Physical Mapping of the Bovine Immunoglobulin Heavy Chain Constant Region Gene Locus

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Bovine antibodies have recently attracted increasing attention, as they have been shown to exhibit prophylactic and therapeutic properties in selected infectious diseases in humans. In the present study, we have isolated bacterial artificial chromosomes and cosmids clones containing the bovine JH, μ, δ, γ, and α genes, which allowed us to make a count of the genes within the bovine IGHC locus. The genes are arranged in a 5’–JH–7 kb–μ–5 kb–δ–33 kb–γ–20 kb–γ1–34 kb–γ2–20 kb–ε–13 kb–α3’ order, spanning ~150 kb DNA. Examination of the bovine germine JH locus revealed six JH segments, two of which, JH1 and JH2, were shown to be functional although there was a strong preference for expression of the former. Sequence alignment of the bovine 5’ Eμ enhancer core region with those of other mammals, demonstrated an absence of the μE3 motif and a shortened spacer between the μA and μB sites within the bovine Eμ enhancer core region. Furthermore, the essential sequence element for class switching, switch μ, spanning ~3-kb repetitive sequence and abundant in the switch region motifs CTGGG (187 repeats) and CTGAG (127 repeats), was identified immediately upstream of the μ gene. A further sequence comparison revealed that the bovine IGHC genes display an extensive polymorphism leading to expression of multiple antibody allotypes.

The mammalian immunoglobulin heavy chain constant region gene (IGHC) loci have only been well characterized in humans and mice (1, 2). Five classes of immunoglobulin heavy chain constant region genes, μ, δ, γ, and α are present in both species, although the number of subclasses of γ and α vary. The locus in the mouse (JH–μ–δ–γ3–γ1–γ2b–γ2a–ε–α) differs from that in the human (JH–μ–δ–γ3–γ1–ψ2–α1–ψγ–γ2–ψy–γ4–ε1–α2) due to a duplication during evolution of the latter. While six functional JH segments and 3 pseudogenes have been identified in the human JH locus (3), the corresponding locus in the mouse only contains four functional genes and two pseudogenes (4). In addition, 27 D segments (5) and 123 VH segments (of which 79 are pseudogenes) (6), have been identified by sequencing of the human VH and DH loci.

In ruminants such as cow and sheep, genes encoding μ, δ, γ, α, ε have all been described (7–14). Like other mammalian species, multiple γ genes, three in the cow (7, 11) and two in the sheep, have been identified (15), whereas the α and ε genes exist as single copy genes (7). The functional bovine IGHC genes have been assigned to the Bos taurus chromosome 21q23-q24 (16, 17), whereas the lambda light chain constant region gene maps to chromosome 17 (18). Interestingly, a bovine μ gene-like sequence, IGHML1, was previously detected on chromosome 11q23 (16, 19). Although all seven bovine IGHC genes have been shown to be transcriptionally active in vivo or in vitro, the δ and γ3 genes are likely to be expressed at a very low level.

The bovine immunoglobulins have been used to a restricted set of VH genes (20), some of which contain unusually long CDR3 regions (21). In addition, an examination of all the available expressed bovine VH sequence in the NCBI GenBankTM suggests that only a single JH gene is employed.

Recently, bovine immunoglobulins, have attracted increasing attention, as they have been shown to exhibit prophylactic and therapeutic effects in selected infectious diseases in humans and animals (22–26). Transgenic calves, expressing human immunoglobulins, have also recently been generated (27), although the restricted transport of human immunoglobulins into colostrum/milk may limit their usefulness. In the present study, we have characterized the bovine IGHC gene locus, aiming to promote a better understanding of the evolution and expression of the mammalian IGHC genes.

EXPERIMENTAL PROCEDURES

Bovine BAC and Cosmid Libraries—Bovine IGHC gene-positive BAC clones were isolated from a previously constructed BAC library (28), which was generated using pBeloBAC11, a BAC vector. A bovine pWE15 cosmids library was purchased from Stratagene (La Jolla, CA). All screenings were carried out using a PCR procedure. The BAC and cosmid DNA was amplified in Escherichia coli, DH10B and XLI-Blue MR, respectively. The preparation of both BAC and cosmid DNA was performed using the Qiagen-tip 500 (Qiagen, Valencia, CA) following the protocol from the manufacturer. Bovine high molecular weight genomic DNA was isolated by phenol-chloroform extraction of proteinase K-digested blood samples.

