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ORIGINAL PAPER

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Demonstration of three *DRB* loci in a domestic horse family

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Abstract Single-strand conformational polymorphism (SSCP) gel electrophoresis and DNA sequencing were used to characterize the second exon of the horse *DRB* homologue as well as to identify eight new *DRB* alleles. The SSCP gels presented a complex pattern, with phenotypes exhibiting between 4 and 13 bands. The *DRB* SSCP patterns were studied for two families (6 to 13 bands per pattern). For both families, the patterns showed simple Mendelian inheritance. The polymerase chain reaction products from two individuals possessing homozygous major histocompatibility complex (*MHC*) alleles by descent were cloned and retested on SSCP gels. All bands derived from the genomic DNA amplification could be accounted for with bands derived from the cloned DNA amplification products. The results were consistent with three *DRB* loci, though this number may be variable within the domestic horse population. Gene sequences were variable among the different products, and we were unable to assign locus designations for particular sequences. Amplification of cDNA library material derived from one of the individuals who is *MHC* homozygous by descent showed an SSCP profile suggesting that all three *DRB* loci are transcribed into mRNA.

Introduction

The major histocompatibility complex (*MHC*) is of primary importance in understanding the immune response. Consequently, we are investigating *MHC* genes in horses. Research in other species has demonstrated that *MHC* class II genes encode the alpha and beta subunits of dimeric proteins which present soluble peptide antigens to $CD4^+$ T cells. Most class II genes show extensive genetic variation for both number of loci and number of alleles among and within species. For example, in humans, *HLA-DRB* genes are assigned to nine different loci. Four of these loci encode expressed *DRB* chain-encoding genes while five are pseudogenes. Variation in the organization of the *DRB* loci among humans exists. Different haplotypes may contain from two to five *DRB* loci, though only one or two are expressed in an individual. Human *DRB1* appears to be common to most haplotypes, but other loci may be contained in only a few haplotype groups (Andersson et al. 1994). Likewise, three *DRB* loci are defined in cattle: *DRB3* is well expressed, *DRB2* is expressed at very low levels by cattle leukocytes (Burke et al. 1991), and *DRB1* is a pseudogene (Muggli-Cockett and Stone 1988). Pigs appear to have three *DRB* loci, one which is expressed and two which are pseudogenes (Brunsberg et al. 1996).

In addition to their role in antigen presentation, *DRB* alleles are of interest because they are associated with a number of diseases. These include susceptibility to the human autoimmune dermatologic disease pemphigus vulgaris (Scharf et al. 1988), protection against collagen-induced arthritis in mice (Gonzalez-Gay et al. 1995), and resistance of cattle to persistent lymphocytosis caused by the bovine leukemia virus (Xu et al. 1993).

To date, three sequences have been reported for the horse *DRB* locus, and are designated *DRB*1, *2*, and **3* (Gustafsson and Andersson 1994). Our current investigation uses single-strand conformational polymorphism (SSCP) analysis and DNA sequencing to identify additional *DRB* alleles as well as to determine the number of *DRB* loci present and transcribed in the horse.

The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database and have been assigned the accession numbers L76972–L76978 and L77079

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Materials and methods

Homozygous offspring

Three true *MHC* homozygous offspring were produced through father-daughter matings using a single stallion. The two offspring reported

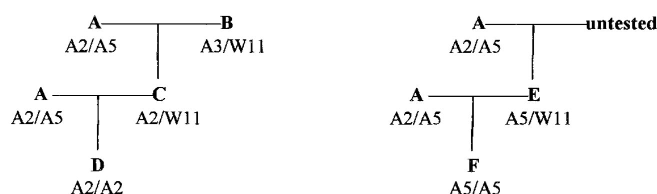

Fig. 1 Pedigrees of families producing *MHC* homozygous offspring by descent. *MHC* class I serological type for the *ELA-A* locus is shown *beneath* the individual's letter designation. Individual A is the sire, and individuals D and F are the homozygous offspring

