The Complete Map of the Ig Heavy Chain Constant Gene Region Reveals Evidence for Seven IgG Isotypes and for IgD in the Horse\textsuperscript{1,2}

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This report contains the first map of the complete Ig H chain constant (IGHC) gene region of the horse (Equus caballus), represented by 34 overlapping clones from a new bacterial artificial chromosome library. The different bacterial artificial chromosome inserts containing IGHC genes were identified and arranged by hybridization using overgo probes specific for individual equine IGHC genes. The analysis of these IGHC clones identified two previously undetected IGHD genes of the horse. The newly found IGHG7 gene, which has a high homology to the equine IGHG4 gene, is located between the IGHG3 and IGHG4 genes. The high degree of conservation shared between the nucleotide sequences of the IGHG7 and IGHG4 genes is unusual for the IGHG genes of the horse and suggests that these two genes duplicated most recently during evolution of the equine IGHG genes. Second, we present the genomic nucleotide sequence of the equine IGHD gene, which is located downstream of the IGHM gene. Both the IGHG7 and IGHD genes were found to be expressed at the mRNA level. The order of the 11 IGHC genes in the IGH locus of the horse was determined to be 5'–M-D-G1-G2-G3-G7-G4-G6-G5-E-A–3', confirming previous studies using \( \lambda \) phage clones, with the exception that the IGHG5 gene was found to be the most downstream-located IGHG gene. Fluorescence in situ hybridization was used to localize the IGHC region to Equus caballus (ECA) 24qter, the horse chromosome corresponding to human chromosome 14, where the human IGH locus is found. The Journal of Immunology, 2004, 173: 3230–3242.

The Ig H chain constant (IGHC)\textsuperscript{4} genes\textsuperscript{5} encode the constant domains of different Ig isotypes. The IGHC genes and the 5'-located variable H chain genes cluster together in the IGH locus. In the IGHC region of the horse, six IGHG genes, one IGHE, and one IGHA gene were identified by investigations on cloned genomic DNA from a \( \lambda \) phage library and genomic DNA obtained from PBMC (1, 2). The equine IGHEM gene was characterized by nucleotide sequencing (3), as were the IGHE (4–6), the IGHA gene (7), and the IGHG1 to IGHG6 genes (8).

The order of the H chain genes in the equine IGH locus was determined by overlapping \( \lambda \) phage clones containing the IGHE and IGHA genes (1) or using deletion analysis of DNA of equine heterohybridomas (9). The deletion analysis aligned the IGHEC genes, which were still detectable in the hybridomas after their genomic DNA had been rearranged by class-switch recombination. This indicated that the IGHEC gene of the horse is located at the 5' end of the IGHC region, followed by the six IGHG genes and the IGHE and IGHA genes at the 3' end. The six equine IGHG genes represent the highest number found to date in any mammalian species, expressing five or fewer different IgG isotypes (10–15). Nucleotide sequence analysis and expression studies of the IGHG1 to IGHG6 genes of the horse have shown that they are all expressed, although their individual functions during immune responses are not yet well characterized.

In this study we provide the first complete map of the equine Ig H chain constant gene region represented by overlapping clones from the new bacterial artificial chromosome (BAC) library of the horse. The large overlapping BAC DNA inserts enabled a clone-based alignment of the IGHC genes of the horse, including the equine IGHD and IGHG7 genes, which are described in this study for the first time.

\textbf{Materials and Methods}

\textit{BAC library of the horse}

The genomic CHORI-241 horse BAC library was constructed by Dr. P. de Jong (Children’s Hospital of Oakland, Oakland, CA; http://chori.org/bacpac/equine241.htm) using genomic DNA isolated from granulocytes of a Thoroughbred stallion in the Baker Institute herd of Cornell University. The library contains \( \sim 190,652 \) clones, with an average insert size of 171 kb, giving an estimated 11.8-fold total genomic representation.

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\textsuperscript{2} The nucleotide sequences reported in this paper have been assigned the EMBL/GenBank accession numbers AY256910 (IGHG3), AY445517 (IGHG7), AY445518 (IGHG4), AY631941 (genomic IGHE, including 3'–untranslated region), AY631942 (genomic IGHE), AY631943 (IGHD from mRNA, transmembrane form), and AY631944 (IGHD from mRNA, secreted form).

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\textsuperscript{4} Abbreviations used in this paper: IGHE, Ig H chain constant; BAC, bacterial artificial chromosome; CH, constant H chain; ECA, Equus caballus; FISH, fluorescence in situ hybridization; IVS, intervening sequence; RFLP, restriction fragment length polymorphism.

\textsuperscript{5} The designation of the IGHC genes of the horse conforms with the official nomenclature of Ig genes of the international ImMunoGeneTics database (http://imgt.cines.fr).
Identification of BAC clones containing IGHC genes

Sequences from the equine IGHM gene (3), the IGHE gene (6), the six IGHG genes (8), the IGHA gene (7), and the IGHD gene sequence described in this study were used to generate specific overgo probes for hybridization. The overgo primer sequences were obtained from the overgo designer webpage (Washington University Medical School, St. Louis, MO; http://genome.wustl.edu). Overgo primers are 24 bp in size. Primer pairs overlap at their complementary 3' ends and create a highly specific overgo probe of 40 bp after radioactive labeling to screen the BAC library. All overgo primers are shown in Table I. The initial screening of the BAC library was performed on nylon membranes, containing one copy of each individual BAC clone, using overgo probes for equine IGHM, IGHG1, IGHG5, and IGHE. All clones that hybridized with at least one of these probes were grown overnight on Luria-Bertani plates containing 20 μg/ml chloramphenicol. The clones were transferred to nylon membranes. Individual membranes were hybridized to the IGHE, IGHG1 to IGHG6, and IGHD overgo probes. The design and radioactive labeling of the overgo probes, treatment, hybridization, and washes of the membranes were performed as described previously in detail (16). The designation used for the IGHC BAC clones corresponds to their original library designation.