Pulsed Field Gel Electrophoresis (PFGE) and Southern Blotting—PFGE was used to separate large DNA fragment (CHEF-DR, III system, Bio-Rad, Hercules, CA). The DNA was run in a 1% agarose gel in 0.5× TBE buffer (6 V/cm). The switch time was adjusted based on the size of DNA, according to the manufacturer’s instruction. The separated DNA was transferred to nylon membrane for hybridization. An oligolabeling kit (Amerham Biosciences) was used to label the probes. All hybridizations were performed with ExpressHyb hybridization solution
RESULTS

Screening of the Bovine BAC and Cosmid Library—Using a PCR screening strategy, 21 μ, 18 γ3, 15 ε, and 1 α positive clones were isolated from the bovine pWE15 cosmid library. Out of these clones, six were shown to be positive for the μ, δ, and γ3 genes, and one clone contained both the ε and α genes.

Four BAC clones were obtained by screening of a BAC library (28), where BAC66R4C11 contained the μ, δ, γ3, γ1 genes, BAC416R4C8 contained the μ, δ, and a part of the Cy3 gene, BAC389R7C7 contained the γ1 and γ2 genes, while BAC412R7C5 contained only the γ1 gene according to results based on Southern blotting and PCR analysis. The NotI-digested BAC DNAs were run on a PFGE agarose gel, showing the size of the respective inserts (185, 115, 55, and 45 kb in BAC66R4C11, BAC416R4C8, BAC389R7C7, and BAC412R7C5) (Fig. 1A).

Cloning and Sequencing of the Bovine Sυ Region—A genomic fragment covering the JH and Cy1 was amplified from BAC66R4C11, and subsequently digested with KpnI and XhoI. The resulting 4.2-kb XhoI-KpnI fragment was initially cloned into the pBluescript II KS (+) vector. The recombinant plasmid was cut again, using SacI and KpnI, to generate an 800-bp SacI fragment, and an ~3.4-kb SacI-KpnI fragment. As the 800-bp SacI fragment was directly cloned into pGEM-T(easy) system (Promega, Madison, WI), the common parameters for the PCR were 94 °C for 30 s; 61 °C for 1 min; and 72 °C for 1 min. These PCR products were separated on an agarose gel and subsequently sequenced.

Computational Analysis of DNA Sequences—DNA sequence homology search was carried out using the NCBI BLAST program. The dot plot comparison was performed using the MegAlign program (DNASTAR, Inc, Madison, WI). The alignment, editing and comparison, was performed using the MegAlign program. The computational analysis was carried out using the MegAlign program (DNASTAR, Inc, Madison, WI). The dot plot comparison was performed using the MegAlign program.
amplification generated a \( -14 \)-kb (Fig. 1B3) band, which was consistent with the analysis of the cosmid clone. These two results also confirmed previous data from analysis of overlapping phage clones (7).

As we did not obtain any clone that contained both the \( \gamma_2 \) and \( \varepsilon \) genes, there was an obvious difficulty to determine the distance between the two genes. However, a comparison with the physical maps of the human and mouse IGHC gene loci, suggested that a less than 25-kb distance between the two genes. Therefore, we attempted to amplify the intron fragment employing a long PCR kit under stringent conditions, using bovine genomic DNA as a template. A weak 20-kb PCR fragment was seen after separating the PCR reactions on an agarose gel, indicating that the size of the bovine \( \gamma_2-\varepsilon \) intron is probably not largely different from those of humans and mice. To confirm this conclusion, we analyzed the clone BAC389R7C7, which was found to contain \(-8\) kb of sequence downstream of the \( \gamma_2 \) gene. We thus sequenced BAC389R7C7 from its \( 3’ \) end to close to the \( \gamma_2 \) gene and designed a new primer BACR7C7-Fas, which was based on the sequence \(-8\)-kb downstream of the \( \gamma_2 \) gene. The primers BACR7C7-Fas and blgELas1 yielded a \( 13\)-kb PCR product when using the bovine genomic DNA (Angus) as a template (Fig. 1B2), suggesting that the size of the \( \gamma_2-\varepsilon \) intron is \( \sim 20 \) kb.