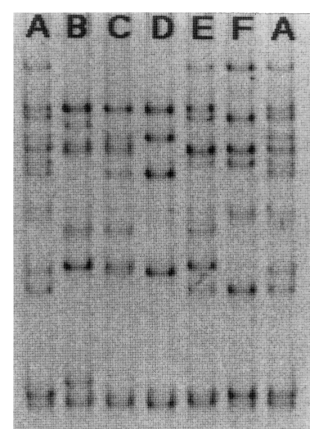

Fig. 2 *DRB* SSCP patterns from genomic DNA amplifications of family samples shown in *reverse contrast* to the ethidium bromide stained gel. Lane A (repeated) contains DNA from the sire, lane B contains DNA from a dam, lanes C and E contain DNA from F_1 females, and lanes D and F contain DNA from F_2 *MHC* homozygotes by descent

here each possessed a different *MHC* haplotype derived from the sire. Homozygosity at the *MHC* was verified by class I serological typing, Southern blot studies of class I and class II genes, and mixed lymphocyte reactivity (class II dependent) against the sire and other offspring (unpublished data), as well as SSCP typing of the *DRA* locus (Albright-Fraser et al. 1996).

DNA isolation

All genomic DNA used for this study was organically extracted from peripheral blood lymphocytes and then ethanol precipitated. The cDNA library in λgt10 bacteriophage was derived from peripheral blood cell RNA of individual D (see Figure 1) as previously described (Albright et al. 1991). Approximately 10^8 PFU of cDNA library material was organically extracted and ethanol precipitated to yield purified phage DNA template for amplification.

Primers

DRB second exon primers used for initial SSCP analysis, and for generating PCR products used in cloning were designed from a combination of cattle flanking intron and/or second exon sequence (Sigurdardóttir et al. 1991), and conserved bases at the 5′ and 3′ ends of three reported horse *DRB* second exon sequences (Gustafsson and Andersson 1994). These primers were designated DRB2a (5′-CTCTGCAGCACATTTCCTGGAG) and DRB2b (5′-CGCCGCTGCACCAGGAA). Primers DRB2a-i (5′-CGAGTGT-CATTTCTYCAACGGRAC) and DRB3 (5′-TCACAGAGCAGAC-CAGGAGGTT) were both used to amplify the cDNA library material. DRB2a-i is a 5′ exon 2 internal primer based on our horse sequences. DRB3 was designed from a consensus of human (Cairns et al. 1985), cattle (Groenen et al. 1990), and mouse (Braunstein et al. 1986) *DRB* exon 3 sequences.

PCR and SSCP

Amplification conditions were identical for all primer sets used, and were as previously reported (Albright-Fraser et al. 1996). When amplifying *DRB* clones or extracted cDNA library material, template DNA quantity was lowered from 50 ng to 5 ng per reaction. SSCP conditions were as previously reported with the following modifications: the gels contained no glycerol, and the run parameters were 18 h at 100 volts at 20 °C (Albright-Fraser et al. 1996).

Cloning

DRB amplification reactions, using the primer set DRB2a and DRB2b, were cloned by the TA-method into ddT-tailed Bluescript SK$^+$ plasmid (Stratagene, LaJolla, CA) as previously described (Holton and Graham 1991). Inserts from positive clones (white on LB, ampicillin, X-gal, IPTG plates) were confirmed by direct amplification of 1 μl of an inoculated 5 ml overnight liquid culture using the DRB2a and DRB2b primers. Clones for sequencing were chosen by running the amplification reactions on an SSCP gel. Only those clones which produced SSCP bands also seen in that individual's genomic SSCP pattern were picked for sequencing. Plasmid DNA was prepared from 1.5 ml of overnight culture by the CTAB mini-prep method (Del Sal et al. 1988). Purified plasmid DNA was resuspended in 9 μl of sterile distilled water, and frozen at –20 °C until sequenced.

