carried out by using the S.N.A.P. Plasmid Purification System (Invitrogen), respectively.

PCR amplification

Promoter regions associated with *DRB* genes were amplified from genomic DNA by PCR amplification using a 5′ primer (5′-GAGAAATACAGACACACCATGC-3′), corresponding to the consensus W box sequence, located 220 bp upstream of the start of transcription (Turco *et al.*, 1990), and a 3′ primer (5′-TGTTTCAGAAAAGGACCTTC-3′), annealing to a sequence located just after the initiation start of transcription (Ripoli *et al.*, 2002). The expected amplification fragment size is ≈ 240 bp.

The PCR was carried out in a total volume of 25 μL comprising 2.5 mM MgCl, 20 mM Tris-HCl, 50 mM KCl, 100 μM each dNTP, 0.2 μM each primer, 0.5 U of *Taq* polymerase (Invitrogen) and 2 μL of purified horse template DNA (50–100 ng). The amplification profile consists of 2 min at 94 °C, followed by 5 cycles of 30 s at 94 °C, 45 s at 45 °C and 30 s at 72 °C, and 30 cycles of 30 s at 94 °C, 30 s at 50 °C and 30 s at 72 °C, and an extension of 10 min at 72 °C. Purified plasmid DNA was screened for the presence of the inserts by PCR using the same oligonucleotide primers and an amplification reaction consisting of: 2 min at 94 °C, 30 cycles of 45 s at 94 °C, 45 s at 58 °C and 45 s at 72 °C, and a final extension of 10 min at 72 °C.

SSCP analysis

By using SSCP gels, amplification products were screened for pattern variation. Typing gels consisted of 10% (w/v) acrylamide:bis-acrylamide (38 : 1), 0.5× tris-borate EDTA (TBE), 0.093% (w/v) ammonium persulfate and 0.08% (v/v) TEMED. Samples plus loading dye [95% (v/v) formamide, 20 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol FF] were heated to 96 °C for 10 min and placed on ice until loaded. Gels were run in 0.5× TBE at 200 V for 16 h at 4 °C. SSCP patterns were visualized by silver staining (Bidler *et al.*, 1982) and photographed by using the Kodak Digital Science Kds 1D Image Analysis Software (Eastman Kodak Company, Rochester, NY, 1994–96). The designations of the different SSCP band patterns was carried out by using URR as a prefix to the name of the pattern, which was identified with capital letters.

Cloning and sequencing

PCR products from individual samples that presented the most common SSCP band patterns were ligated into TopoTA cloning vectors, according to the manufacturer's instructions (Invitrogen). Inserts from ≈ 20 positive clones for each animal were confirmed by PCR using the URR primers mentioned above. SSCP variations were determined in each positive clone, and PCR products corresponding to different URR SSCP band patterns were chosen for DNA sequencing. The DNA isolated from multiple clones was separately sequenced on an Applied Biosystems 377 automated sequencer (BioResource Center, Cornell University, Ithaca, NY, USA), using the T7 universal primer (Invitrogen).

Sequence analysis

The multiple alignment of the nucleotide sequences of the *ELA-DRB* URR was carried out using CLUSTAL W, version 1.7 (Thompson *et al.*, 1994). The similarity for DNA sequences was estimated by pairwise sequence alignment. Phylogenetic analysis of the equine URR sequences was performed by neighbour-joining trees constructed from Jukes-Cantor distance (Jukes & Cantor, 1969) and Kimura's two-parameter estimates (Kimura, 1980). To test the topology of the tree, the bootstrap statistical test was used. All the parameters were estimated by using the MEGA program Version, 2.1 (Kumar *et al.*, 1994). Nucleotide diversity was estimated by means of Kimura's two-parameter method included in the ARLEQUIN analysis software (Schneider *et al.*, 1997).