Preparation of BAC DNA

The BAC DNA was isolated from Luria-Bertani cultures containing 20 μg/ml chloramphenicol using the protocol from the Advanced Center for Genome Technology, University of Oklahoma (http://genome.ou.edu/ BAC_iso/200ml_culture.html).

PCR, nucleotide sequencing, and relative expression of the IGHG4 and IGHG7 genes

The PCR conditions and primers to amplify the IGHG genes of the horse were described previously (6, 8). In brief, all PCR products were amplified from 500 ng of BAC DNA using 1.25 U of Pfu polymerase (Stratagene, La Jolla, CA) and 2 mM MgSO4 at 55°C annealing temperature. The following primers were used for amplification of the entire IGHG4 and IGHG7 genes: sense, 5'-GGCTCCACCCAGGGCCGGAAG-3'; and antisense, 5'--GGGAAGGTCATACCCAGGAGACTTGAG-3'. For PCR of the hinge exons, equine consensus primers, amplifying the respective regions of all seven IGHG genes, were used. The sense primer corresponded to the 3' end of the CH1 exon, 5'--GCCAGCACTTCAAGGGAAG-3', and the antisense primer corresponded to the 5' end of the CH2 exon, 5'--GGGGAAAGATGAAAGAGAGGGGAGG-3'. All PCR products were cloned in the pCR4 TopoBlunt vector (Invitrogen Life Technologies, Carlsbad, CA). Nucleotide sequencing was performed using an ABI automatic sequencer at the BioResource Center, Cornell University.

To determine the relative expression rates of the IGHG4 and IGHG7 genes, total RNA was isolated from PBMC of three Thoroughbred horses, and cDNA was obtained as described previously (6). The entire IGHG4 and IGHG7 genes were obtained by PCR using a primer pair that amplified both genes (see above) and cloned in pCR4 TopoBlunt, and 20 cloned PCR products of each horse were analyzed by nucleotide sequencing.

| Primer | Orientation | Primer Sequence (5'→3') | Corresponding Genbank Accession No. |
|--------|-------------|--------------------------|-----------------------------------|
| IGHM   | F           | CTCAACCCAGAAAGTGTTGCATTGC | L49414 |
|        | R           | CTTGTTGTCCACATGGCAAGTGGA | |
| IGHG1  | F           | CCACCAAAAGGTGCGACATGAG   | AJ302055 |
|        | R           | GCATTGGGACACTTCATCACGC   | |
| IGHG2  | F           | ACTTGGGTGTGTTGAACTTGAGG  | AJ302056 |
|        | R           | CATCGGGGTTATGATGCTCAAGTC | |
| IGHG3  | F           | TGTGGAACGGCACCAGGTTGAAGAC | AJ312379 |
|        | R           | CCGCATATGCTTTGAGGACACAC | |
| IGHG4  | F           | CAATCCTCACCAGGGAATACTCCT | AJ302057 |
|        | R           | ACCGGGGAGACCTTGGGACGAGTT | |
| IGHG5  | F           | CTCGGAACCTTGATGCCTGGAAGA | AJ312380 |
|        | R           | AAGGCTCTGTATGCTCCGACCTTG | |
| IGHG6  | F           | GGAGGACTTCCAGGACCAAAAAGTG | AJ312381 |
|        | R           | TGGGTGGGCCAGATGCACACCCTTG | |
| IGHE   | F           | TACAGTGCTGCCTGTAACACCTGCC | AJ305046 |
|        | R           | GCTGTCTGTCTTTGATGAGCACAT | |
| IGHA   | F           | TGAAGCCCTCCAGGAAATGCTGACCTCAG | AY247966 |
|        | R           | ATGACACCAGGGTCCACCTGAGTCA | |
| IGHD   | F           | AGTGTCAAGAGCCACACCCACACCTC | AY631942 |
|        | R           | ACAGGAGTGGATATCGAGCAAGGTT | |

*a* Overgo primers are each 24 bp in length. Corresponding forward (F) and reverse (R) primer pairs contain a complementary sequence of 8 bp at their 3' ends. The complementary sequences are underlined. The primers create a highly specific probe of 40 bp for each of the genes investigated.
strategy was used for different appropriate restriction enzyme and primer combinations to obtain the sequence of the entire genomic IGHD gene. In particular, for the large introns of the genomic IGHD gene, this procedure resulted in nucleotide sequences with distinctly higher qualities than using the respective primers for sequencing directly from the BAC DNA. The following restriction enzymes and primers were used. For the region upstream of the CH2/CH3 region: circle 1) 781-bp *Pst*I circle using the primers 5'-GGTGTTGGCTTGACACT-3' (antisense) and 5'-CCAGTATCTACCTGCGACC-3' (sense); circle 2) 1009-bp *Nco*I circle and primers 5’-CTCATAGGGTTTCCATCTTC-3’ (antisense) and 5’-CCATCCACGGGATGTTCGTG-3’ (sense); circle 3) 1671-bp *Pst*I circle using primers 5’-GGACTCTCCTGCTGCTGTG-3’ (antisense) and 5’-CAATACCGGCGCAGCCCTCTG-3’ (sense); circle 4) 1992-bp *Pst*I circle using the primers 5’-CAGGAACATCCCGTTG-3’ (antisense) and 5’-GAAAGCTTGAACAACTGGGAAATG-3’ (sense). The nucleotide sequences of these PCR products were aligned using their overlapping regions to obtain the sequence of the genomic equine IGHD gene. In addition, the exon/intron boundaries were confirmed by PCR of cDNA obtained from equine PBMC as described previously for other equine IGHC genes (6–8).