Thus, in summary, the bovine IGHC gene locus is arranged sequentially in the following order: \( 5’-\mu-5 \) kb–\( -\delta-33 \) kb–\( \gamma_3-20 \) kb–\( \gamma_1-34 \) kb–\( \gamma_2-20 \) kb–\( -\varepsilon-13 \) kb–\( -\alpha-3’ \), spanning roughly \( 150 \) kb contiguous DNA on chromosome 21 (Fig. 2A).

**The Three \( \gamma \) Genes Can All Be Functionally Expressed but at Different Levels**—It is well known that the \( \gamma_1 \) and \( \gamma_2 \) genes are functionally expressed at the protein level, as both the IgG1 and IgG2 can be detected in bovine serum (29). The \( \gamma_3 \) gene has only shown to be functionally expressed \textit{in vitro} (7), but the gene can be at least transcribed \textit{in vivo}, as the \( \gamma_3 \) encoding cDNA could be cloned from a bovine spleen cDNA library (12).

A large bovine EST data base containing \( \sim 319,775 \) EST clones (Jun 8, 2003), is available in NCBI GenBank™. Principally, the abundance of genes in the EST data base would roughly reflect their transcriptional levels, as almost all sequences in the EST data base have been obtained by sequencing of randomly picked clones from cDNA libraries. Although the EST sequences are derived from a variety of tissues, we do not expect transcription of immunoglobulin genes in non-lymphoid cells. By searching the NCBI bovine EST data base, more than \( 55 \) \( \gamma_1 \), 27 \( \gamma_2 \), and 11 \( \gamma_3 \) EST clones were identified. In addition, 35 clones were found that could not unambiguously be identified as \( \gamma_1 \) or \( \gamma_2 \). These data indicate that the \( \gamma_3 \) gene is transcribed at a lower level than those of the other \( \gamma \) genes. In addition, more than \( 82 \mu, 5 \delta, 74 \alpha \), and 2 \( \varepsilon \) EST clones could be identified in the data base, suggesting that both the \( \delta \) and \( \varepsilon \) genes are expressed at very low levels in cows.

**The Bovine JH Locus Has Two Functional JH Genes**—To analyze the bovine JH germline sequence and \( 5’-\mu \) intronic enhancer region, a \( -7\)-kb DNA fragment, spanning a part of the JH locus and \( C_\mu \) from the BAC clone 66R4C11 was amplified and sequenced (AY158087). Another \( 2.6 \) kb further upstream sequence was also cloned to ensure that the germline JH locus was identified in its entirety. In the \(-2\)-kb sequence (AY158087), only two potentially functional JH genes, termed JH1 and JH2, were identified, 862 bp apart, encoding 15 and 17 amino acids respectively (Fig. 3). The two JH segments share a five amino acid motif, VTVSS, at their \( 3’ \)-ends.

A BLAST search of the NCBI GenBank™ and bovine EST data base suggests that the JH1 is the only JH used in hitherto reported bovine VH sequences. More than 84 clones in the NCBI Genbank™, and 150 clones from the bovine EST data base were found to contain the JH1 segment, suggesting that the JH2 may represent a pseudogene. However, a close examination of the flanking sequences of JH1 and JH2 shows that they exhibit almost the same \( 5’-\)recombination signal sequences and \( 3’-\)-donor splice sites, although a large sequence difference can be observed in the spacer regions between the heptamer and nonamer. To prove whether the JH2 gene is at all functional or not \textit{in vivo}, bovine genomic DNA and cDNA were both subjected to JH specific PCR as indicated in Fig. 4. Primer P1 (blgVH-Leaders) is derived from the leader exon of the bovine VH genes and supposed to be conserved within most expressed bovine VH genes based on a comparison of VH se-