Nucleotide sequences were screened for the presence of transcriptional regulatory target sequences by using the

NSITE program (Shahmuridov *et al*., 1986; Solovyev y Kolchanov, 1994; Heinemeyer *et al*., 1999; Solovyev, 2002), which uses the Transfac Database (http://transfac.gbf.de). Sequence data from some other class II genes were retrieved from the literature: *HLA-DQB* (Andersen *et al*., 1991), *HLA-DRB1*, *-DRB2*, *-DRB3*, *-DRB4*, *-DRB5* and *-DRB7* (Louis *et al*., 1993), *BoLA-DRB3* (Ripoli *et al*., 2002, 2004) and *H2-Eb* (Janitz *et al*., 1997; Mitchinson & Roes, 2002).

Results

ELA-DRB URR organization

In this work we described the organization of the equine *DRB* upstream regulatory region (URR), and we analysed the promoter polymorphism by PCR-SSCP and DNA sequencing.

DNA sequencing of the 240-bp PCR products showed that the proximal promoter region of horse *DRB* genes encompasses ≈ 220 bp upstream to the initiation of transcription. As in humans and mice, the equine *DRB* promoter sequence showed the same organization of the conserved regulatory elements (Singal *et al*., 1993; Singal & Qiu, 1996; Louis *et al*., 1993; Louis *et al*., 1994; Mitchinson & Roes, 2002). Location and length of the conserved motifs were determined by comparison of the sequence alignment data together with the *HLA-DRB* promoter sequences. Thus, horse URR contains the three classical *DRB* regulatory elements known as Y, X and W boxes, as well as the CAAT and TATA motifs (Fig. 1).

The Y consensus sequence of horse DRB is 12-bp long and is located between nucleotides –143 and –154. The X region is located between nucleotides –168 and –187 upstream of the transcription initiation site. This conserved box contains two overlapping regulatory elements: first, a 12-bp motif (called X1), located at a conserved distance of 20 bp (nucleotides –176 to –187) from the sequence of the Y box; and second, an 8-bp motif (the X2 box) located between nucleotides –168 and –175. The W region is located between nucleotides –219 and –199 upstream of the transcription initiation site. As in the human regulatory regions, it overlaps the sequence of the S box (nucleotides –206 to –209). The TATA conserved element was detected at nucleotides –84 to –91 upstream of the transcription initiation site, with high homology to all functional DRB TATA boxes studied to date. In addition, a CCAAT sequence was observed between nucleotide positions –123 and –128 upstream of the start transcription site. Nevertheless, the nucleotide sequences of the *ELA-DRB* URR did not show the presence of the classical CCAAT sequence in these genes at an alternate position.

Computational search for the consensus transcriptional regulatory targets in the *ELA-DRB* promoter sequences revealed the presence of the recognition sites of the regulatory specific proteins of *DRB* Class II genes (Ting & Trowsdale, 2002), i.e. NF-Y, NF-YB, χ^2 BP, RFX5.

ELA-DRB URR polymorphism

ELA-DRB URR polymorphism was examined by using two different approaches: PCR-SSCP and DNA sequencing. PCR-SSCP analysis of the promoter amplified from the 90 analysed animals identified five different URR SSCP band patterns. All individual horses produced only

