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Genomic characterization of MHC class I genes of the horse

Abstract The availability of a contig of bacterial artificial chromosome (BAC) clones spanning the equine major histocompatibility complex (MHC) made possible a detailed analysis of horse MHC class I genes. Prior to this study, only a single horse MHC class I gene had been sequenced at the genomic level. Although many (~60) MHC class I cDNA sequences had been determined and published, from this information, it was not possible to determine how many class I loci are expressed in horses or to assign individual sequences to allelic series. In this study, 15 MHC class I genes were identified in BAC subclones and fully sequenced. Because the BAC library donor horse had been bred for homozygosity at the MHC, these 15 genomic clones represent distinct MHC class I genes and pseudogenes and not alleles at a smaller number of loci. For five of the genes, cDNA sequences from these loci had previously been identified. Two additional expressed class I genes were discovered, bringing the known total of different equine MHC class I genes (loci) expressed as mRNA to seven. Expression of all seven loci was detected by reverse transcriptase–polymerase chain reaction in adult, fetal, and placental tissues. The remaining eight genes were designated as pseudogenes. This work resulted in moderate expansion of the horse MHC BAC contig length, and the remaining gap was shortened. The information contained in these equine MHC class I sequences will permit comparison of MHC class I genes expressed across different horse MHC haplotypes and between horses and other mammalian species.

Keywords Horse · MHC class I gene · Genomic sequence · Noncoding regions

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Introduction

Major histocompatibility complex (MHC) class I molecules are membrane-bound glycoproteins that complex with a variety of peptides derived from intracellular degradation of endogenous and exogenous proteins. MHC class I molecules complexed with nonself peptides and β-2-microglobulin that are recognized by the T-cell receptor (TCR) on T lymphocytes initiate a specific immune response, thus providing immune surveillance against intracellular pathogens (Germain and Margulies 1993). MHC class I molecules also regulate innate immunity by serving as ligands for inhibitory receptors on natural killer (NK) cells, effecting inhibition of NK-mediated cytolytic activity (Lanier 1997).

Major histocompatibility complex class I molecules of humans and mice are often separated into two main classes: the classical, "Ia," and the nonclassical, "Ib." The nonclassical molecules are often distinguished by their shorter cytoplasmic tail and their low levels of polymorphism in the 5′ end (Shawar et al. 1994). The classical genes have many alleles: for *HLA-A*, 372; for *HLA-B*, 661; and for *HLA-C*, 190, whereas the nonclassical genes have fewer: for *HLA-E*, 5; for *HLA-F,* only 2; and for *HLA-G,* 15 (as of April 2005, http://www.ebi.ac.uk/imgt/hla/intro.html).

Early studies indicated the presence of at least 16, and up to 33, MHC class I genes in the equine genome (Vaiman et al. 1986; Alexander et al. 1987; Guerin et al. 1987). This estimate includes both expressed genes and pseudogenes, and the total number of bands varied between horses. The horse MHC was localized to horse chromosome 20q14–q22 by in situ hybridization (Ansari et al. 1988) and was confirmed to position 20q21 with fluorescent in situ

hybridization (FISH) of bacterial artificial chromosome (BAC) clones containing MHC class I genes (Gustafson et al. 2003).

Variation in horse MHC class I genes was initially assessed serologically rather than by molecular and sequence methods. Approximately 15 distinct specificities can be discerned, and the designated nomenclature identifies them as ELA-A1, ELA-A2, ELA-A3, ..., ELA-A19 (Lazary et al. 1988). These are considered to be markers of MHC haplotypes, implying a distinctive collection of inherited alleles.

Several studies have described horse MHC class I cDNA sequences from horses of different ELA haplotypes, based on either cDNA library screening or reverse transcriptase–polymerase chain reaction (RT-PCR) experiments (Barbis et al. 1994; Ellis et al. 1995; Chung et al. 2003; McGuire et al. 2003). The full-length MHC class I gene sequences reported here may provide the genomic equivalent of some of the cDNA sequences obtained by Ellis et al. (1995) from an ELA-A3/ELA-A7 pony because of the shared ELA-A3 haplotype. Ellis and coworkers reported seven expressed MHC class I transcripts from this MHC heterozygous pony. *ECMHCB1* through *ECMHCB4* are considered classical MHC class I genes, and the remaining three transcripts, *ECMHCA1*, *ECMHCC1*, and *ECMHCE1* are considered putative nonclassical genes (Holmes and Ellis 1999).

The present study was fueled by the combination of the genomic characterization of a classical MHC class I gene of the horse (Carpenter et al. 2001) and the construction of a BAC clone contig spanning the horse MHC (Gustafson et al. 2003). We present genomic sequence for 15 MHC class I genes of the horse from the ELA-A3 haplotype. Several correspond to previously published genes and provide a noncoding and flanking, extragenic sequence. The others are newly identified genes, some of which are expressed, and others which are pseudogenes. In total, we detected mRNA expression of seven distinct MHC class I genes from an MHC homozygous individual. Sequence variations in the promoter region regulatory elements are presented, comparing classical and nonclassical loci, as well as pseudogenes. This collection of data can be compared to MHC class I gene sequences from other equine MHC haplotypes and from other species.

Materials and methods

Animals, tissue samples, and cDNA preparation

The horses sampled for this study were from either the Equine Genetics Center herd at Cornell University or from local farms in the Ithaca, NY area. Peripheral blood lymphocytes were isolated from heparinized samples of venous jugular blood using methods described previously (Antczak et al. 1982). Conceptus tissues were recovered by nonsurgical uterine lavage from lightly sedated mares between days 33 and 34 of pregnancy. Conceptus tissues were dissected under a stereoscopic microscope (Nikon Instrument Group, Melville, NY) at 150X magnification

and snap-frozen in liquid nitrogen. RNA was isolated from lymphocytes and conceptus tissues following homogenization by QIAshredder (Qiagen, Valencia, CA) as directed by the RNeasy kit (Qiagen). One microgram of RNA was treated with DNase I (Invitrogen, Carlsbad, CA) to degrade any contaminating genomic DNA. cDNA synthesis reactions were primed with oligodT (PE Biosystems, Foster City, CA) and transcribed by Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, USB, Cleveland, OH).

Horse bacterial artificial chromosome library and screening

The CHORI-241 BAC library was constructed at the Children's Hospital Research Institute, Oakland, CA, using neutrophil DNA from a Thoroughbred stallion in the Cornell herd bred to be homozygous for known MHC region genes. The CHORI-241 horse BAC library was screened by hybridization with an MHC class I overgo probe as previously described (Gustafson et al. 2003). Additional overgo probes were generated from other genes in the MHC class I region, BAC end sequences, or subclones derived from BAC clones (see Table 1). The sequences were analyzed by RepeatMasker for the presence of repetitive elements (A.F.A. Smit and P. Green, unpublished data, http://repeatmasker.genome. washington.edu/cgi-bin/RepeatMasker). Overgoes were then designed from nonrepetitive sequence by Overgo Maker http://genome.wustl.edu/tools/?overgo=1-input). Overgoes were synthesized by Integrated DNA Technologies (Coralville, IA) http://www.idtdna.com).