**FIGURE 1.** BamHI RFLPs of IGHC genes of various BAC clones were identified by Southern blotting. DNA of different BAC clones, containing various numbers of IGHC genes, were digested with BamHI, separated in 0.8% agarose gels, transferred onto two nylon membranes, and hybridized to the equine IGHG1 cDNA probe (IGHG). The two membranes correspond to the left panel shown in the figure. The equine IGHG1 cDNA probe hybridized to all IGHC genes of the horse, but not to the IGHM, IGHE, or IGHA gene. Using BamHI for digestion of genomic DNA, each IGHC gene is located on a single BamHI fragment. The only exception is the IGHG2 gene, which contains a BamHI site within the gene, resulting in two hybridizing fragments. The designation of the individual BAC clone and the IGHC genes that were identified on the clone, according to the second library screen are given under each lane. For example, 38403 (M-G6) means that BAC clone 38403 contains the IGHM to IGHG6 genes. The designation of the BAC clones corresponds to the original nomenclature of the BAC library. G7, BAC clones 109K05 and 456N04 hybridized to the IGHG4 overgo probe, which identified both the IGHG4 and IGHG7 genes of the horse. Further characterization of these two clones revealed that both contained the IGHG7 gene only, but no IGHG4 gene. The BamHI RFLPs linked individual BAC clones to their corresponding IGHC haplotypes, designated IGHG1* and IGHG7* for the IGHG1, IGHG5, and IGHG6 genes.
FIGURE 2. BamHI RFLP of the IGHA gene of the horse. DNA of six different BAC clones, five of them containing the IGHA gene, were digested with BamHI. After gel electrophoresis and Southern blotting, the membrane was hybridized to an equine IGHA cDNA probe. Two hybridizing fragments of different sizes were identified, corresponding to the IGHC<sup>a</sup> and IGHC<sup>b</sup> haplotypes.

Southern blotting

For gel electrophoresis, 5 μg of each BAC DNA or 8 μg of genomic DNA from PBMC of horses of different breeds (Thoroughbred, Standardbred, Quarterhorse, Appaloosa, Warmblood, Hannoverian, and Pony) were digested with BamHI and separated on 0.8% agarose gels. Southern blotting and hybridization were performed as described in standard protocols (17). The cDNA of the entire equine IGHG1 gene and the equine IGHA cDNA probe containing the CH2 and CH3 exons were obtained by PCR as described previously (7, 8). The IGHC overgo primers used to design probes for Southern blotting are listed in Table I.

Long-template PCR and amplification of the genomic IGHM gene

The intron between the equine IGHM and IGHD genes of BAC clone 52M17 was amplified by PCR using the Expand Long Template PCR System (Roche, Indianapolis, IN), following the manufacturer’s instruction. Primers were designed from the CH2 exon of the equine IGHM gene (L49414; 5′-CTTCATACGGAAGAGGTGC-3′, sense) and the CH1 exon of the IGHD gene (5′-CGCTGTATCTTCGAGGGACCC-3′, antisense). The 9.3-kb PCR product was cloned in the pGEM-T Easy vector (Promega, Madison, WI) and used for nucleotide sequencing. The remaining 5′ end of the genomic IGHM gene of BAC clone 52M17 was amplified by a regular PCR using primers obtained from the CH1 and CH3 exons of the IGHM gene (L49414): 5′-GAGGATCGAAGACCCCCAGAT-3′ (sense) and 5′-ACTTTAACCTGTCTTAGGTCG-3′ (antisense).

Fluorescence in situ hybridization (FISH)

Metaphase chromosome preparation (18) and FISH (19) were performed as described previously; however, all posthybridization washes were performed at 42°C. The chromosomes were counterstained with 31.5 ng/ml 4′,6-diamidino-2-phenylindole solution (Vysis, Downers Grove, IL). Chromosome images were captured and analyzed using Cytovysion/Genus Application software version 2.7 (Applied Imaging, Santa Clara, CA).

Results

Identification of clones from the BAC library of the horse containing IGHC genes

The BAC library of the horse was screened for clones containing equine IGHC genes using overgo probes for the equine IGHM, IGHE, IGHA, and IGHG1 to IGHG6 genes. We identified a total of 34 clones containing IGHC genes of the horse. Because the individual BAC clone inserts spanned an average of 171 kb of genomic DNA, most of them contained more than one IGHC gene. Using the nine overgo probes, large overlapping regions were determined on various BAC clones carrying identical IGHC genes (Table II). The overlapping regions were used to align the IGHM, IGHG1 to IGHG6, IGHE, and IGHA genes in the IGHC region of the horse.

Compared with previous data obtained from A phage clones containing single IGHG genes and by deletion analysis of equine murine heterohybridomas, one discrepancy was found for the order of the IGHG5 and IGHG6 genes. The hybridization data obtained from overlapping BAC clones clearly indicated that the order of these two genes is 5′-IGHG6-IGHG5-3′. This is obvious for the BAC clones 384I03 (M-G6)<sup>a</sup> and 50M21 (G1–G6) containing the IGHG4 and IGHG6 genes, but no IGHG5 gene, and for the clone 339A13 (G5–A), which hybridized to the IGHG5, IGHGE, and IGHA overgo probes, but did not hybridize to the IGHG6 probe (Table II).