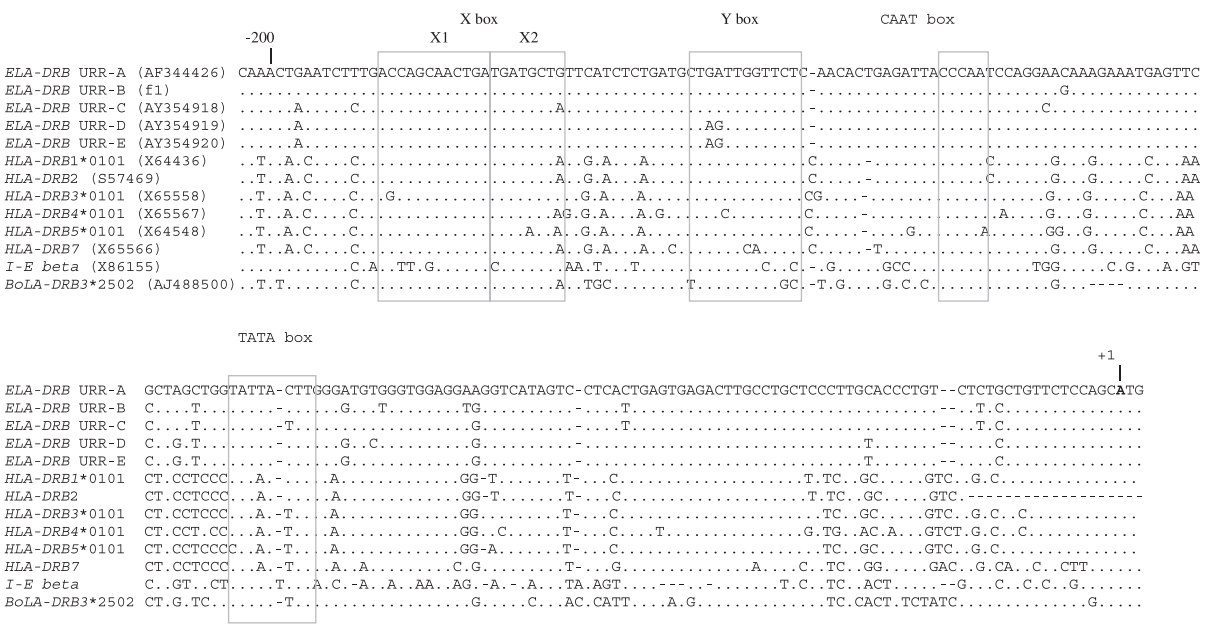

Figure 1. Comparison of nucleotide sequences of the upstream regulatory regions (URR) of *ELA-DRB*, *HLA-DRB*, *BoLA-DRB* and *H2-Eb* genes. GenBank accession numbers are indicated in parentheses. The A of ATG is designated as +1. Boxed sequences represent the conserved consensus boxes (X, Y, CAAT and TATA). Dashes represent nucleotide identity to the *ELA-DRB* URR sequence (AF344426). Sequences from human, murine and bovine class II DRB promoters were retrieved from the literature as described in the Materials and methods.