Isolation of BAC DNA was performed using Plasmid Midi kits (Qiagen). For sequencing, 2 µg of BAC DNA was digested with *Bam*HI at 37°C overnight. The enzyme was heat-inactivated, and the BAC DNA was sequenced with 40 pmol of primer.

Southern blot hybridization

Bacterial artificial chromosome clones from the published equine MHC class I region (Gustafson et al. 2003) and additional BAC clones identified previously in this laboratory were subjected to Southern blot hybridization with an MHC class I probe to determine the number and location of MHC class I genes.

For Southern blot analysis, 2–5 µg BAC DNA was digested with *Eco*RI, *Bam*HI or *Hin*dIII restriction enzymes at 37°C overnight. The digested BAC DNA was run on a 1% Tris borate ethylenediaminetetraacetic acid (EDTA) (TBE) gel for 24–44 h at 45 V and then visualized. The BAC DNA was transferred to a nylon membrane (Hybond-N, Amersham Biosciences, Piscataway, NJ) in alkaline transfer buffer (1.5 M NaCl and 0.25 M NaOH) by capillary action overnight. The membrane was then neutralized (0.5 M EDTA, 1 M Na_2HPO_4, pH 7.4) and hybridized as described above.

Subcloning and sequencing of MHC class I genes from BAC clones

Restriction fragments containing horse MHC class I genes identified by Southern blot hybridization were selected for subcloning. Insert DNA was purified with the QIAEX II gel extraction kit (Qiagen). Ligation into a modified pCR2 vector was performed at a ten insert to one vector ratio with T4 Ligase (Invitrogen) and incubated overnight at 15°C. Transformation of 2–5 μl of ligation reaction into One Shot TOP10 chemically competent *Escherichia coli* was performed according to supplied instructions (Invitrogen). Colonies were grown at 37°C overnight and were screened by colony blot hybridization to identify those containing MHC class I genes. Methods were similar to those described above. Positive colonies were grown, and plasmids were purified with the QIAprep Spin MiniPrep Kit (Qiagen) and analyzed by restriction digest, PCR, or sequencing. Full sequencing was performed with universal vector primers M13F and M13R, MHC class I gene-specific primers, and primer walking.

Polymerase chain reaction amplification and sequencing

To determine mRNA expression for potentially expressed genes, locus-specific primers were designed (Table 1)

Table 1 Oligonucleotides used for hybridization and amplification

Oligonucleotide	Sequence (5–3′)		Product (bp)
Overgo probes	Forward	Reverse	
013K08T7	AAGATGCTGCTAGGACTGATGTCA	GAGGCAGTACACTCTTTGACATCA	
024A136-1	CACTGCATGTCACAGACAGAGTTC	CCTAGCAGAAGTGTGAGAACTCTG	
024A13SP6	ACTGAAGCAGGAGAGTTCTGGTTG	CCATTCCTCAAGGAGACAACCAGA	
024A13T7	TTCCCATGGATGTGCTTGGGGATG	ACTGGAGCTCCAACCCCATCCCCA	
101I16SP6	GAATTCCAAGTGGTATCTCTCATG	CTCTTGCATCTTTGTTCATGAGAG	
157M01SP6	CAAATGTGAATGTTGTTTGACTAC	TGTGCATTTGCTAGGAGTAGTCAA	
190H04SP6	AGGTAATCCGCCGCCAGGAGCTT	AATCCAGAACAAAGGGAAGCTCCT	
212E21SP6	AGCTCTGCCTTCCTTTGGAAGCTA	ATGGGCTCTGTCAGTATAGCTTCC	
220B06SP6	CTCTGGTGAATGGCATTCTGACTG	GTTGCAAGTGCTCATGCAGTCAGA	
274K01SP6	TCCAAGGGAATCTGAGGTCCTGAT	GGGGCTTCATGCAAAAATCAGGAC	
345J21SP6	CTAGGGTTTTCAGGCACAAAGACC	CTCACAACCTGTGTCAGGTCTTTG	
360H03T7	TTAGTCTCCACCACCCTCTCACTC	TGGTGCCACAATTCAGGAGTGAGA	
394D17T7	TCTGGAGTCCTTAAATAACAGAGC	GTCCTCCGTAGTATCGGCTCTGTT	
454N08SP6	TAGAAACCACGTGTCTATCAACCT	GAAGTGGGTGAGCATTAGGTTGAT	
464F03SP6	GACCTGCAAACCTATGAGAGGTCA	TGGCTAATCAGCTGAGTGACCTCT	
499E16T7	TGCTTGTGCCTTGGAATCAAAGGC	AAGACATTCCCTTCCGGCCTTTGA	
519G06T7	CAGGTGCCCCAATATAATATGCAC	CTGACACCTAAGGGATGTGCATAT	
BAT1	Reported in Gustafson et al. 2003		
C1subcloneB	CACCACACACATGTACCTAATCAC	GAAGAGGGTGAAAGAGGTGATTAG	
E1subclone	TTTGGCTTGCTTCCCTCTACTAGA	TATCTCGTTTTCCTCCTCTAGTAG	
MHCclassIα1	GCTTCATCTCCGTCGGCTACGTGG	ACGAACTGCGTGTCGTCCACGTAG	
MHCclassIα3	TGGTGCCTTCTGGGGAGGAGCAGA	ACATGGCACGTGTATCTCTGCTCC	
MICA	Reported in Gustafson et al. 2003		
POU5F1	Reported in Gustafson et al. 2003		
TNFA	Reported in Gustafson et al. 2003		
PCR primer Locus-specific amplification			
3.1	GGGCCACGCACAGACTTT	CCTCCAGGTAGTTCCTGTGC	283
3.2	ACGCGGATCTACAAGGAAGC	CTCCAGGTAGTTCCTCCAGC	293
3.3	GTGGACGACACGCAGTTC	TCCACGCACGTGCCCTCT	416
3.4	TATTGGGAGATGAGCACAC	CAGGAAGTTCCTCCAGTC	306
3.5	GTGGACGACACGCAGTTC	CCTCTGGAGAGTGTGG	210
3.6	GAGTCCTGGTACCTCGAA	CAGTACTTCGCATTACCC	413
3.7	GAGGAGAACACGCAGACCA	CCAGGTAGGCCCTGTCTTG	301
3.8	CGAGAGTCCGAGGATGC	CGTCGTAGGCGAACTGAGTA	239
3.10	GTCTCACACCCTCCAGGAC	TGCCAGGACAGGCTGATG	381
3.11	CACAGAGTTTCCGAGTGG	CTGCCCTCCAGGTAGTTT	258

within the hypervariable region when possible and used in RT-PCR experiments. Oligonucleotide primers were designed with Primer3 on the Web at: http://www.genome.wi.mi.edu/cgi-bin/primer/prime3.cgi and synthesized by Integrated DNA Technologies. Amplification of 50 ng cDNA or genomic DNA was performed either with *Taq* (Invitrogen) or *Pfu* DNA polymerase (Stratagene, La Jolla, CA). Amplification with *Taq* DNA polymerase was performed in a Robocycler 96 (Stratagene) with the following conditions: 1 cycle at 95°C for 5 min, 33 cycles of 95°C for 30 seconds, 55°C for 30 s, and 72°C for 1 min, and 1 cycle

of 72°C for 10 min. Amplification with *Pfu* DNA polymerase was performed with the following conditions: 1 cycle at 95°C for 45 s, 33 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 2 min, and 1 cycle of 72°C for 10 min. Products were separated by electrophoresis on 1% agarose gels, stained with ethidium bromide, and visualized under a UV Transilluminator (UVP, Upland, CA).