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**Fig. 2. Physical mapping of the bovine IGHC gene locus.** A, a physical map of the bovine IGHC gene locus. B, structural analysis of BAC389R7C7.
quences (data not shown). The two JH-specific primers, P2 (bIgJH1as) and P3 (bIgJH2as) were designed based on the JH1 and JH2 sequences (Fig. 4B). Theoretically, if VH and DH genes recombine to the JH1 gene, the primer combination P1 and P2 should generate \( \frac{1}{\text{H11011}} \) 500-bp PCR products (Fig. 4A1). In this case, the primer combination P1 and P3 should also be able to yield \( \frac{1}{\text{H11011}} \) 1.3-kb PCR products (Fig. 4A1). On the other hand, if the JH2 gene is functional in vivo and a recombination of VH and DH genes to the JH2 occurs, the primers P1 and P3 would also amplify \( \frac{1}{\text{H11011}} \) 500 bp rearranged genomic VDJ fragments (Fig. 4A2). As shown in Fig. 4C, the PCR results clearly showed that at the genomic level, VH and DH genes recombine to both the JH1 and JH2 genes in bovine B cells. However, the recombination rate to the JH2 is much lower than that of the JH1 according to the intensity of the PCR bands generated using bovine spleen, blood, and bone marrow genomic DNA as templates (Fig. 4C). Both the recombined VDJH1 and VDJH2 genomic fragments can be normally transcribed and spliced, as we could amplify VDJH1 and VDJH2 fragments at the cDNA level. The nature of the PCR products in Fig. 4C was proven by hybridization using a bovine VH gene probe (data not shown). These data thus indicate that the JH2 is expressed but at a low level.

Four ovine JH pseudogenes have previously been identified in a \(-2\)-kb DNA region containing the two functional JH genes (30). In the bovine JH locus, there are also four JH pseudogenes corresponding to the ovine JH segments (Fig. 3), where the RSS and 3’-donor splice sites for the four pseudo-JH segments have diverged from the canonical sequences (Table I).

**The Bovine 5’ Ig Intronic Enhancer**—The putative bovine 5’ \( E_\mu \) intronic enhancer was identified through a comparison of the bovine JH-C\( _\mu \) intron sequence with other well studied mammalian \( E_\mu \) enhancers. As shown in Fig. 5A, the bovine 5’ \( E_\mu \) enhancer core region shows a conserved organization, where several nuclear binding motifs, \( E1 – E5 – E2 – A – B – E4 – O \) (octamer motif), are tightly clustered in a very short region. The most striking feature of the bovine 5’ enhancer core region is that the space between the \( E1 \) and \( E2 \) motifs is shorter than in other mammalian 5’ \( E_\mu \) enhancers (Fig. 5B). Furthermore, AT-rich sequences, which are supposed to be matrix attachment region (MAR), could be identified at either side of the bovine \( E_\mu \) enhancer core region as previously noted both in humans and mice.

**Characterization of the Bovine Switch \( \mu \) (S\( _\mu \)) Region**—The S\( _\mu \) region is located upstream of the \( C_\mu \) gene in mammals. The region is involved in switching to the production of other anti-
body classes, downstream of the Cμ, through non-homologous recombination. A dot plot analysis of the bovine JH-Cμ genomic intron revealed a 3-kb repetitive region (4380–7380, accession number: AY158087) abundant in switch region motifs (CTGGG and CTGAG) similar to both the human and mouse Sμ regions (Fig. 6, A, D, and E). The 3-kb bovine Sμ, containing –187 CTGGG and 127 CTGAG repeats, is slightly shorter than the human Sμ (3.5 kb) but double as long as the mouse Sμ (1.5 kb). The longest repeats that contain the 123-bp DNA fragment (5865–5987, 6053–6175, accession number: AY158087). Another 87-bp long repeat is located from 7018 to 6004 and again from 6012 to 6098. In addition, a 24-bp long sequence (GACA-GAGTGGTGGACTGTCGTCACCTGTCCTCTCCTCA) appears dispersed as 9 repeats within the bovine Sμ.

It was previously proposed that immunoglobulin switch regions are rich in palindromic and stem-loop structures, which serve as targets of switch recombination (31). A dot plot comparison of the bovine JH-Cμ intron and its reverse complementary sequence indicates that the bovine Sμ is indeed abundant in palindromic sequences (Fig. 6B).

**Bovine Immunoglobulin Alotypes**—The bovine IGHC gene sequences obtained in this study were found to contain polymorphic sequences as compared with previously published cDNA or genomic DNA, suggesting allotypic variants. Most strikingly, a bovine δ heavy chain cDNA transcript lacking the CH2 exon, probably resulting from alternative RNA splicing, was identified based on RT-PCR amplification of bovine spleen-derived RNA (32), indicating that there could be two different IgD molecules expressed on the surface of the bovine B cells. The CH2 domain, lacking IgD molecule has previously been only found in rodents (where the CH2-encoding exon has been deleted in the rodent germline Sμ sequences). The biological significance of the co-presence of these two IgD heavy chain molecules in cows remains unclear.