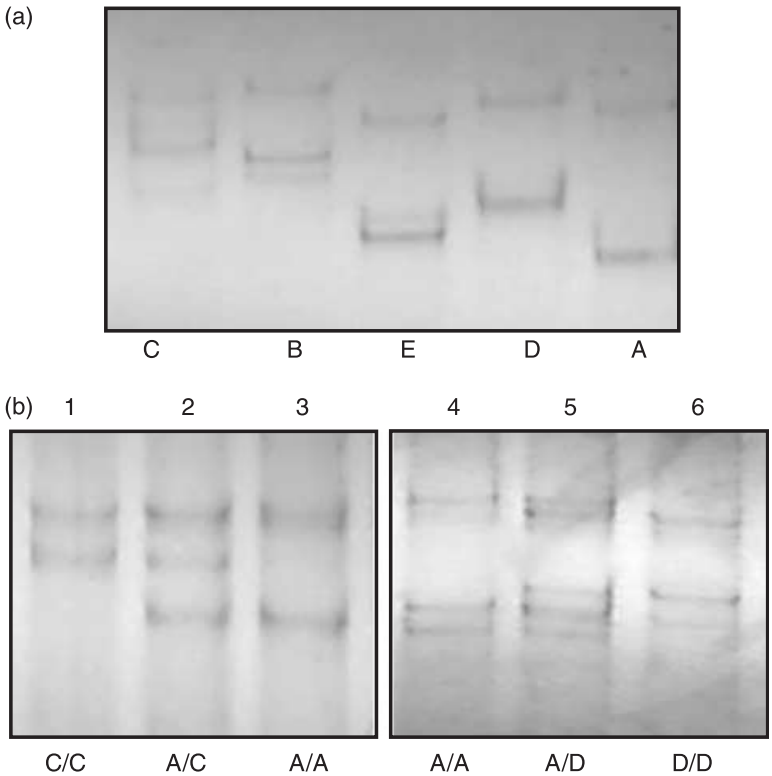

Figure 2. (a) Upstream regulatory region single-strand conformation polymorphism (URR SSCP) band patterns from cloned DNA polymerase chain reaction (PCR) amplifications; (b) URR SSCP band patterns from genomic DNA amplifications showing the Mendelian inheritance. The alleles are indicated at the bottom of the figure. Family relationships were as follows. Lanes 1–3, Argentine Creole horses: lane 1, dam; lane 2, offspring; and lane 3, sire. Lanes 4–6, Thoroughbred horses: lane 4, dam; lane 5, offspring; lane 6, sire.

Table 1. Average nucleotide diversity in the upstream regulatory region (URR) of *ELA-DRB*, *HLA-DRB* and *H2-Eb* promoter regions, estimated by using the Kimura two-parameter method; nucleotide compositions are also indicated

	Nucleotide composition (%)					
	C	T	A	G	Mean number of pairwise differences	Average nucleotide diversity
ELA-DRB	23.12	30.09	23.48	23.30	10.41 ± 5.73	0.046 ± 0.029
HLA-DRB	26.43	27.04	22.77	23.76	32.17 ± 14.48	0.085 ± 0.042[b]
HLA-DRB1	26.88	27.27	21.04	24.81	31.97 ± 14.86[a]	0.085 ± 0.044[c]
H2-Eb	26.42	27.63	24.73	21.23	4.48 ± 2.51	0.021 ± 0.013

[a] Includes the different *HLA-DRB*1 gene specificities; [b] includes comparisons between loci; and [c] includes comparisons within loci.

two or four SSCP bands (Fig. 2a), suggesting that the oligonucleotide primers employed amplified only a single polymorphic *DRB* locus or various loci with a high degree of sequence identity. Family studies documented Mendelian inheritance for URR SSCP patterns (Fig. 2b). Different SSCP pattern combinations were observed in the breeds studied (Fig. 2b). Thus, the URR-A and URR-D alleles were observed in all breeds, URR-C in all breeds except TB, while the URR-B and URR-E were found only in PP and Spanish breeds (SP and SN).

Nucleotide polymorphism

Figure 1 shows nucleotide sequences for the *ELA-DRB* URR. Sequence information was obtained after PCR-SSCP, cloning and sequencing of 240 bp upstream of the first exon of *ELA-DRB* genes for the five most common SSCP patterns. Each selected SSCP band pattern corresponded to a unique DNA sequence (GenBank accession number AF344426, AY354918, AY354919, AY354920 and AY572852).

The alleles of the promoter region of *DRB* genes showed a total of 20 variable sites located in the conserved consensus boxes and also in spacers between various consensus sequences. Variations in the W box were not considered because the *5′* primer was complementary to its sequence. Polymorphic sites identified among *ELA-DRB* URR sequences resulted in an average nucleotide diversity of 4.6%, with a mean number of pairwise differences of 10.41 (Table 1). However, URR-D and URR-E differed only in one base change, at position –77. No deletions were detected among the horse URR sequences.

Sequences of X1 and CAAT boxes were completely conserved among the five *ELA-DRB* promoter alleles identified. Likewise, sequences between X and Y boxes, and between Y and CAAT conserved boxes, showed no base substitutions. Nevertheless, polymorphism was observed in the remaining consensus boxes. Polymorphism in the X2 box was observed at position –168, where an A is present instead of a G in allele URR-C (GenBank accession no.: AF354918). The Y box showed two polymorphic sites in alleles URR-D (GenBank accession no.: AF354919) and URR-E (GenBank accession no.: AF354920): at position –151, A was substituted by G, and at position –152, G was substituted by A, respectively. These base substitutions are unique for horse promoter sequences and involve the loss of the CCAAT reverse sequence of the Y conserved element. The TATA box presented a specific polymorphism at position –86, where a T occurred instead of a C in URR-C (Fig. 1).