DNA sequencing

DNA sequencing was done on an Applied Biosystems 377 automated sequencer (Division of Perkin-Elmer, Foster City, CA) using their PRISM ready reaction dye-terminator kit according to the manufacturer's suggested protocol. Three clones of each SSCP pattern were sequenced in both the forward and reverse directions using M13 (–47) forward and (–48) reverse primers (New England Biolabs, Beverly, MA). Sequence was accepted if at least four of six reactions produced identical results at a given base. Sequence was identical in all six replicates in most cases. Because of poor cloning efficiency, only two clones were sequenced for *DRB*10* and only one clone for *DRB*11*.

Results

DRB SSCP patterns

SSCP gels testing the 276 base pair (bp) amplification product of *DRB* second exon primers DRB2a and DRB2b resulted in fragment patterns containing as many as 13 distinct bands as resolved by our gel conditions. In families, these *DRB* fragments were inherited in a simple Mendelian fashion as shown in Figure 2. Family relationships are shown schematically in Figure 1. Individual A is the sire, and individuals D and F are the *MHC* homozygous offspring by descent. All bands present in the sire's *DRB* SSCP profile can be accounted for in the profiles of offspring D and F. The SSCP band patterns in each offspring were, with one exception, mutually exclusive, and offspring F inherited two more bands than offspring D by total count.

DRB genomic amplification reactions from sire A and offspring D and F, as well as an unrelated individual (X), were each cloned into plasmid vectors. Both D and F possessed three unique clonal patterns by SSCP analysis, and the summation of bands in the three patterns accounted for all bands seen in the genomic amplification (Fig. 3).

Fig. 3 *DRB* SSCP patterns from genomic DNA amplifications of individuals A, D, and F, and from cloned DNA amplifications of individuals D and F (denoted by D1, F1, etc.) shown in *reverse contrast* to the ethidium bromide stained gel

DRB exon 2 sequences

DRB consensus sequences, based on individual clone sequences, are presented in Figure 4. *DRB*1, *2*, and *3*, are those sequences previously reported by Gustafsson and Andersson (1994). *DRB*4, *5*, and *6* are the three sequences from offspring D. *DRB*7, *8*, and *9* are the three sequences from offspring F. *DRB*10* and *11* are sequences from individual X. The consensus sequence for *DRB*9* also included a clonal sequence from the sire (A) to verify that identical SSCP patterns in sire and offspring clones indeed indicated identical DNA sequence. The predicted amino acid sequences are shown in Figure 5. Most base substitutions were non-synonymous, thus resulting in an amino acid change. Only *DRB*5* contained a premature stop codon (#). The question marks in *DRB*10* and *DRB*11* indicate an unresolved base sequence at that site which differs in its predicted amino acid residue. Underlined residues are those which are thought to contact antigen (Brown et al. 1988). Among all of the predicted amino acid sequences reported, 36 of 83 sites (43%) were variable. For residues thought to contact antigen, 14 of 16 sites (87.5%) were variable. For non-contact residues, 22 of 67 sites (33%) were variable.

Fig. 4 *DRB* exon 2 DNA sequences. Identity to *DRB*1* is shown by a *dash*. GenBank accession numbers are as follows: *DRB*1* (L25644), *DRB*2* (L25645), *DRB*3* (L25646), *DRB*4* (L77079), *DRB*5* (L76978), *DRB*6* (L76972), *DRB*7* (L76973), *DRB*8* (L76975), *DRB*9* (L76974), *DRB*10* (L76976), and *DRB*11* (L76977)

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                                                                                               64
DRB*1       G GAG TAT AGT ACG TCC GAG TGT CAT TTC TTC AAC GGA ACA GAG CGA GTG CGG TAC TTG GAC AGA
DRB*2       - --- GC- GTG -A- -T- --- --- -G- --- -C- --- --G --T --- --- --- --- -T- C-- --G ---
DRB*3       - --- CTG GT- -AA CA- --- --- --- --- -C- --- --G --T C-- --G --- --- -T- C-- --- ---
DRB*4 (D1)        --- -C- --- --- --- --- --- --- -C- --- --G --T C-- --G --- --- -T- C-- --- ---
DRB*5 (D2)        --- --- --- --- --- --- --- --- -C- --- --G --T C-- --- --- --- -T- C-- --- ---
DRB*6 (D3)        --- --- --C -T- --- --- --- --- -C- --- --G --T C-- --G --- -TA --- --- C-- ---
DRB*7 (F1)        CTG GT- -AA CA- --- --- --- --- -C- --- --G --T C-- --G --- --- -T- C-- --- ---
DRB*8 (F2)        CTG GT- -AA CA- --- --- --- --- -C- --- --G --T C-- --G --- --- -T- C-- --- ---
DRB*9 (F3)        --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DRB*10            CTG GT- -AA CA- --- --- --- --- -C- --T --G --G --- A-G --- -R- --T G-A TT- ---
DRB*11            --- --- --C -T- --- --- --- --- -C- --- --G --T C-- --G --- -TA --- --- C-- ---