Horse IGHC region haplotypes defined by BamHI restriction fragment length polymorphisms (RFLP)

To link the individual BAC clone inserts to their corresponding IGHC haplotypes, we used the previously observed BamHI RFLPs of the IGHG1, IGHG6, and IGHA genes of the horse (1, 2). Southern blot analysis of BamHI-restricted genomic DNA from PBMC of the horse used for construction of the BAC library indicated that this animal is heterozygous for the IGHC region. Thus, each of the BAC clone inserts contained the genomic information of one IGHC haplotype. We isolated DNA from 19 clones containing different IGHC genes and digested them with BamHI. After gel electrophoresis and Southern blotting, the membranes were hybridized to equine IGHG1 or IGHA cDNA probes. The IGHG1 cDNA probe contained the coding sequence of the entire equine IGHG1 gene. Due to the high nucleotide sequence homology between the equine IGHG genes, this probe hybridized with all IGHG genes of the horse. Accordingly, one or more fragments were found for each BAC clone that contained IGHG genes, but not for clones 354M20, 301P10, and 88J13, which carried the IGHM gene, or for clone 165E04, which contained the IGHE and IGHA genes (Fig. 1). The BamHI RFLPs of the equine IGHG1 and IGHG6 genes were clearly detectable on BAC DNA. The IGHG1 gene corresponded to the 10.0- or 10.8-kb BamHI fragment, and the IGHG6 gene to the 4.6- or 4.9-kb fragment depending on the IGHC haplotype of the respective BAC clone. An additional RFLP was observed for the IGHG5 gene with BamHI fragments of 6.6 or 6.9 kb. The IGHA probe identified a BamHI RFLP in all BAC clones containing this gene (Fig. 2). The hybridizing fragments were either 8.0 or 9.6 kb.

The different sizes of the BamHI fragments of the IGHG1, IGHG6, IGHG5, and/or IGHA genes of the horse were used to link each individual BAC clone to its corresponding chromosome. The IGHC haplotypes defined by BamHI RFLPs of these four IGHC genes were designated IGHC<sup>a</sup> and IGHC<sup>b</sup> (Table III).

Evidence for a seventh IGHG gene of the horse

Each BAC clone insert contains the genomic DNA of an individual chromosome, corresponding to one defined IGHC haplotype. Thus, the number of BamHI fragments hybridizing to the IGHG1...
The IGHG1 to IGHG6 overgo probes were used for identification of their corresponding BamHI fragments on the BAC clone 60B11 (G1–G5). The IGHG overgo probes were designed from nonconserved parts of the IGHG1 to IGHG6 genes. Thus, they should identify their corresponding IGHG gene only, i.e., hybridize to one BamHI fragment each. The presence of all IGHG genes on their respective BamHI fragments was confirmed in that way. All IGHG overgo probes hybridized to one BamHI fragment only, with the exception of the IGHG4 overgo, which identified two fragments (Fig. 3). These two BamHI fragments corresponded to the 6.0-kb BamHI and the 6.2-kb BamHI fragments of the BAC clone 60B11 shown in Fig. 1. The IGHG genes on the 6.0- and 6.2-kb BamHI fragments were subsequently sequenced and shown to represent the IGHG4 and IGHG7 genes of the horse (see below). The first fragment represented the IGHG4 gene, and the latter represented the newly identified seventh IGHG gene of the horse. Regarding the different BAC clones shown in Fig. 1, the 6.2-kb BamHI fragment was identified on most, but not all, clones, containing the IGHG4 gene and on clones 109K05 (M-G7) and 456N04 (G1–

![FIGURE 3. Southern blot of BamHI-digested DNA of the BAC clone 60B11 (G1–G5). Each membrane (lane) was used for hybridization with a different probe, linking the individual IGHG gene to its corresponding BamHI fragment. The probes used were either the IGHG1 cDNA probe (IGHG), hybridizing to all IGHG genes of the horse or overgo probes for the equine IGHG1 to IGHG6 genes. The IGHG7 gene was identified by subsequent nucleotide sequence analysis of the 6.2-kb BamHI fragment.

| Haplotype      | IGHG1 | IGHG6 | IGHG5 | IGHG7 | IGHA |
|----------------|-------|-------|-------|-------|------|
| IGHC^a         | 10.8  | 4.6   | 6.9   | 9.6   | 5.0  |
| IGHC^b         | 10.0  | 4.9   | 6.6   | 8.0   | 5.0  |

The haplotype of each individual BAC clone was determined by BamHI RFLPs of the IGHG1, IGHG6, IGHG5, and/or IGHA gene fragments. Each haplotype is located on one chromosome carrying the equine IGH locus.

![Table III. BAC clones and corresponding IGHC haplotypes (IGHC^a or IGHC^b)](image)

| BamHI Fragments (kb) | BAC Clones, Corresponding to the Respective Haplotype |
|----------------------|-----------------------------------------------------|
| 60B11                | 52M17, 417C10, 427L03, 109K05, 351M20, 50M21, 271F08, 163G06, 339A13, 165E04 |
| 67G21                | 384I03, 456N04, 60B11, 316A15, 67G21, 223C15 |

According to data obtained from cloned horse DNA, it was previously assumed that the eighth IGHG2 BamHI fragment of 6.2 kb represented the 5' part of the IGHG3 gene (2, 9). Although the available nucleotide sequence of IGHG3 cDNA contained no BamHI site (8), the restriction site could be located within the unknown intervening sequences of this gene. Nucleotide sequencing of the genomic IGHG3 gene of BAC clone 60B11 (2.6-kb BamHI fragment in Fig. 1) was performed in this study and revealed no BamHI site in the equine IGHG3 gene (EMBL/GenBank accession no.AY256910). Consequently, the entire IGHG3 gene is located on the 2.6-kb BamHI fragment, and we suggested that the 6.2-kb BamHI fragment might represent a seventh IGHG gene.