Polymerase chain reaction products were either purified and directly sequenced or cloned. QIAquick kits (Qiagen) were used for PCR product purification. The pGEM-T Easy vector was used for TA-cloning *Taq* DNA polymerase
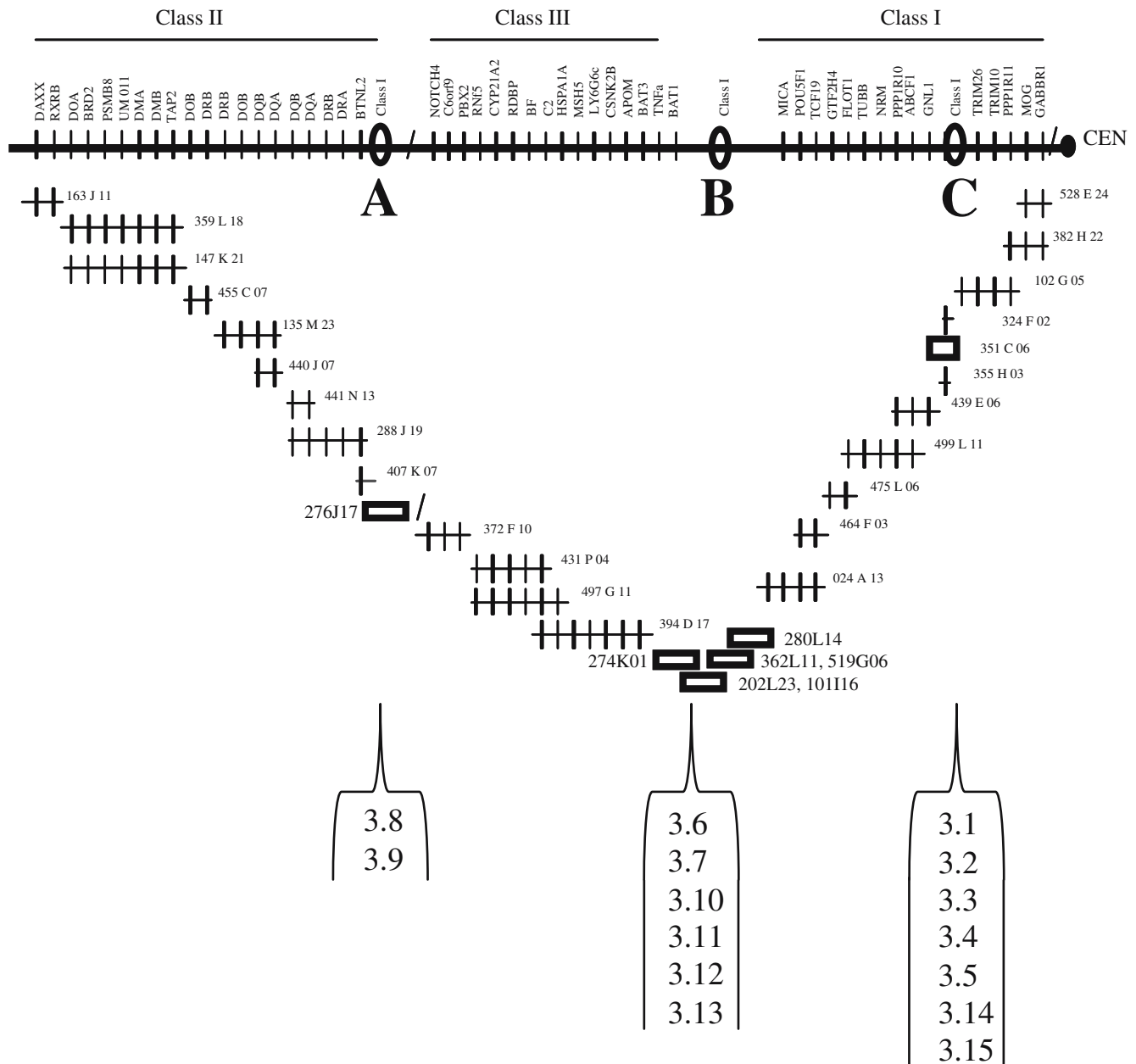
Fig. 1 Supplemented minimal tiling path of the BAC contig of the equine MHC. Genes are shown in *sequential order*, as determined by hybridization. MHC class I gene clusters are shown as *ovals* along the *top*, and new BAC clones are shown as *open boxes* in the contig. MHC class I gene subclones are listed under the corresponding cluster. Genes are spaced equally because distance between genes has not been determined. / indicates a gap between the MHC class II and III regions. Chromosomal orientation as determined by FISH is indicated by *CEN* (centromeric) at the MHC class I end. Refer to Table 2 for subclone information

products as described in the cloning kit (Promega, Madison, WI). The pCR 4Blunt-TOPO vector was used for cloning *Pfu* DNA polymerase products as described in the Zero Blunt TOPO PCR cloning kit (Invitrogen).

Sequencing was performed by the Biotechnology Resource Center at Cornell University, Ithaca, NY, with an ABI 3700 automated sequencer (PerkinElmer, Foster City, CA). Templates (PCR products or plasmids) were sequenced at least once in each direction. All sequences generated for this work have been submitted to GenBank.

Sequence analysis: subclone sequences, MHC class I genes, and RT-PCR products

Sequencing electropherograms were viewed with EditView 1.0.1 software from Applied Biosystems (Foster City, CA). Sequences were analyzed with VectorNTI (Invitrogen). Alignments and sequence identity tables were generated by MegAlign from the LaserGene package (DNASTAR, Inc., Madison, WI). Promoter elements were identified by comparison with the previously published horse *ELA-A2* gene promoter (Carpenter et al. 2001).

Fluorescent in situ hybridization

Metaphase chromosome preparation and FISH were performed as previously described (Lear et al. 1998).