                                                                                            127
DRB*1       TAC TTC TAT AAC GGG AAG GAG TAC GTG CGC TTC GAC AGC GAC GTG GGC GAG TAC CGG GCG CTG
DRB*2       CG- --- C-- --- --- G-- --- --- -C- --- --- --- --- --- --- --G --- --- --- --- G--
DRB*3       --- --- --- --- A-- G-- --- --- --- --- --- --- --- --- --- --G --- --- --- --- G--
DRB*4 (D1)  --- --- --- --- --- --- --- AC- --- --- --- --- --- --- --- --G --- --- --- --- G--
DRB*5 (D2)  --- --- -C- --- --- G-- --- AC- --- --- --- --- --- --- --- --G --- --- --- --- G--
DRB*6 (D3)  CT- --- --- --- --- --- --- --- --- --- --- --- --- --- C-- --- --- --- --- --- ---
DRB*7 (F1)  --- --- --- --- A-- G-- --- --- --- --- --- --- --- --- --- --G --- --- --- --- G--
DRB*8 (F2)  --- --- C-- --- --- --- --- -T- --- --- --- --- --- --- --- --G --- -T- --- --- G--
DRB*9 (F3)  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DRB*10      G-- G-- --C --- C-- G-- --- C-- --- --- --- --- --- --- --- --G --- -T- --- --- G--
DRB*11      CT- --- --- --- --- --- --- --- --- --- --- --- --- --- C-- --- --- --- --- --- ---