A Southern blot analysis of BamHI-digested genomic DNA of 25 unrelated horses of seven different breeds using the IGHG1 cDNA probe for hybridization was performed to investigate whether the 6.2-kb BamHI fragment is detectable throughout the horse population or whether it appeared only in a selected breed or an individual horse, e.g., the horse used for construction of the library. Using the genomic DNA of seven different horse breeds, the hybridization pattern for the IGHG genes corresponded to those observed previously for Trakehner and Icelandic horse families (2, 9). To date, the 6.0- and 6.2-kb BamHI fragments have been detected in all horses and breeds we investigated (data not shown).

To confirm the existence of individual IGHG genes on the respective BamHI fragments hybridizing to the IGHG1 cDNA probe, the IGHG1 to IGHG6 overgo probes were used for identification of their corresponding BamHI fragments on the BAC clone 60B11 (G1–G5). The IGHG overgo probes were designed from nonconserved parts of the IGHG1 to IGHG6 genes. Thus, they should identify their corresponding IGHG gene only, i.e., hybridize to one BamHI fragment each.
FIGURE 4. Comparison of the IGHG4 and IGHG7 genes of the BAC clone 60B11 with the IGHG4 gene described previously (AJ302057). A, Nucleotide sequence comparison of the genomic DNA. The splice signals are underlined. B, Predicted amino acid sequences. The IGHG4 and IGHG7 genes of BAC clone 60B11 have EMBL/GenBank accession numbers AY445518 and AY445517, respectively.
G7), containing the IGHG3 gene, but no IGHG4 gene. This indicated that the equine IGHG7 gene is located between the IGHG3 and IGHG4 genes.

Overgo probes are only 40 bp in length; thus, it is very likely that both BamHI fragments hybridizing to the IGHG4 overgo probe represent one IGHG gene each. In the case of an RFLP that creates a new BamHI site within the 40-bp sequence where the hybridization occurred, it might be possible to obtain two hybridizing fragments from a single gene on one BAC clone. According to the nucleotide sequence of the genomic equine IGHG4 gene, which was described previously and originated from an Arabian horse (EMBL/GenBank accession no. AJ302057), no BamHI site occurred within the IGHG4 gene.

To verify that the seventh IGHG fragment really represented an additional IGHG gene, we isolated a mixture of the 6.0- and 6.2-kb BamHI fragments from BAC clone 60B11 and used them as a template for amplification of the entire IGHG4 gene by PCR. A total of 10 plasmid clones containing the amplified 1.5-kb PCR product were analyzed by nucleotide sequencing. Five of the PCR products had a homology of 99% to the nucleotide sequence of the prototype IGHG4 gene from an Arabian horse, showing five single base exchanges over the entire genomic nucleotide sequence. Five additional PCR products had a homology of only 96% to the IGHG4 gene, with 45 single base exchanges, three deletions, and six insertions and the most distinct sequence divergence in the hinge region (Fig. 4A). This newly found IGHG gene was designated the IGHG7 gene of the horse.

The BAC clones 109K03 (M-G7) and 456N04 (G1–G7) represented the (IGHp) and (IGHβ) haplotypes, respectively. Both clones contained the 6.2-kb BamHI fragment, but no 6.0-kb BamHI IGHG4 fragment (Fig. 1). To confirm that the IGHG7 gene of clone 60B11 is identical with the IGHG gene located on the 6.2-kb BamHI fragment for both IGHC haplotypes, the 6.2-kb BamHI fragments of BAC clones 109K03 and 456N04 were isolated. As the most prominent divergences between the IGHG4 and IGHG7 genes were found in the hinge region, fragments containing the genomic CH1–CH2 region were amplified by PCR from the 6.2-kb BamHI fragments of both BAC clones and sequenced. Both PCR products were 100% identical with the IGHG7 gene sequence shown in Fig. 4A. This confirmed that the 6.2-kb BamHI fragment corresponds to the equine IGHG7 gene and that both the 6.0- and 6.2-kb BamHI fragments represent individual IGHG genes, namely the IGHG4 and IGHG7 genes of the horse.

The IGHG4 overgo probe, which has been shown in Fig. 3 to hybridize with the IGHG4 and IGHG7 genes of clone 60B11 (G1–G5), is located in the CH3 exon at position 1430–1469 of the IGHG4 gene (Fig. 4A). This region is conserved between the IGHG4 and IGHG7 genes, explaining the hybridization of the probe with both genes.

Although the IGHG4 and IGHG7 genes represent individual genes, their overall nucleotide sequence homology of 96% is high compared with their homologies with the five remaining IGHG genes of the horse, ranging between 79 and 85% (Table IV). The comparison of individual exon and intron sequences of the IGHG4 and IGHG7 genes also indicated a high homology ranging from 94–99% for the CH1 to CH3 exons as well as for the IVS1 to IVS3. However, the only exception was the hinge region, with only 74% homology between both genes, which was even lower than the homology between the IGHG7 and IGHG6 hinge regions (83%). The hinge region of Igs is known to be the part of highest diversity, i.e., fastest evolutionary changes (8, 20, 21). The equine IGHG4 and IGHG7 genes, with their high degree of conservation throughout their genomic sequences, except for the hinge exons, underline this fact in a remarkable way.