Results

Physical map of MHC class I genes

Bacterial artificial chromosome (BAC) clones from the published equine MHC class I region contig (Gustafson et al. 2003) were digested with restriction enzymes and subjected to Southern blot hybridization with an MHC class I probe to define the location and restriction fragment size of the horse MHC class I genes. Fifteen horse MHC class I genes were identified in this way, and because the donor horse of the BAC library is an ELA-A3 MHC homozygote, these genes represent 15 distinct loci rather than alleles at a smaller number of loci.

Three regions containing MHC class I genes were found, designated A, B, and C, from left to right (Fig. 1). Region A is at the *BTNL2* end of the MHC class II contig and is contained within one BAC clone. This BAC clone was not included in the initial characterization of the horse MHC BAC contig but was mapped in close proximity to the *BTNL2* gene by BAC end-sequence hybridization in this study, extending into the gap described by Gustafson et al. (2003). Two MHC class I genes are located in region A.

Region B is at the junction of the classes III and I regions, between the *BAT1* and *MIC* genes, and is comprised of five overlapping BAC clones. Four of these clones close a previously undetected gap in the horse MHC contig. This was further supported by FISH mapping of BAC clones 41M02, 69N18, and 101I16, placing them all within the established MHC region (data not shown). Establishing this supplemental minimal tiling path was difficult because of the high prevalence of repetitive elements in BAC end sequences (54%) as identified by RepeatMasker analysis (data not shown). The major repetitive elements included long interspersed nucleotide elements (LINE), long terminal repeats (LTR), DNA/MER repeats, and short interspersed nucleotide elements (SINE). These repetitive elements are also present in the human MHC sequence, although the SINE families differ between species. Six MHC class I genes were found in this region.

Region C is in the class I region between the *GNL1* and *TRIM26* genes and spans four overlapping BAC clones. Seven MHC class I genes are located in this region.

Genomic structure of horse MHC class I genes

Sequences were obtained for all 15 of the identified horse MHC class I genes. MHC class I genes consist of eight

Fig. 2 Comparison of exon and intron lengths of horse MHC class I genes. Nucleotide length of exons, introns, and total genes listed. Expressed genes are listed on *top*, pseudogenes are listed *below*. Asterisk (*) indicates previously published cDNA sequence exists for this gene, *ND* indicates not detected, *NS* indicates not sequenced, and *Underline* indicates region was truncated in subclone

```
5'UTR, Promoter
        Leader      α 1      α 2          α 3      TM      CP      CP        3' UTR
                                                                                    Gene
                                                                                    length
Expressed
3.1*  64  317  270 230 276  610  276 134 105 418 33 146 45 162 5          3091
3.2*  73  346  270 229 276  615  276 123 105 421 33 135 45 162 5          3114
3.3   73  244  270 238 276  600  276 104 105 419 33 144 48 165 5          3000
3.4   73  319  270 238 276  623  276 123 105 422 33 143 43  NS      NS    2944
3.5*  61  324  270 245 276  612  276 117 120 413 33 144 48 159 5          3103
3.6*  73  340  270 240 276  606  276 124 108 439 33 142 42 165 5          3139
3.7*  64  316  270 242 276  633  276 123 114 422 33 132 31  NS      NS    2932

Pseudogenes
3.8   38  308  270 251 276  371  276 131 121  NS    NS NS  NS NS   NS     2042
3.9   74  308  267 220 271  377  276 123 120 358   NS NS  NS NS    NS     2394
3.10  NS  253  270 239 276  629  276 123 113 444   33 58  NS NS    NS     2714
3.11  73  300  270 213 276  627  276 123 114 442 33 143  42 165 5         3102
3.12  ND  ND   ND  ND  276  573  276 124 120 430 33  76  NS NS     NS     1908
3.13  NS  NS   172 207 276  618  276 123  67  NS    NS NS  NS NS   NS     1739
3.14  72 1138  270 228 281  617  276 124 120 422 33 145  47 159 5         3937
3.15  67  305  270 235 276  627  275 131  96 423 33 141  44  NS      NS   2923
```

exons and extend approximately 4,000 bases (Steinmetz et al. 1981; Moore et al. 1982). Exon 1 encodes the leader sequence; exons 2, 3, and 4 encode the alpha 1, 2, and 3 domains, respectively. The transmembrane-spanning domain is encoded by exon 5; exons 6, 7, and 8 encode the cytoplasmic tail. The previously identified horse MHC class I genomic sequence of the *ELA-A2* gene confirmed that this genomic structure is conserved in the horse (Carpenter et al. 2001), and this sequence was used as a guide to identify intron/exon boundaries of the new class I genes. Overall, the length of each exon or intron was highly conserved among the 15 class I genes (Fig. 2).

The coding region of the 15 new sequences were compared to cDNA or expressed sequence tag (EST) sequences deposited previously into the GenBank (Ellis et al. 1995; McGuire et al. 2003). Six of the new MHC class I genes were previously characterized at the cDNA level, and two were sequenced as ESTs (Table 2). The remaining unidentified genes were analyzed for potential expression by determining and translating the putative coding sequences.

The genes can be assigned to one of two categories: (a) genes encoding a transmembrane length of 35 amino acids which are considered to be members of highly polymorphic series and therefore classical MHC class I genes, or (b) genes encoding longer transmembrane domains (36–40 amino acids) and that tend to be either monomorphic or oligomorphic and are classified as putative nonclassical MHC class I genes (Holmes and Ellis 1999). Eight MHC class I gene subclones contained a functional coding sequence with appropriate intron/exon boundaries, splicing sequences, and stop codons. Four of these encoded molecules with a transmembrane length of 35 amino acids are therefore considered classical MHC class I genes; the other four genes encoded a longer transmembrane domain and are putative nonclassical MHC class I genes.

Each subclone was assigned a number with the prefix 3, indicating the equine ELA-A3 MHC haplotype carried by the MHC homozygous horse from which the BAC library was constructed, followed by a period and a sequential number. Table 2 provides a description of each subclone.