                                                                                            190
DRB*1       ACC GAG CTG GGG CGG CCG GAC GCC GAG TAC TGG AAC GGG CAG CAG GAC ATC CTG GAG CAG AAG
DRB*2       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --T --A CG-
DRB*3       --- A-- --- --- --- A-- --- --- --- --- -A- --- --- --- A-- --- G-- --- --T G-C GC-
DRB*4 (D1)  --- --- --- --- --- --- --- --- --- --- --- --- --- --- A-- --- T-- --- --T G-C GC-
DRB*5 (D2)  --- --- --- --- --- --- --- --- --- --- --- --- --- --- A-- --- G-- --- --T G-C GC-
DRB*6 (D3)  --- --- --- --- --- -G- AG- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DRB*7 (F1)  --- --- --- --- --- --- --- --- --- --- --- --- --- --- A-- --- G-- --- --T G-C GC-
DRB*8 (F2)  --- --- --- --- --- --- --- --- --- --- --- --- --- --- A-- --- G-- --- --T G-C GC-
DRB*9 (F3)  --- --- --- --- --- -G- AG- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DRB*10      --- --- --- --- --- --- --- --- --- --- --- --- --- --- A-- --- G-- --- --- -G- ---
DRB*11      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                                                                                        250
DRB*1       CGG GCC AAG GTG GAC ACG TAC TGC AGA CAC AAC TAC GCC GTC AGC GAG AGC TTC CTG GTG
DRB*2       --- --A G-- --- --- --- --- --- --- --- --- --- -GG --T -TT --T G-- --- --- ---
DRB*3       --- --- G-- --- --- --- --- --- --- --- --- --- -G- --- -CG --C -CG --- --- ---
DRB*4 (D1)  --- --- GC- --- --- --- -TG --- --G --- --- --- -G- A-- --- --- ---
DRB*5 (D2)  --- --- GC- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DRB*6 (D3)  --- --G G-- --- --- --- GTG --- --G --- --- --- -G- --- --- --- ---
DRB*7 (F1)  --- --- GC- --- --- --- --- --- --- --- --- --- -G- --- --- --C ---
DRB*8 (F2)  --- --- C-- --- --- --- --- --- --- --- --- --- -G- A-- --- --C ---
DRB*9 (F3)  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DRB*10      --- --- GC- --- --- --- --- --- --- --- --- --- -GS --- CTT --C -A-
DRB*11      --- --G G-- --- --- --- GTG --- --- --- --- --- -S- --- --- --- ---
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                       10         20         30         40         50         60         70         80
DRB*1       EYSTSECHFF NGTERVRYLD RYFYNGKEYV RFDSDVGEYR ALTELGRPDA EYWNGQQDIL EQKRAKVDTY CRHNYAVSES FLV
DRB*2       -AVKF--R-S -------F-E -R-H--E--A ---------- -V-------- ---------- D-R--E---- -----G-IDG ---
DRB*3       -LVKH----S ---Q---F-- -----RE--- ---------- -V-K---T-- --#---K-V- DDA--E---- -----G-TDT ---
DRB*4 (D1)   -T------S ---Q---F-- --------T- ---------- -V-------- ------K-F- DDA--A---L -----GI---
DRB*5 (D2)   --------S ---Q---F-- ---S--E-T- ---------- -V-------- ------K-V- DDA--A---- ----------
DRB*6 (D3)   ---F----S ---Q--L--H -L-------- -----L---- -------RS- ---------- -----E---V -----G----
DRB*7 (F1)   LVKH----S ---Q---F-- -----RE--- ---------- -V-------- ------K-V- DDA--A---- -----G--D-
DRB*8 (F2)   LVKH----S ---Q---F-- ---H----F- --------F- -V-------- ------K-V- DDA--Q---- -----GI-D-
DRB*9 (F3)   --------- ---------- ---------- ---------- -------RS- ---------- ---------- ----------
DRB*10       LVKH----S ------x-VF -DV--RE-H- --------F- -V-------- ------K-V- -R---A---- -----G-LDN
DRB*11       ---F----S ---Q--L--H -L-------- -----L---- ---------- ---------- -----E---V -----x----
```

Fig. 5 Predicted amino acid sequence of *DRB* exon 2 alleles. Identity to *DRB*1* is shown by a dash. A predicted termination codon in *DRB*3* is shown by a '#'. The x in sequences *DRB*10* and *DRB*11* indicate an unresolved predicted amino acid sequence due to DNA sequence ambiguity. *Underlined* residues are those which are thought to contact antigen (Brown et al. 1988)

DRB transcription

To determine which *DRB* sequences are transcribed, primers DRB2a-i and DRB2b were used to amplify phage DNA extracted from a cDNA library derived from leukocytes of individual D. This primer pair was also used to amplify clones D1, D2, and D3 (which contain only *DRB* exon 2 sequence), as well as genomic DNA from individual D. On SSCP analysis, the fragment patterns of the cloned DNA once again added up to the fragment pattern seen with genomic DNA amplifications. Amplifications from cDNA library material showed an identical pattern to the genomic DNA pattern, suggesting that all three *DRB* sequences are present in the library (data not shown). To control for genomic DNA contamination, the cDNA library material was also amplified with the primer pair DRB2a-i and DRB3. Only 320 bp fragments were observed which is the length expected from a fully spliced transcript assuming conserved exon lengths between the horse and other mammalian species.

Discussion

Although *DRB* polymorphism has been examined in many species, it has been difficult to determine the number of *DRB* loci, and to assign sequences to allelic groups. Our use of individuals who are homozygous by descent at the *MHC* allowed us to address these questions. The different clonal patterns within offspring D and F, as seen by SSCP, are assumed to represent products of distinct *DRB* loci, since these offspring are homozygous for the *MHC*. Therefore, discovery of three clonal patterns for each haplotype is consistent with three *DRB* loci in these individuals.