Phylogenetic relationships among URR sequences

The overall identity was found to be 99.1–96.6% among *ELA-DRB* URR sequences. The degree of identity for this region of *ELA-DRB* in comparison to other class II genes extended from 82 to 71%. *BoLA-DRB* (87–80%) and the functional *HLA-DRB* genes (82–71%) showed higher identities to the *ELA-DRB* promoter. In addition, comparison with *H2-Eb* revealed identity values from 71 to 68%. As expected, the lowest similarity was observed when compared to *HLA-DQB* and murine DQB homologous (*H2-Ab*) genes (≈ 37–25% and 20–7%, respectively).

Figure 3 shows the neighbour-joining tree for the five *ELA-DRB* URR nucleotide sequences and the *HLA-DRB*, *BoLA-DRB*, and *H2-Eb* published sequences. In addition, the paralogous *DQB* and *H2-Ab* promoter sequences were included. In general, the evolution of *DRB* genes, as deduced from the polymorphism comparative analysis of the regulatory regions, appears to reflect true ancestral relationships. The phylogenetic tree showed two main clusters of sequences supported by high bootstrap values (bootstrap = 99). The first cluster consists of all the *DRB* promoter sequences, and the second cluster included the *HLA-DQB* and orthologous *H2-Ab* promoter sequences. Within the *DRB* group, upstream regulatory sequences of the same species are clustered together. Consequently, horse *DRB* promoter sequences clustered together but separately from these same promoter sequences from *HLA*, *BoLA* and *H2* genes (bootstrap > 88). Similar results were observed within the *DQB* cluster. As expected, the topology of the tree is in accordance with the identity estimated values.

Discussion

The results obtained in this study allowed us to perform the first characterization of the URR of the *DRB* genes in domestic horses. The PCR fragment size obtained corresponds to previously published human, murine and bovine data (Turco *et al*., 1990; Singal *et al*., 1993; Louis *et al*., 1993; Louis *et al*., 1994; Mitchinson & Roes, 2002; Ripoli *et al*., 2004), comprising the proximal 220 bp between the transcription start site and the W box.

Nucleotide sequences obtained from horse *DRB* promoters account for the presence of the boxes W, X, Y, CCAAT and TATA in the 5′–3′ direction (Fig. 1) at conserved distances. Thus, the horse upstream regulatory region exhibited the typical organization of *DRB* gene promoters.

To explore promoter variation, polymorphism within different horse breeds was screened by means of PCR-SSCP. Five SSCP band patterns of the promoter region of *DRB* genes were identified in the present study. These results demonstrated the potential utility of the SSCP method as a rapid screen of the URR *DRB* variability in domestic horses. Although each SSCP band pattern corresponded to a unique DNA sequence, further studies are necessary to confirm the existence of additional promoter sequences corresponding to the same SSCP band pattern.

The analysis of the detected alleles within each breed showed the presence of different allele combinations in each one. These results evidenced the genetic differentiation among them, and probably differences in the number and extent of expressed *DRB* genes. However, additional studies are needed to assign allelic relationships in order to make it possible to investigate the populational distribution of URR polymorphism.

Nucleotide sequences of the different URR alleles from domestic horses, as defined by SSCP, are shown in Fig. 1. Comparative analysis of the different horse URR nucleotide sequences revealed four nucleotide changes within the conserved elements X2, Y and TATA boxes, and 16 nucleotide changes in the interconsensus regions, e.g. between the W and the X box, CCAAT and TATA boxes, and downstream of the TATA box. On the contrary, X1 and CAATT boxes remained invariable in all horse URRs, as well as in the interconsensus regions X–Y and Y–CAAT. Interestingly, base changes unique for horse URRs in Y and TATA boxes were detected (Fig. 1).