Table 2 Description of horse MHC class I subclones[a]

Subclone	BAC ID	Contig region	Subclone size (nt)	Transmembrane length (AA)[b]	mRNA detected by RT-PCR[c]	GenBank matches	Comments	Citation
3.1	351C06	C	5877	35	Yes	*ECMHB2* (X79891)		Ellis et al. 1995
3.2	355H03	C	14239	35	Yes	*8-5* (Ay225157), EST CD469867 DN504809		McGuire et al. 2003
3.3	355H03	C	13464	35	Yes	New sequence		This study
3.4	324F02	C	4556	35	Yes	EST DN511137		This study
3.5	102G05	C	4559	40	Yes	*ECMHCA1* (X71809)		Ellis et al. 1995
3.6	274K01	B	9384	36	Yes	*ECMHCC1* (X79893)		Ellis et al. 1995
3.7	519G06	B	3039	38	Yes	*ECMHCE1* (X79894)		Ellis et al. 1995
3.8	276J17	A	2024	37	No	New sequence		This study
3.9	276J17	A	2532	35	Not tested	New sequence	Stop codons in exons 2, 4, and 5	This study
3.10	274K01	B	2714	37	No	New sequence	Two stop codons in exon 5	This study
3.11	101I16	B	4310	38	No	EST CX603790	Stop codon in exon 6, exon 8 lacks stop codon	This study
3.12	24A13	B	7200	40	Not tested	New sequence	Lack exons 1 and 2	This study
3.13	24A13	B	1739	35	Not tested	*7-7* (AY225156)	Lacks stop codon in exon 8	McGuire et al. 2003
3.14	355H03	C	8093	40	Not tested	New sequence	Stop codons in every exon, microsatellite TKY22 (AB048332) in intron 1	This study
3.15	324F02	C	3953	32	Not tested	New sequence	Multiple stop codons in exons 4 and 5	This study

[a]Refer to Fig. 1 for corresponding region

[b]Length of transmembrane region distinguishes classical and nonclassical genes: classical MHC class I genes have a transmembrane length of 35 amino acids, whereas nonclassical genes have an insertion that increases the length to 36–40 amino acids (Holmes and Ellis 1999)

[c]RT-PCR performed on adult peripheral blood lymphocytes and conceptus tissues

Classical MHC class I gene subclones

Subclone 3.1 contains the genomic equivalent of the *ECMHCB2* cDNA sequence (X79891). Alignment of the previously published cDNA with the exons from the genomic sequence is in 100% agreement (deduced amino acid alignment shown in Fig. 3). Subclone 3.2 provides the genomic sequence for the *8-5* transcript (AY225157). Alignment of the previously published cDNA with the exons from the genomic sequence has a 97.7% identity (deduced amino acid alignment shown in Fig. 3). Nucleotide differences between these sequences are found in exons 1 through 4. The *8-5* sequence was originally identified from a horse of the A1/A4 haplotype (McGuire et al. 2003). Subclones 3.3 and 3.4 both contained novel and potentially functional coding sequence, with a transmembrane length indicative of classical loci. All four subclones, 3.1–3.4, are located in region C (Fig. 1).