Gauging by the number of bands seen in SSCP patterns for most horses tested (6 to 13), three loci may be the usual number present, but a few samples showed fewer bands using *DRB* SSCP analysis. One of these individuals, an Andalusian stallion (X), consistently showed only four bands regardless of the SSCP gel conditions used. Only two sequences were found in cloned *DRB* amplification products from this stallion, and their SSCP fragment patterns accounted for all the bands seen in the genomic DNA amplification. Because of poor cloning efficiency with this sample, we cannot rule out the possibility that more sequences exist which happen to have identical SSCP patterns. However, this seems unlikely, since altering SSCP gel conditions (run temperatures and addition of glycerol to some gels) resulted in remarkably different SSCP patterns for every other individual tested. The gel condition we chose for these studies was the one which gave us the maximum number of fragments in all samples screened. It is also possible that our primer pair DRB2a and DRB2b did not amplify some *DRB* alleles. However, sequence data from other equid species and rhinoceros show complete conservation across the perissodactyls for the nine bases on the 3′ end of primer DRB2b and the four bases on the 3′ end of primer DRB2a (M. Breen, personal communication).

The three *DRB* sequences present in offsprint D are presumably allelic to the three *DRB* sequences present in offspring F, but no conclusions could be made as to which sequences belong to allelic pairs. In both offspring, two of their three alleles are more similar to one another than the third, but the similar pairs are quite different between offspring. In individual X, the two sequences present show little similarity, but we do not know whether these are alleles of a single locus (heterozygote) or two different loci (homozygote). Interestingly, a similar observation was made for horse class I gene sequences. Ellis and co-workers (1995) sequenced *MHC* class I genes from a horse cDNA library. Seven unique full-length sequences were identified, indicating at least four class I loci are transcribed in the horse, but cladistic analysis revealed no clear relationships between the sequences and allelic pairs could not be discerned. Therefore, a common mode of evolution may be acting on the horse *MHC* as a whole, using parallel locus duplication as a precursor to locus diversification.

Variant sequences due to PCR artifacts resulting from heteroduplex formation have been reported when amplifying *DRB* loci in humans (L'Abbé et al. 1992). These artifacts are generally a small proportion of the total products, so we would not expect to observe them on our genomic-derived SSCP patterns. Several clones produced aberrant SSCP patterns when compared with those observed using genomic DNA templates. These clones may have contained sequence artifacts from heteroduplex formation but this was not verified, since we sequenced only those clones which, when amplified, produced SSCP bands seen in the genomic amplification of that individual. We did encounter evidence for heteroduplex artifacts in one of the

clones we sequenced (data not reported). This particular clone, by chance, gave an SSCP pattern indistinguishable from one of the alleles under our gel conditions.

All three *DRB* loci present in offspring D appear to be transcribed into mRNA as indicated by SSCP analysis of cDNA library amplifications. We cannot completely exclude the possibility of genomic DNA contamination in our cDNA library. However, the amplifications from the library material were unlikely to be contaminants for two reasons. First, the SSCP patterns perfectly matched genomic DNA amplifications of the individual from which the library was made. Since all horses initially screened with our *DRB* primers had distinct SSCP patterns, it is unlikely that DNA from a different individual was a contaminant. Second, a primer was made using a consensus sequence to the third exon of *DRB* (DRB3). Amplification of the cDNA library material with primers DRB2a-i and DRB3 produced only 320 bp fragments, the length expected from a fully spliced transcript. No amplification was seen in our genomic DNA samples, but this is probably due to the limitations of our thermocycling protocol for amplifying large DNA fragments. The length of the second intron of *DRB* in cattle is variable, with lengths of around 3 kilobases (kb) and greater than 6 kb reported (Groenen et al. 1990), so a similar size intron in the horse would prevent amplification with this primer set under our current conditions. Since we do not have full-length mRNA sequence generated from each *DRB* locus, we do not know whether one or more of the three transcripts is the product of a pseudogene. Which of these transcripts result in expressed, functional cell surface proteins remains undetermined.

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