The nucleotide diversity estimated within *ELA-DRB* promoter sequences was, on average, 4.6%. In interspecies comparisons, horse URR sequences showed diversity values that were intermediate between mouse and human promoter sequences (Table 1), exhibiting a higher genetic diversity than *H2-Eb* and a lower nucleotide variability than *HLA-DRB* promoters. Observation of our DNA sequences showed that both the identity comparisons and phylogenetic analyses support the idea that these sequences corresponded to a *DRB* gene. *ELA-DRB* URR sequences exhibited a higher identity with human, murine and bovine *DRB* genes than with their paralogous *DQB* genes, and they clustered together with orthologous *DRB* genes instead of with *DQB* sequences. Furthermore, phylogenetic tree topology data supported that the URR polymorphisms within each species probably occurred after the origin of these orders.

The horse DR region contains at least three transcriptionally active beta chain genes (Fraser & Bailey, 1996). For this reason, it is difficult to assign the polymorphic sequences

Figure 3. Neighbour joining tree for the five horse URR sequences and *HLA-DRB*, *BoLA-DRB*, *SLA-DRB* and *H2-Eb* gene URR sequences. The numbers indicate bootstrap values for the internal nodes.

in the URR detected here to a particular DRB gene and, consequently, to discriminate whether the variations identified belong to a single (but very polymorphic) promoter, or to different and less variable DRB promoters.

We could hypothesize that the observed polymorphism in conserved boxes in horse affects the level and/or pattern of *ELA-DRB* gene expression. Such polymorphism may play a role in the level of expression of the *DRB* genes

and, perhaps, in the susceptibility to *ELA*-associated and autoimmune diseases.

Consequently, it is necessary to study the effect of these polymorphisms in the conserved boxes on constitutive and cytokine-mediated transcription of *ELA-DRB* genes. Moreover, it would be necessary to investigate the effects of the base changes involving the inverted CAAT sequence in the Y box in induced expression of *ELA-DRB* genes, for better understanding of the mechanisms of *DRB* expression in horses.

In conclusion, we have shown that the equine URR of *DRB* genes is highly conserved and is composed of the conserved motifs observed on the MHC class II gene promoters. In addition, we reported the existence, in some critical consensus boxes, of nucleotide polymorphism that is crucial for the coordinate control of *DRB* gene expression in horses and, thus, in the regulation of the levels of expression of the different *DRB* genes. In addition, phylogenetic comparisons, identity analysis and sequence organization supported that the reported sequences would correspond to an expressed *ELA-DRB* loci. As additional horse *DRB* URR sequences become available, it may be possible to identify locus-specific sequences that will aid in assigning URR alleles to specific loci.

Variability in the promoters would probably enhance the diversity of the coding region, having the potential for differential expression of class II proteins. Further studies are needed to determine the molecular mechanisms influencing *DRB* gene expression in horses. Such investigations would allow us to achieve a better understanding of the regulation of the immune response in normal as well as in pathological conditions, giving additional tools for the complete knowledge of the mechanisms underlying the complexity of the *ELA* class II region genes.

Acknowledgements

The authors thank Dr D. G. Fraser for comments on the manuscript. We thank Dr José Luis Vega-Pla for providing the SP and SN DNA samples. This work was supported by grant PICT 99 No.0805216 from the Agencia de Promoción Científica y Tecnológica de Argentina (SeCyT). S. Díaz is a fellow from CONICET.

References

Andersen, L.C., Beaty, J.S., Nettles, J.W., Seyfried, C.E., Nepom, G.T. & Nepoom, B.S. (1991) Allelic polymorphism in transcriptional regulatory regions of HLA-DQB genes. *Journal of Experimental Medicine*, **173**, 181.

Ansari, H.A., Hediger, R., Fries, R. & Stranzinger, G. (1988) Chromosomal localization of the major histocompatibility complex of the horse (ELA) by *in situ* hybridization. *Immunogenetics*, **28**, 362.

Auffray, C., Lillie, J.W., Korman, A.J., Boss, J.M., Frechin, N., Guillemot, F., Cooper, J., Mulligan, R.C. & Strominger, J.L. (1987) Structure and expression of HLA-DQα and DXα genes: interallelic alternate splicing of the HLA-DQα gene and functional splicing of the HLA-DXα gene using a retroviral vector. *Immunogenetics*, **26**, 63.