Nonclassical MHC class I gene subclones

Subclone 3.5 contains the genomic equivalent of the *ECMHCA1* cDNA sequence (X71809) and is located in region C. Alignment of the previously published cDNA with the exons from the genomic sequence is in 100% agreement (deduced amino acid alignment shown in Fig. 3). Subclone 3.6 contains the full-length gene encoding the *ECMHCC1* cDNA (X79893) and is located in region B. Alignment of the previously published cDNA with the exons from the genomic sequence is also in 100% agreement (deduced amino acid alignment shown in Fig. 3). Subclone 3.7 contains the genomic sequence of the *ECMHCE1* cDNA (X79894) and is also located in region B. Alignment of the previously published cDNA with the exons from the genomic sequence differs by 3% and provides exon 1 (deduced amino acid alignment shown in Fig. 3). The differences between the coding sequences occur in exons 1, 2,
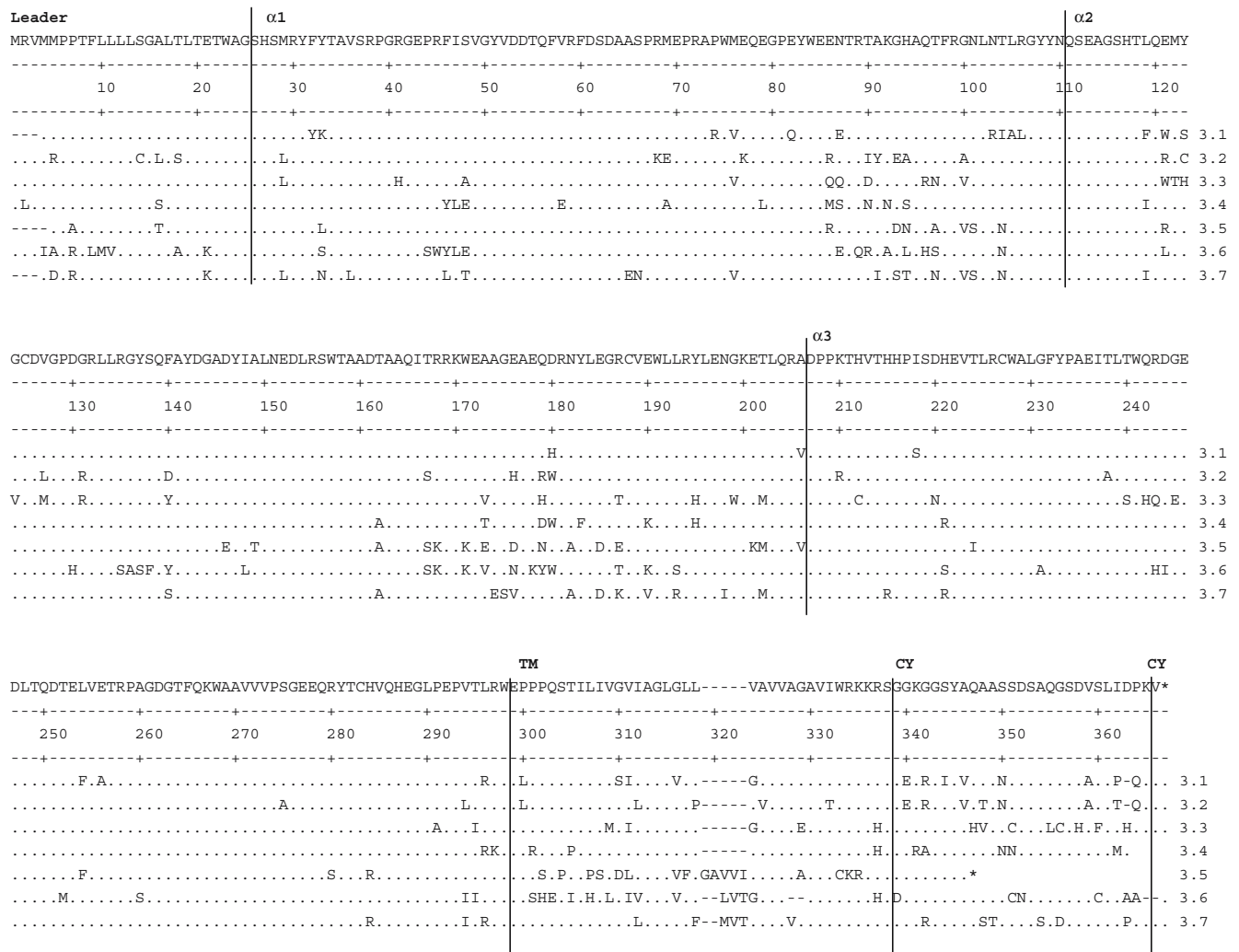
```
Leader                               α1                                                                                                          α2
MRVMMPPTFLLLLSGALTLTETWAGSHSMRYFYTAVSRPGRGEPRFISVGYVDDTQFVRFDSDAASPRMEPRAPWMEQEGPEYWEENTRTAKGHAQTFRGNLNTLRGYYNQSEAGSHTLQEMY
---------+---------+-----+---+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---
         10        20        30        40        50        60        70        80        90       100       110       120
---------+---------+-----+---+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---
---...............|.....YK..................................R.V....Q....E.............RIAL....|.........F.W.S 3.1
....R.......C.L.S.......|...L..............................KE......K.....R...IY.EA.....A.........|.........R.C 3.2
........................|..L.....H......A..................V......QQ..D....RN..V...............|.........WTH 3.3
.L...........S..........|.................YLE....E.......A......L.....MS..N.N.S..............|.........I....S 3.4
-----..A......T.........|.......L.........................R....DN..A..VS..N................|.........R.. 3.5
...IA.R.LMV......A..K....|.......S......SWYLE.........................E.QR.A.L.HS......N.......|.........L.. 3.6
---.D.R..........K....|..L...N..L.........L.T.........EN......V...........I.ST..N..VS..N......|.........I... 3.7
```

```
                                                                                                α3
GCDVGPDGRLLRGYSQFAYDGADYIALNEDLRSWTAADTAAQITRRKWEAAGEAEQDRNYLEGRCVEWLLRYLENGKETLQRADPPKTHVTHHPISDHEVTLRCWALGFYPAEITLTWQRDGE
------+---------+---------+---------+---------+---------+---------+---------+---------+------+---+---------+---------+---------+------
        130       140       150       160       170       180       190       200       210       220       230       240
------+---------+---------+---------+---------+---------+---------+---------+---------+------+---+---------+---------+---------+------
.......................................H.................|.V.......S.............. 3.1
...L...R......D............S........H..RW.............|..R....................A..... 3.2
V..M...R.......Y..............V....H......T......H..W..M.....|...C.....N.............S.HQ.E. 3.3
.......................A.........T....DW..F.....K...H...........|...R............. 3.4
.............E..T..........A....SK..K.E..D..N..A..D.E.......|.KM..V...............I........ 3.5
......H....SASF.Y.......L.........SK..K.V..N.KYW......T..K..S..............|..S.........A..........HI. 3.6
.............S...............A........ESV.....A..D.K..V..R....I...M.....|.....R.....R.............. 3.7
```

```
                                            TM                                                   CY                          CY
DLTQDTELVETRPAGDGTFQKWAAVVVPSGEEQRYTCHVQHEGLPEPVTLRWEPPPQSTILIVGVIAGLGLL-----VAVVAGAVIWRKKRSGGKGGSYAQAASSDSAQGSDVSLIDPKV*
---+---------+---------+---------+---------+--------+---+---------+---------+---------+--------+---+---------+---------+-----+--
   250       260       270       280       290       300       310       320       330       340       350       360
---+---------+---------+---------+---------+--------+---+---------+---------+---------+--------+---+---------+---------+-----+--
......F.A..............................R..|.L.........SI....V..-----G.............|.E.R.I.V...N........A..P-Q... 3.1
...............A................L....|.L..........L....P-----.V......T......|.E.R....V.T.N........A..T-Q... 3.2
....................A...I....|.R...P...........M.I.....-----G....E.......H.........HV..C...LC.H.F..H... 3.3
..........................RK..|..R..P..................-----.........H..|.RA.......NN..........M. 3.4
......F...............S...R...............|..S.P..PS.DL...VF.GAVVI.....A...CKR....|.......* 3.5
....M....S.....................II..|..SHE.I.H.L.IV...V..--LVTG...-----..........H.D.........CN.......C..AA--. 3.6
................................R..........I.R..|........L....F--MVT....V.........|...R....ST....S.D......P...| 3.7
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Fig. 3 Predicted amino acid sequence alignment of expressed horse MHC class I genes from the ELA-A3 haplotype. Predicted amino acid alignment of MHC class I gene sequences determined in this study. *Dots* (.) indicate identity, *dashes* (-) indicate gaps inserted to optimize alignment, *asterisks* (*) indicate stop codon, and *vertical bars* (|) indicate exon/intron boundaries

3, and 7, leaving the characteristic transmembrane domain (exon 5) unchanged. In total, there are 32 nonsynonymous positions that translate into 22 amino acid changes. However, the genomic sequence identified here matches two GenBank sequences from an independent cDNA library (CD465113 and CD467042), with only one nucleotide difference. Therefore, we suggest that the new sequence is a second allele at the *ECMHCE1* locus.

Pseudogenes

Pseudogenes were identified by either the presence of one or more premature stop codons, the lack of a stop codon, or lack of mRNA expression in lymphocytes and conceptus tissues. Five of the horse MHC class I genes were determined to be pseudogenes by either the presence of one or more premature stop codons or the lack of a stop codon (Table 2). Only one pseudogene subclone, 3.12, lacked entire exons. MHC class I genes contained in subclones 3.9, 3.14, and 3.15 encoded stop codons in exons before the transmembrane region (exon 5), which would presumably disrupt the conformation of any resulting proteins, and were categorized as pseudogenes without further analysis. Subclone 3.13 lacked a stop codon and provided genomic sequence for a pseudogene that is expressed as mRNA, but not protein, as described previously for the 7-7 sequence (AY225156) (McGuire et al. 2003). Subclones containing MHC class I genes that encoded premature stop codons but could produce soluble or truncated cell-surface proteins (3.10 and 3.11) were assayed for expression by RT-PCR.

Expression of MHC class I genes