The predicted amino acid sequences obtained from the IGHG4 and IGHG7 genes of BAC clone 60B11 indicated a total of eight amino acid changes, seven in the CH2 domain and one in the hinge region (Fig. 4B). Due to one additional amino acid deletion and two insertions, the hinge region varied in length between 11 and 12 aa in the IgG4 and IgG7 H chains. Between the IgG4 H chain constant region obtained from the sequence of the BAC library clone 60B11 (G1–G5) and the IgG4 H chain sequence of the Arabian horse described previously, only two amino acid changes in the CH3 domain were detected. These changes were conserved between the CH3 domains of IgG4 and IgG7 from the Thoroughbred horse used for construction of the BAC library (Fig. 4B).

Regarding the nucleotide sequences of the remaining five IGHG genes (Table IV), sequence homologies to the IGHG4 and IGHG7 genes >90% were found only for the CH1 exons of the IGHG3 (92%) and IGHG6 (96%) genes and for the IVS1 of the IGHG7 and IGHG5 genes (91%). Our previous investigations indicated that several gene conversion or unequal crossing-over events occurred during evolution of the IGHG genes of the horse, resulting in a high degree of segmental homology (8). This means that in addition to IGHG gene duplications, generating the seven IGHG genes of the horse we observe today, various segments of these genes were exchanged during evolution. The high degree of segmental homology between the IGHG1 to IGHG6 genes of the horse makes it very difficult, if not impossible, to determine the frequency of their development or a clear relationship between

| Table IV. Nucleotide sequence comparison of the IGHG4 and IGHG7 genes of the BAC clone 60B11 with the IGHG1–IGHG6 genes of the horsea |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | IGHG1 (AJ302055) | IGHG2 (AJ302056) | IGHG3 (AY256010) | IGHG4 (AJ302057) | IGHG5 (AJ312380) | IGHG6 (AJ312381) |
| Overall         | 83/82            | 79/79            | 93/83            | 99/96            | 83/84            | 85/84            |
| Genomic         | 83/83            | 80/79            | 80/80            | 99/96            | 83/83            | 87/86            |
| Coding          |                 |                 |                 |                 |                 |                 |
| Exon/IVS        |                 |                 |                 |                 |                 |                 |
| CH1 exon        | 87/86            | 88/88            | 92/92            | 99/99            | 78/78            | 96/96            |
| IVS1            | 86/85            | 83/82            | 81/79            | 100/95           | 89/91            | 90/89            |
| Hinge exon      | 35/43            | 27/35            | 18/23            | 100/74           | 48/72            | 61/83            |
| IVS2            | 76/75            | 69/68            | 77/75            | 100/96           | 87/85            | 67/65            |
| CH2 exon        | 84/80            | 74/72            | 82/80            | 100/95           | 86/84            | 84/82            |
| IVS3            | 73/73            | 71/72            | 88/90            | 100/94           | 64/68            | 67/70            |
| CH3 exon        | 88/89            | 83/84            | 86/87            | 98/97            | 86/86            | 83/83            |

a The numbers indicate the percent homology between the IGHG4 and IGHG7 genes (IGHG4/IGHG7) of 60B11 and the respective IGHG1 to IGHG6 gene sequences. All calculated homologies >90% are underlined. The EMBL/GenBank accession numbers of the IGHG1–IGHG6 genes used for the calculation are given in parentheses.
individual IGHG genes of the horse. In contrast, the highly conserved nucleotide sequences of the IGHG4 and IGHG7 genes we found in this study and the absence of segmental homologies in between them suggested that these two IGHG genes duplicated most recently during evolution of the equine IGHG genes.

In previous studies the IGHG4 gene was found to be expressed at the mRNA and protein levels (8, 9). To determine whether the equine IGHG7 gene is also expressed at the mRNA level and what the relative usage rates of the IGHG4 and IGHG7 genes are, both genes were amplified from cDNA obtained from PBMC of three different horses. A PCR was performed using a common primer pair of the IGHG4 and IGHG7 genes and resulted in a PCR product of ~1 kb. A total of 20 cloned PCR products from each horse were sequenced and analyzed to distinguish between the IGHG4 and IGHG7 genes. The mRNA expression of both genes could be detected in all three horses. Consistently, the IGHG4 gene was the predominant gene (Table V). The average mRNA expression ratio was calculated from all 60 cDNA clones and resulted in the IGHG7:IGHG4 ratio of 1:1.6.

Table V. Relative mRNA expression rates of the IGHG4 and IGHG7 genes in PBMC from three different horses

| Horse | No. of cDNA Clones | Ratio IGHG7:IGHG4 |
|-------|-------------------|------------------|
| 3157  | 11 9              | 1:1.2            |
| 3454  | 14 6              | 1:2.3            |
| 3474  | 12 8              | 1:1.5            |
| Total | 37 23             | 1:1.6            |

*Both IGHG genes were amplified from cDNA by PCR using a common primer pair from conserved regions of both genes. The PCR products were cloned in a plasmid vector, and nucleotide sequences of 20 cDNA clones from each horse were analyzed. Horse 3474 was the animal used to generate the BAC library.*

To date, no IGHD gene encoding IgD has been described in the horse. Until recently, it was assumed that the IGHD gene, which is located downstream of the IGHM gene, exists in humans, mice, and rats and a couple of lower vertebrates only, but not in other mammalian species. Zhao and coworkers (21) recently described the IGHD genes of cattle, sheep, and pig. In this study the nucleotide sequence of the genomic equine IGHD was obtained from BAC clone 52M17 (M-G1). The entire genomic IGHD gene spans ~9.1 kb and is composed of eight exons, including the CH1, CH2, and CH3 exons, two hinge exons, a secretion exon, and two membrane exons (Fig. 5). The exon/intron boundaries of the IGHD gene were confirmed by PCR of cDNA from equine PBMC. The secreted form of the equine Igδ H chain constant region is encoded by 1175 bp, and the transmembrane form is encoded by 1256 bp, resulting in H chain constant regions of 391 and 418 aa for the secreted and transmembrane IgD, respectively (Fig. 6). The coding nucleotide sequence containing the CH1 to CH3 exons of the equine IGHD gene was compared with those of other mammalian species and showed overall homologies of 64% to human and porcine and of 61% to bovine and ovine IGHD genes. Within the IGHD genes, highest sequence homologies were found between the CH2 and CH3 exons (68–79%) and for the transmembrane exon (74–78%), whereas the CH1 exon, the two hinge exons, and the secretion exon were less conserved between these species (Table VI).