Benoist, C. & Mathis, D. (1990) Regulation of major histocompatibility complex class II genes: X, Y and another letters of the alphabet. *Annual Review of Immunology*, **8**, 681.

Bidler, L.L., Hilliard, P.R. & Rill, R.L. (1982) Ultrasensitive staining of nucleic acids with silver. *Analytical Biochemistry*, **126**, 374.

Del Pozzo, G., Perfetto, C., Ombra, M.N., Ding, G.Z., Guardiola, J. & Maffei, A. (1992) DNA polymorphisms in the 5′-flanking region of the HLA-DQA1 gene. *Immunogenetics*, **35**, 176.

Díaz, S., Giovambattista, G., Dulout, F. & Peral-García, P. (2001) Genetic variation of the second exon of the ELA-DRB genes in Argentine Creole horses. *Animal Genetics*, **32**, 257.

Fraser, D.G. & Bailey, E. (1996) Demonstration of three DRB loci in a domestic horse family. *Immunogenetics*, **44**, 441.

Glimcher, L.H. & Kara, C.J. (1992) Sequences and factors: a guide to MHC class II transcription. *Annual Review of Immunology*, **10**, 13.

Hake, S.B., Tobin, H.M., Steimle, V. & Denzin, L. (2003) Comparison of the transcriptional regulation of classical and non-classical MHC class II genes. *European Journal of Immunology*, **33**, 2361.

Harton, J.A. & Ting, J.P. (2000) Class II transactivator: mastering the art of major histocompatibility complex expression. *Molecular and Cell Biology*, **20**, 6185.

Hedrick, P.W., Parker, K.M., Miller, E.L. & Miller, P.S. (1999) Major histocompatibility complex variation in the endangered Przewalski's horse. *Genetics*, **152**, 1701.

Heinemeyer, T., Chen, X., Karas, H., Kel, A.E., Kel, O.V., Liebich, I., Meinhardt, T., Reuter, I., Schacherer, F. & Wingender, E. (1999) Expanding the TRANSFAC database towards an expert system of regulatory molecular mechanisms. *Nucleic Acids Research*, **27**, 318.

Janitz, M., Mitchison, A., Reiners-Schramm, L. & Lauster, R. (1997) Polymorphic MHC class II promoters exhibit distinct expression pattern in various antigen-presenting cell lines. *Tissue Antigens*, **49**, 99.

Jukes, T.H. & Cantor, C.R. (1969) Evolution of protein molecules. In: *Mammalian Protein Metabolism* (ed. H. N. Munro), p. 21. Academic Press, New York.

Kappes, D. & Strominger, J.L. (1988) Human class II major histocompatibility complex genes and proteins. *Annual Review of Biochemistry*, **57**, 991.

Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, **16**, 111.

Kumar, S., Tamura, K. & Nei, M. (1994) MEGA: Molecular evolutionary genetics analysis software for microcomputers. *Computer Applications in the Biosciences*, **10**, 189.

Louis, P., Eliaou, J.-F., Kerlan-Candon, S., Pinet, V., Vincent, R. & Clot, J. (1993) Polymorphism in the regulatory regions of HLA-DRB genes correlating with haplotype evolution. *Immunogenetics*, **38**, 21.

Louis, P., Pinet, V., Cavadore, P., Kerlan-Candon, S., Clot, J. & Eliaou, J.-F. (1994) Differential expression of HLA-DRB genes according to the polymorphism of their regulatory region. *CR Academy of Science Paris*, **137**, 161.

Måkinen, A., Chowdhary, B., Mahdy, E., Andersson, L. & Gustavsson, I. (1989) Localization of the equine major histocompatibility complex (ELA) to chromosome 20 by *in situ* hybridization. *Hereditas*, **110**, 93.