Five of the eight potentially expressed horse MHC class I genes have been previously characterized at the cDNA level. To investigate the expression of all eight class I genes, locus-specific primers were used in RT-PCR experiments (Table 1). Nearly all primers were designed within the hypervariable regions for optimal specificity. We assayed cDNA from four tissues, all of the ELA-A3 haplotype: (1) adult lymphocytes from four horses, including the BAC library donor, (2) day 33 invasive trophoblast (chorionic girdle), (3) day 33 noninvasive trophoblast (allantochorion), and (4) day 33 fetus. Seven of the eight genes, *3.1* through *3.7*, were expressed in all four tissues tested (adult lymphocyte gel shown in Fig. 4). RT-PCR products were purified and directly sequenced, confirming that a single gene was amplified. Further, all sequences were identical to the expected ELA-A3 coding sequences. No evidence of mRNA expression was found for the eighth gene, *3.8*, in either lymphocytes or conceptus tissues. Although the full MHC class I gene sequence was not determined for the *3.8* gene, it is designated as a pseudogene in this haplotype based on the lack of mRNA expression in these tissues.

Specific primers were also designed for RT-PCR analysis of class I genes with premature stop codons that could produce soluble or truncated cell-surface proteins. The MHC class I gene contained in subclone 3.10 encoded two stop codons in the transmembrane region (exon 5). The MHC class I gene contained in subclone 3.11 encoded a stop codon in exon 6 and lacked a stop codon in exon 8, and an EST match was found in GenBank (CX603790). No mRNA expression was detected from peripheral blood lymphocytes or conceptus tissues for these genes (*3.10* result shown in Fig. 4), but both primer sets produced the expected product when tested on the respective genomic subclone. Accordingly, class I genes *3.10* and *3.11* are designated as pseudogenes in this haplotype.

Previous transfection studies confirmed the protein expression of *3.1*, *3.2*, *3.5*, and *3.6* (D.F. Antczak and S. Ellis, unpublished; Holmes and Ellis 1999; McGuire et al. 2003).

In summary, subclones 3.1 through 3.4 contain expressed classical loci, subclones 3.5 through 3.7 contain expressed putative nonclassical loci, and subclones 3.8 through 3.15 contain pseudogenes in this haplotype.

MHC class I gene regulatory element sequence analysis

Eight regulatory elements have been described in the 5′ upstream sequence of MHC class I genes, and they are conserved in multiple species (Howcroft et al. 2003; van den Elsen et al. 2004), including the horse (Carpenter et al. 2001). At least three and up to eight of these regulatory elements were present in 11 of the horse MHC class I gene subclones (Table 3). The order and spacing of the regulatory elements is conserved among all 11 genes.

High sequence conservation of the listed elements is evident for all of the expressed MHC class I loci, although a few nucleotide substitutions occur in each regulatory element. Some variations are shared among all expressed loci, such as the T to C change in the X2 box, and some are unique to a single locus. It is not possible to determine at this time whether any of these variations manifest locus specificity, as sequences from alleles of other haplotypes
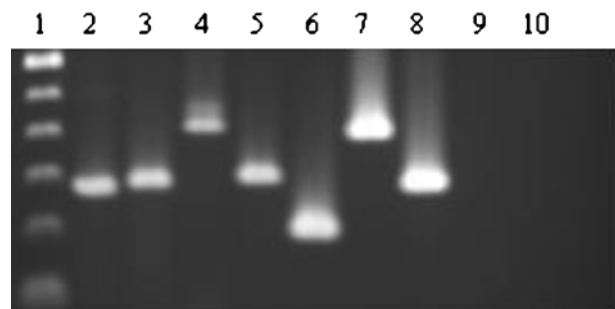

Fig. 4 RT-PCR analysis of horse MHC class I gene mRNA expression. Peripheral blood lymphocytes express mRNA of seven distinct MHC class I genes as determined by locus-specific RT-PCR. Primers listed in Table 1. *Lane 1* indicates 100-bp ladder (top band 600 b); *Lane 2*, 3.1 (283 b); *Lane 3*, 3.2 (293 b); *Lane 4*, 3.3 (416 b); *Lane 5*, 3.4 (306 b); *Lane 6*, 3.5 (210 b); *Lane 7*, 3.6 (413 b); *Lane 8*, 3.7 (301 b); *Lane 9*, 3.10 (381 b), and *Lane 10*, no template control

Table 3 Comparison of promoter elements among horse MHC class I genes, horse β-2-m, and human HLA-A2*0101

Published horse MHC class I gene	NFκB-2	ISRE	W/S	X1/X2	Enhancer B	CCAAT	TATAA
8-9	TGGGGAGTCCCCG	AGTTTCACTTCT	CAACCTG	CCTGGAGACTCA-<u>TGACGTG</u>	CCATTGCGAGTC	CCAAT	TATAA
Expressed loci							
3.1	............T	....	....	............-.....C.	........T...	.....	.....
3.2	....	..A........	....	............-.....C.	........T...	.....	.....
3.3	...........T.	........C...	.....C.	............-.....C.	.G......T...	.....	.....
3.4	....	....	....	............-.....C.	...C...AT...	.....	.....
3.5	....	....	..CA.GC	............-.....C.	....	.....	C
3.6	C.C....C..TG.	....	....	...........-A....C.	....	.....	C.A..
3.7					......G.T..T	.G...	..G..
Pseudogenes							
3.9				.A.TT...C-...A.AT	........C...	T....	.....
3.11	..C...TC.ATGA	--..TG...	...A...	............-....A..	........T...	TTC..	ND
3.14	AAAA....G.ATT	TAA.G-GT.AAG	AC.TA..	T.A..CTTTTT.GT.C.A.CC	AG.A.AGTTAC.	TAGG.	.....
3.15	.......T.T.T	....G.G.....	......	............-.....C.	......G.T...	.....	.....
Horse β-2-m	G.TAA........	GAAAC.GAAAAG	T......	.TA......AAG-.....GC	.G....G...CA	ND	.....
HLA-A2*0101	......T......A	......TT....	......A	......T.....-C....C.	......G.T.G	.....	.C...

X1 and X2 boxes overlap; X2 box is underlined in 8-9 sequence
Dots Nucleotide identity, *Dashes* gaps inserted to optimize the alignment, *ND* not detected

are necessary for this analysis. Varying levels of sequence conservation are found in these elements among the horse MHC class I pseudogenes. Pseudogenes *3.9*, *3.11*, and *3.15* have retained recognizable elements, and notably, the *3.11* gene is transcribed (Table 2). In contrast, the *3.14* gene only has one detectable element.

Major histocompatibiliy complex class I expression on the cell surface is dependent on stabilization from β-2-microglobulin (Zijlstra et al. 1990), thereby requiring co-ordinate regulation of these genes. A comparison of the regulatory elements contained in the promoter of the horse MHC class I gene *ELA-A2* and the horse β-2-microglobulin is shown in Table 3, as previously published (Tallmadge et al. 2003). High sequence conservation is detected in all of the elements also found in the β-2-microglobulin promoter, with exceptions of sequence variation in the interferon-stimulated response element (ISRE), and the lack of the CCAAT box.

Finally, the sequence from the human allele HLA-A2 (L36528) is included for comparison and shows very high sequence conservation of MHC class I gene regulatory elements between these species.

Discussion

This study describes the physical map locations and sequences of 15 equine MHC class I genes. BAC clones from the published MHC class I region contig (Gustafson et al. 2003) served as the template for subcloning individual class I genes. The horse class I genes were located in three clusters: (A) at the *BTNL2* end of the class II region, (B) at the junction of the class III and I regions, and (C) between the *GNL1* and *TRIM26* genes in the class I region.

The BAC clone containing region A shortened the gap between the MHC class II and III regions, and the BAC clones placed in region B lengthened the original horse MHC BAC contig. It had been noted previously that most mammals have at least three distinct class I duplication blocks (Kulski et al. 2002). However, all three blocks are generally found within the class I region. Horse class I regions B and C correspond to the defined beta (β) and kappa (κ) duplication blocks (between *BAT1* and *POU5F1*, and between *GNL1* and *TRIM26*, respectively) (Kulski et al. 2000). The specific MHC class I gene content within these regions varies between human and horse. The human classical genes *HLA-B* and *HLA-C* and pseudogenes *HLA-S* and *HLA-X* are found in the β block, while the horse β block contains two putative nonclassical genes and four pseudogenes. In the κ block, one human nonclassical gene is found, *HLA-E*, and two pseudogenes, *HLA-L* and *HLA-N*, while in the horse, all four classical genes, one putative nonclassical gene, and two pseudogenes are found. In fact, the organization of the horse MHC class I genes is more similar to that of the pig than the human, with the exception of horse class I region A (Velten et al. 1999).