We have previously reported the genomic sequences of both the bovine μ and ε genes (8, 13). Extensive DNA polymorphisms, as summarized in Table II, were found in the coding regions of both genes. Furthermore, sequence variants of the γ1 and γ2 hinge regions were also identified based on a BLAST search of the bovine ETS data base at NCBI (Fig. 7).
Mapping of the Bovine IGHC Gene Locus

The combination of restriction mapping of bovine IGHC genes, positive BAC clones and long PCR amplifications, have allowed us to conclude that the bovine IGHC gene locus is contained within an ~150 kb contiguous DNA as a gene cluster (5’–JH–7 kb–μ–5.1 kb–6–33 kb–γ3–20 kb–γ1–34 kb–γ2–20 kb–ε–13 kb–α–3’), which essentially resembles the structure of the human and the mouse IGHC loci. Consistent with the lesser number of genes however, the bovine IGHC locus is smaller in size than that of human (~350 kb) and mouse (~200 kb) (1, 2).

Investigations on the human IGHC locus, suggests that duplications of entire genes or even a large block of DNA has occurred during its evolution. Interestingly, duplication of individual exon sequences has also recently been observed within the bovine locus (14). The transposition of these exons probably occurred through a process involving reverse transcription and re-integration into the genome and may help explain the location of a Cμ gene like sequence on chromosome 11 (16, 17).

Several publications have suggested that there may be four subclasses of γ genes in the bovine IGHC locus (7, 11). However, we could only identify three γ genes using the available BAC library, as the results derived from Southern blotting and sequencing of PCR products clearly showed that both the BAC66R4C11 and BAC389R7C7 contained the γ1 and one additional γ gene (γ2 and γ2, respectively). Furthermore, as indicated in Fig. 2, there is only ~12 kb DNA that is not covered by the BAC389R7C7 and Cosmid 3 clones. Hybridization of this region with a γ probe did not generate a positive signal, indicating that the suggested fourth γ gene is most probably a polymorphic allele of γ1, γ2, or γ3.

The number of γ genes in mammals varies from one in the rabbit (33), two in sheep (15), three in cow (7), and four in human (34), mouse and rat (2, 35), to 6 in horse (36), suggesting that duplication of the γ gene has occurred at different time points during evolution of the IGHC locus in mammals. As only γ1 and γ2 have been identified in sheep, it is likely that the bovine γ3 gene appeared after the speciation of cows and sheep ~20 million years ago (37). The bovine γ1 gene is most probably the ancestral gene, since it shows an 87.1% similarity to the ovine γ1 at the protein level, which is higher than the similarity between the γ2 in the two species (79.8%). This suggests that, during evolution, the γ1 gene was initially duplicated to form the γ2 gene, then later again duplicated in the cow to generate the γ3 gene. This notion is supported by the greater homology of the γ3 gene with the γ1 gene (85.1%) than with the γ2 gene (83.4%).

The dot plot comparison of the bovine JH-Cμ with that of human and mouse shows that the region from JH locus to the Cμ enhancer, despite some discontinuous sequences with a low degree of homology, is still largely linear (Fig. 6, D and E), indicating a common origin for the mammalian JH and Cμ loci. The JH gene locus, partially contributing to the diversity of the immunoglobulins, has however diverged in mammals not only with regard to the sequences but also the number of the JH genes. The latter vary from only one in pigs (38), four in mice (4), to six in humans and rabbits (3, 39). The bovine JH locus is quite similar to the locus in another ruminant species, the sheep (30). Both loci contain four pseudogenes with abnormal RSS or without normal 3′-splicing sites in addition to the two functional JH genes. An interesting point regarding the JH genes in both ruminant species is that the JH1 gene appears to be extremely preferentially used. The mechanism mediating the quantitatively selective recombination is however not clear as yet. A sequence examination of JH genes and their flanking sequences in cow and sheep did not provide a clue as to why the JH2 gene is only rarely involved in VDJ recombination even in fetal stages, as a varied utilization pattern of JH genes in human and mouse has been demonstrated during different developmental stages (41–43).