An IGHD overgo probe (Table I) was designed from the CH2 exon of the equine IGHD sequence. The IGHD and IGHM overgo probes were used for hybridization of BamHI-digested equine IGHBC DNA. All BAC clones, which contained the IGHM gene, also hybridized with the IGHD overgo probe on a single 8.5-kb BamHI fragment (Fig. 7A). The genomic IGHM gene was located on a BamHI fragment of ~20 kb, suggesting a BamHI site in the intron between the IGHM and IGHD genes (Fig. 7B). To confirm the position of the IGHD gene downstream of the equine...
IGHM gene, a long template PCR was performed to amplify the intron sequence between both genes from BAC clone 52M17. A 9.3-kb PCR product was obtained, spanning the CH2 exon of the equine IGHM gene to the CH1 exon of the IGHD gene (Fig. 7C). Nucleotide sequencing of this PCR product indicated that the equine IGHD gene is located 5 kb downstream of the IGHM gene (Fig. 7D). No evidence of any switch region was found within the IGHM-IGHD intron sequence. In addition, the 5’ end of the genomic IGHM gene was amplified by PCR from BAC clone 52M17. The equine genomic IGHM gene is composed of six exons, encoding the CH1, CH2, CH3, and CH4 exons (the latter also includes the secretory regions), and two transmembrane exons (Fig. 7D). The coding sequence of the CH1 to CH4(S) exons of the genomic IGHM gene from BAC clone 52M17 (GenBank accession no. AY631941) has 96% nucleotide sequence homology to the secreted form of the IGHM gene obtained from cDNA described previously (3). The data confirmed that the horse IGHC region contains an IGHD gene that is expressed at the RNA level and is located 5 kb downstream of the IGHM gene.

The complete IGHC region is located on horse chromosome 24. The IGHM, IGHD, seven IGHG, IGHA, and IGHE genes were aligned using the overlapping BAC clones to generate a map of the entire IGHC region of the horse (Fig. 8). In summary, the IGHC region of the horse contains 11 Ig H chain constant genes per haploid genome, which are arranged in the order 5’-M-D-G1-G2-G3-G7-G4-G6-G5-E-A-3’. The entire IGHC region was found to be located on a minimum of two overlapping BAC clones, e.g., 351M20 (M-G4) and 163G06 (G3-A). The average size of the equine insert per BAC clone is 171 kb. Thus, the size of the entire IGHC region can be estimated as 250–350 kb, resulting in an average intron size between individual IGHC genes of 25–35 kb. The size of the intron between the equine IGHE and IGHA genes was determined previously and is ~11 kb (1). The intron between the IGHM and IGHD gene is 5 kb (see above). For all other equine IGHC genes, we suggest that the intron between the IGHD and the IGHG1 gene is the largest and that the intron size decreases from the 5’ to the 3’ direction, as in the murine IGHC region (11).

**FIGURE 6.** Nucleotide and predicted amino acid sequence of the equine IGHD gene from BAC clone 52M17, including its secretion and transmembrane exons. Only the coding nucleotide sequences of the IGHD gene are shown in the figure. The complete genomic nucleotide sequence, including the intervening sequences and 5’- and 3’-untranslated regions, is available from EMBL/GenBank (AY631942). The sizes of the individual exons are given in parentheses and correspond to the 10,487-bp EMBL/GenBank sequence. Potential N-glycosylation sites are boxed.
The chromosomal location of the equine IGHC region was determined by FISH (Fig. 9) using the BAC clone 456N04, containing the IGHG1 to IGHG7 genes. The equine IGHC region was identified on horse chromosome 24 (Equus caballus (ECA) 24qter). ECA24 has been identified before to be the homologue of human chromosome 14, which carries the human IGH locus (22).

Discussion

The principle of different Igs classes (IgM, IgG, IgA, IgE, and IgD) sharing individual effector functions during the immune response is generally conserved between the mammalian species. Nevertheless, the number of existing Ig isotypes varies considerably in different mammals. For example, rabbits express 13 IgA isotypes (12), whereas humans have two IgA Abs (23), and most other mammalian species have only one IgA (7, 13, 15, 23–27). In addition to variations in the number of isotypes, a structural diversification of IgG occurred in llama and camel, where functional H chain Abs are expressed as well as the normal IgG, composed of two H and L chains each (28; reviewed in Ref. 29).

The evolution of the IGHG genes is believed to have begun ~600 million years ago (30). The seven IGHG genes found today in the horse provide another remarkable example for Ig H chain constant diversity. To date, no other species has been described to contain so many IGHG genes in the IGH locus. Probably all seven

| Nucleotide Sequence Homology to Equine IGHD (%) | Overall (CH1–CH3)\(^a\) | CH1 | H1 | H2 | CH2 | CH3 | S | M |
|-----------------------------------------------|-------------------------|-----|----|----|-----|-----|---|---|
| Human                                        | 64                      | 58  | 46 | 36 | 73  | 71  | 27|74 |
| Porcine                                      | 64                      | 48  | 39 | 39 | 74  | 79  | 63|NA |
| Bovine                                       | 61                      | 46  | 48 | 60 | 70  | 71  | NA|78 |
| Ovine                                        | 61                      | 47  | 54 | 58 | 68  | 73  | NA|76 |

\(^a\) The overall homologies of the coding regions of the CH1 to CH3 exons, excluding the secretion and transmembrane exons, were compared. In addition, the homologies between individual CH1, CH2, CH3 exons, hinge exons (H1 and H2), secretion exons (S) and transmembrane exons (M) were calculated. NA, not available.