Mitchinson, N. & Roes, J. (2002) Patterned variation in murine MHC promoters. *Proceedings of the National Academy of Sciences of the USA*, **99**, 10561.

Perfetto, C., Zacheis, M., McDaid, D., Meador, J.W. & Schwartz, B.D. (1993) Polymorphism in the promoter region of HLA-DRB genes. *Human Immunology*, **36**, 27.

Peterlin, M.B., Anderson, G., Lotscher, E. & Tsang, S. (1990) Transcriptional regulation of HLA clas II genes. *Immunological Research*, **9**, 164.

Pinet, V., Eliau, J.F., Clot, J. (1991) Description of polymorphism in the regulatory region of the HLA-DRA gene. *Human Immunology*, **32**, 162.

Reichstetter, S., Krellner, P., Meenzen, C., Kalden, J. & Wassmuth, R. (1994) Comparative analysis of sequence variability in the upstream regulatory region of the HLA-DQB1 gen. *Immunogenetics*, **39**, 207.

Reith, W., Steimle, V. & Mach, B. (1995) Molecular defects in the bare lymphocyte syndrome and regulation of MHC class II genes. *Immunology Today*, **16**, 539.

Ripoli, M.V., Díaz, S., Peral-García, P. & Giovambattista, G. (2002) Nucleotide sequence of the upstream regulatory region of BoLA-DRB. *European Journal of Immunogenetics*, **29**, 537.

Ripoli, M.V., Peral-García, P., Dulout, F.N., & Giovambattista, G. (2004) Polymorphism in the bovine BoLA-DRB3 upstream regulatory regions detected through PCR-SSSP and DNA sequencing. *Gene*, **339**, 71.

Schneider, S., Roessli, D. & Excoffier, L. (1997) *ARLEQUIN: A Software for Population Genetics Data Analysis* Version 2.000. University of Geneva, Geneva, Switzerland.

Shahmuridov, K.A., Kolchanov, N.A., Solovyev, V.V. & Ratner, V.A. (1986) Enhancer-like structures in middle repetitive sequences of the eukaryotic genomes. *Genetics*, **22**, 357.

Sindwani, S. & Singal, D.P. (2001) Polymorphism in the Y box controls level of cytokine-mediated expression of HLA-DRB1 genes. *Tissue Antigens*, **58**, 315.

Singal, D.P. & Qiu, X. (1994) Polymorphism in the upstream regulatory regions and level of expression of HLA-DRB genes. *Molecular Immunology*, **31**, 1117.

Singal, D.P. & Qiu, X. (1996) Polymorphism in both X and Y box motifs controls level of expression of HLA-DRB1 genes. *Immunogenetics*, **43**, 50.

Singal, D.P., Qiu, X., D'Souza, M. & Sood, S.K. (1993) Polymorphism in the upstream regulatory regions of HLA-DRB genes. *Immunogenetics*, **37**, 143.

Solovyev, V.V. (2002) Structure, properties and computer identification of eukaryotic genes. In: *Bioinformatics from Genomes to Drugs V 1. Basic Technologies* (ed. T. Lengauer), p. 59. World Scientific Co., London.

Solovyev, V.V. & Kolchanov, N.A. (1994) Search for functional sites using consensus. In: *Computer Analysis of Genetic Macromolecules* (eds N.A. Kolchanov & H.A. Lim), p. 16. John Wiley & Sons Inc., Hoboken.

Thompson, J.D., Higgins, D.G. & Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, **22**, 4673.

Ting, J.P.Y. & Baldwin, A.S. (1993) Regulation of MHC gene expression. *Current Opinions in Immunology*, **5**, 8.

Ting, J.P.Y. & Trowsdale, J. (2002) Genetic control of MHC class II expression. *Cell*, **109**, 21.

Turco, E., Manfras, B.J., Ge, L., Rudert, W.A. & Trucco, M. (1990) The X boxes from promoters of HLA class II B genes at different loci do not compete for nuclear protein-specific binding. *Immunogenetics*, **32**, 117.