Comparison of the JH locus sequence obtained in this study with the sequence derived from NCBI GenBankTM (accession
number: AY149283) suggests that the bovine JH locus is highly polymorphic in different breeds or individuals. All the six JH genes in the two haplotypes differ from each other. Strikingly, the two functional JH genes, JH1 and JH2, showed even greater sequence diversities from their corresponding genes than the four JH pseudogenes. The bovine VH sequences deposited in the NCBI GenBank™ (including the EST database) showed a highly biased usage of the JH1 gene as described in this study. However, neither the JH1 nor JH2 corresponding genes in the previously reported haplotype (accession number:

**Fig. 6. Dot plot analysis of the bovine JH-Cμ intron sequence.** A, a self-comparison of the bovine sequence, window: 30, percentage: 80; B, a dot plot comparison of the bovine JH-Cμ intron with its reverse complementary sequence, window: 30, percentage: 65; C, a dot plot comparison of the bovine JH-Cμ intron with the bovine Cμ-Cδ intron sequences, window: 30, percentage: 80; D, a dot plot comparison of the bovine sequence with the human JH-Cμ sequence, window: 30, percentage: 85; E, a dot plot comparison of the bovine JH-Cμ intron with the mouse sequence, window: 30, percentage: 80.
The sequence positions are based on the \( \mu \) (derived from BAC664RC11, GenBank® accession number: Y2230207) and \( \epsilon \) sequences (GenBank® accession number, Y221098) which have been deposited into the NCBI GenBank®. \( \Delta \) denotes deletion.

| Gene   | Sequence position | DNA variance | Amino acid substitution | Compared to |
|--------|-------------------|--------------|-------------------------|-------------|
| \( \mu \) |                  |              |                         |             |
| 26 CH1 | A → G             | M → V        | U63637                  |             |
| 34 CH1 | C → G             | No change    | U63637                  |             |
| 75 CH1 | A → G             | Q → R        | U63637                  |             |
| 83 CH1 | A → G             | M → V        | U63637                  |             |
| 131 CH1 | G → A         | G → S        | U63637                  |             |
| 159 CH1 | C → A             | A → E        | U63637                  |             |
| 299 CH1 | G → A             | V → I        | U63637                  |             |
| 302 CH1 | A → G             | T → A        | U63637                  |             |
| 305 CH1 | C → A             | P → T        | U63637                  |             |
| 388 CH2 | A → G             | No change    | U63637                  |             |
| 403 CH2 | C → A             | No change    | U63637                  |             |
| 404 CH2 | A → G             | I → V        | U63637                  |             |
| 547 CH2 | C → A             | No change    | U63637                  |             |
| 622 CH2 | A → G             | No change    | U63637                  |             |
| 627 CH2 | A → G             | K → R        | U63637                  |             |
| 710 CH2 | A → G             | N → D        | U63637                  |             |
| 1113 CH3 | T → G         | No change    | U63637                  |             |
| 1139 CH3 | G → A             | G → E        | U63637                  |             |
| 1141 CH3 | A → C             | No change    | U63637                  |             |
| 1264 CH3 | A → G             | T → A        | U63637                  |             |
| 219 CH1 | T → G             | S → P        | U63640                  |             |
| 247 CH1 | C → G             | P → A        | U63640                  |             |
| 293 CH2 | T → C             | No change    | U63640                  |             |
| 338 CH2 | A → C             | No change    | U63640                  |             |
| 730 CH3 | T → C             | No change    | U63640                  |             |
| 904 CH3 | A → G             | No change    | U63640                  |             |
| 1100–1101 CH4 | ACCA | T3       | U63640                  |             |
| 1213 CH4 | C → T             | No change    | U63640                  |             |

**Fig. 7. The hinge region sequences of the bovine \( \gamma \) genes.** A hinge region variation of the \( \gamma_1 \) (10) (also exhibited by EST clones, BE753591, BG688283, BE477986, BG691099, \( \gamma_2 \) (11) (also exhibited by EST clones, BF231464, BG691729), \( \gamma_3 \) (3) (EST clones, BE479646, BG689264), \( \gamma_4 \) (4) (EST clones, BG691650, CA034906, BE489880), \( \gamma_5 \) (5) (EST clones, BE484750, BE589048, BE692035, AW689600, BE483348). (A) \( \gamma_1 \) a DPRECK–TCCDCF. \( \gamma_2 \) a GVB6DCCCKXPNQ. \( \gamma_3 \) a ****EP****, ****EP****, ****EP****, ****EP****, ****EP****. \( \gamma_4 \) a ****E****, ****E****, ****E****, ****E****, ****E****. (B) | REFERENCES |

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