Region A is unique to the horse MHC when compared to the human or pig MHC map. Although both mice and rats encode MHC class I genes within the MHC class II genomic region (Kulski et al. 2002), the placements are not similar to that found in the horse. Horse class I region A is outside of the class II region, next to the *BTNL2* gene, while the rodent class I genes map between the *SACM2L* and *RING1* genes within the class II region. The current study describes the MHC class I physical map for the ELA-A3 haplotype only. Other ELA haplotypes may have differences in MHC class I gene content as has been reported in other species (Wroblewski et al. 1994; Ellis et al. 1999).

The horse MHC class I gene sequences characterized in this study include the genomic equivalents of previously published horse MHC class I cDNA sequences and newly identified genes and pseudogenes. This work confirms the previously published cDNA sequences and provides sequences of introns, untranslated regions, and extragenic-flanking regions. It also provides evidence for horse MHC class I mRNA expression in four tissues, indicating that at least seven different loci are expressed by horses carrying the ELA-A3 haplotype of the BAC library donor horse. We did not find a gene encoding an expressed soluble MHC class I (ESCI) gene matching the one described previously (Lew et al. 1986). The horse ESCI molecule is analogous to the mouse Q10 molecule (Lew et al. 1986), which is produced exclusively in the liver and yolk sac (David-Watine et al. 1990). Because these tissues were not included in this study, and because not all horses express soluble MHC class I molecules, it is not known whether the donor stallion expresses a soluble class I gene.

The total number of expressed MHC class I loci in horses may be higher than the seven identified thus far in the ELA-A3 haplotype. While this total of seven expressed class I loci exceeds that of human and mouse (six loci each), the rhesus macaque expresses at least nine and up to 22 class I genes (Daza-Vamenta et al. 2004). The optimal number of expressed MHC class I and II genes has been suggested to be 12, as the expression of additional MHC genes would deplete the T-cell receptor repertoire excessively (Takahata 1995).

The regulatory elements identified in the expressed MHC class I genes are likely to be functional as demonstrated by *ELA-A2* reporter construct (Carpenter et al. 2001). For several class I subclones, a large amount of flanking sequence in the 5′ upstream region was obtained. This may be useful in the identification of additional regulatory elements, especially those known to be more than 1,000 bases upstream (Mavria et al. 1998). Introns have also been shown to harbor regulatory elements (Drezen et al. 1995), which could also be investigated using these subclones.

Differential regulation between the classical and putative nonclassical loci can also be pursued with the sequences provided. For example, the nonclassical *HLA-G* gene is regulated by different transcriptional control mechanisms than the other HLA class I genes (Gobin and van den Elsen 1999). Also, the tissue distribution of MHC class I nonclassical genes remains to be thoroughly studied in the horse. In humans, nonclassical MHC class I loci have a restricted tissue distribution and lower levels of expression, similar to the *H2-Q10* gene of the mouse (David-Watine et al. 1990). In contrast, the *H2-Qa2* gene in the mouse and the nonclassical genes in the pig, *SLA-6*, *SLA-7*, and *SLA-8*, have a wide tissue distribution at the transcriptional level (Ungchusri et al. 2001; Crew et al. 2004). Sequences for both classical and putative nonclassical loci have been detected in both horse lymphocytes and trophoblast (this study and Bacon et al. 2002), but additional somatic tissues have not yet been tested. At this time, the protein expression and function of the putative nonclassi-cal genes of the horse is not known and warrants further investigation.

Previously, at least two classical MHC class I loci and the nonclassical loci *ECMHCA1* (*3.5*) and *ECMHCC1* (*3.6*) were identified in the invasive horse trophoblast of the chorionic girdle that expresses high levels of cell-surface MHC class I protein (Donaldson et al. 1990; Bacon et al. 2002). In this study, we detected two additional classical loci and the third putative nonclassical locus (*ECMHCE1*, *3.7*) in the invasive trophoblast and all seven MHC class I genes in the noninvasive trophoblast of the allantochorion. The allantochorion trophoblast expresses only about one tenth of the mRNA level of the chorionic girdle (Bacon et al. 2002) and has little to no detectable MHC class I protein expression (Donaldson et al. 1990).

We designated MHC class I genes *3.8–3.15* as pseudogenes in this haplotype based either on inappropriate placement of stop codons, lack of mRNA expression in lymphocytes and conceptus tissues, or identity to a previously described pseudogene. However, it is possible that genes *3.8*, *3.10*, and *3.11* are not pseudogenes but have a restricted tissue distribution that does not encompass the tissues included in this study. This can explain the mRNA expression of *3.11* in a horse cartilage cDNA library (CX 603790; J. MacLeod, University of Kentucky) and lack of expression in the tissues assayed in this study.

Common regulatory elements were detected for horse MHC class I and β-2-m genes (Enhancer A/NF-κB, ISRE, S, X1, X2, Enhancer B/Y, and TATA box), which may effect coordinate transcriptional control (Tallmadge et al. 2003). Both genes, however, have additional separate regulatory elements that might allow for disparate expression (MHC class I, NFκB2 site of Enhancer A and CCAAT; β-2-m, PAM). This information will facilitate further studies on the regulation of MHC class I and β-2-microglobulin genes in the horse.

While this study provides the sequence of 15 MHC class I genes from the ELA-A3 haplotype, further studies including multiple haplotypes are necessary to make intra-locus comparisons. With additional sequence information, molecular typing methods may be devised for horse MHC class I genes. Despite the clarity of locus-specific residues in human and mouse MHC class I genes (Koller et al. 1984; Parham et al. 1989; Pullen et al. 1992; Cereb and Yang 1994; Cereb et al. 1995), such indicators have not been found in cattle or horse MHC class I genes (Ellis et al. 1995, 1999). Further, the number of loci varies among haplotypes of other species, including mice, cattle, and possibly, horse (Lew et al. 1986; Wroblewski et al. 1994; Ellis et al. 1999). The flanking extragenic sequence obtained from the subclones has the potential to serve as a unique marker, which may be useful for mapping MHC class I loci between haplotypes. A more complete understanding of the regulation of MHC class I genes may have applications in equine medicine in areas such as immunity to infectious diseases, pregnancy, autoimmunity and allergies, where the state of expression of MHC molecules is crucial.

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