\(^b\) The entire sequence of the porcine hinge region was compared to the entire equine hinge region.

Table VI. Nucleotide sequence homologies between the equine IGHD gene and those of human (K02878), porcine (AF411239), bovine (AF411240), and ovine IGHD (AF411238)

**FIGURE 7.** The IGHD gene of the horse is located 5 kb downstream of the IGHM gene. A and B, Southern blot analysis of BamHI-digested DNA obtained from 12 BAC clones, nine of which contain the IGHM gene. The membrane was hybridized to the IGHD (A) or IGHM (B) overgo probes. C, A long template PCR was performed to amplify the intron between the IGHM and IGHD genes of BAC clone 32M17. The primers were located in the CH2 exon of the IGHM (sense) and the CH1 exon of the IGHD genes (antisense); lane 1, 1-kb ladder; lane 2, long template PCR product; and lane 3, λ/HindIII ladder. D, A PCR was performed to amplify the CH1 to CH3 exons of the IGHM gene. This 1187-bp PCR product and the 9295-bp IGHM-IGHD intron PCR fragment shown in C were analyzed by nucleotide sequencing and resulted in the map of the equine genomic IGHM gene and the intron spanning the IGHM and IGHD genes of the horse (EMBL/GenBank AY631941).
IGHG genes of the horse are expressed (see below), implicating their functional relevance. In other mammalian species the numbers of IgG isotypes vary considerably depending on the number of functional IGHG genes, ranging from one in the rabbit (12); three in cattle (14); four in the human (10), mouse (11), and rat (13); and five in the pig (15). However, these numbers also describe the usual number of IGHG genes in the haploid genome, and exceptions have been found in both humans and mice.

For the human IGHG genes the usual number is five per haploid genome, including one pseudogene that is not expressed (10, 31). In different populations, polymorphic multiple gene deletions, duplications or triplications, have been described (32), resulting in a variation in the number of human IGHG genes ranging from one to nine (33). In BALB/C mice, four IGHG genes are found (11). Due to a duplication of the IGHG2 gene, five IGHG genes were identified in other mouse strains or wild mice (34–36).

In contrast, the duplication event that resulted in the IGHG4 and IGHG7 genes of the horse is very widespread in this species. The corresponding 6.0- and 6.2-kb BamHI fragments have been detected in each individual and breed investigated to date. This includes Thoroughbred horses, which have been bred without any foreign influences by other breeds for at least 1000 years. Thus, it is likely that the IGHG4 and IGHG7 genes are present in most, if not all, horse breeds.

The phylogenetic analysis of the IgG H chain constant regions shows a strong relationship within most mammalian species, as reflected by bootstrapping values of >800 (8). In contrast, the previously described evolutionary clustering of the IgG1 to IgG6 H chain regions of the horse resulted in a higher degree of variation than usually observed for the IgG H chains of one species (8). This was indicated by remarkably low bootstrapping values (<500) as a result of multiple gene conversion events within the genomic IGHG gene sequences of the horse. Nevertheless, the IGHG4 and IGHG7 genes share high sequence homologies at both the nucleotide and amino acid sequences. The high sequence homology of both genes and the lack of gene conversion between the IGHG4 and IGHG7 and the remaining equine IGHG genes indicate that they duplicated most recently during evolution of IGHG genes of the horse.

Decades ago, the Ig isotypes of the horse were investigated by serological and biochemical analyses (reviewed in Ref. 37) and in the past 6 years have also been studied using mAbs (38, 39). In these studies, five IgG subclasses of the horse were described, designated IgGa, IgGb, IgGc, IgG(T), and IgG(B), with the last initially named equine aggregating Ig. The molecular characterization of the IGHG genes indicated that the Abs of the horse might be even more complex than previously assumed. The IgG1 (IgGa), IgG3 (IgG(T)), and IgG4 (IgGb) isotypes were linked to their corresponding IGHG genes by deletion analysis of equi-murine heterohybridomas (9). The former IgG(T) of the horse was found to be composed of two different isotypes, namely IgG3 and IgG5. The latter was identified by comparing a C-terminal 18-aa sequence of purified equine IgG(T) (40) with the predicted amino acid sequences of IgG1 to IgG6 H chain constant regions (8). In addition, equine IgG2, IgG5, and IgG6 have been expressed recently in mammalian cells (A. Wege, W. Leibold, and B. Wagner, unpublished observations) using a system to generate recombinant hapten-specific Igs (41). The IGHG7 gene encodes for a complete H chain constant region, and its corresponding genomic 6.2-kb BamHI fragment, which was suggested in earlier studies to represent the 5′ part of the IGHG3 gene, also contains a switch region (2, 9). In the current study the expression of IGHG7 mRNA was detected in equine PBMC. The relative expression ratio of the
The existence of an equine IGHD gene indicates that the ancestral IGHC gene duplication leading to the IGHD gene also occurred in the horse, and thus most likely took place early in mammalian evolution. This is consistent with the results reported by Zhao and coworkers (21), suggesting the presence of the IGHD gene in the genome and a biological function for IgD in most mammalian species.

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