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Artiodactyl IgD: The Missing Link[1,2]

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IgD has been suggested to be a recently developed Ig class, only present in rodents and primates. However, in this paper the cow, sheep, and pig Ig δ genes have been identified and shown to be transcriptionally active. The deduced amino acid sequences from their cDNAs show that artiodactyl IgD H chains are structurally similar to human IgD, where the cow, sheep, and pig IgD H chain constant regions all contain three domains and a hinge region, sharing homologies of 43.6, 44, and 46.8% with their human counterpart, respectively. According to a phylogenetic analysis, the Cδ gene appears to have been duplicated from the Cμ gene >300 million yr ago. The ruminant μCH1 exon and its upstream region was again duplicated before the speciation of the cow and sheep, ~20 million yr ago, inserted upstream of the δ gene hinge regions, and later modified by gene conversion. A short Sδ (switch δ) sequence resulting from the second duplication, is located immediately upstream of the bovine Cδ gene and directs regular μ-δ class switch recombination in the cow. The presence of Cδ genes in artiodactyls, possibly in most mammals, suggests that IgD may have some as yet unknown biological properties, distinct from those of IgM, conferring a survival advantage. ***The Journal of Immunology,*** **2002, 169: 4408–4416.**

Immunoglobulin D was first discovered as a minor component of serum Igs in humans and is mainly expressed on the surface of B cells (1, 2). Subsequently, it was also found in mice and rats (3). Although IgD has been suggested to be present in other mammals (4, 5), no conclusive evidence has been obtained to date, suggesting that IgD is a newly developed Ig class. Homologues of the δ gene, however, were recently found in teleosts, including channel catfish (*Ictalurus punctatus*), Atlantic cod (*Gadus morhua*), and Atlantic salmon (*Salmo salar*) (6–8), suggesting a more ancient origin. In these species the μCH1 exon is spliced onto δ gene-like sequences, yielding chimeric H chains containing a large number of constant domains, but devoid of a hinge segment (6–8).

A comparison of the δ genes in different species suggests that, unlike the μ gene, considerable structural alterations have occurred during evolution, where exon duplications in fish (7, 8) and exon deletions, resulting in a lack of δCH2, in rodents (9, 10) have played key roles in their phylogeny.

The currently held view is that the Cδ genes have been evolutionarily deleted in all other mammalian species except primates and rodents (11, 12). This conclusion is based on experiments that have failed to detect either the IgD protein or the Cδ gene in additional species (11–13). In recent years we have witnessed a rapid progress of research on large scale analysis of animal genomes, where databases for expressed sequence tags (ESTs),[4] facilitate the identification of new genes. In this paper we have identified and characterized the cow, sheep, and pig Cδ genes using an EST-based approach.

Materials and Methods

First-strand cDNA synthesis and 5′,3′RACE PCR

Total RNA was extracted from animal spleen or blood using TRIzol (Life Technologies, Gaithersburg, MD) following the manufacturer's instructions. About 5 μg total RNA was used to synthesize first-strand cDNA with a First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The primers used for the bovine IgD RACE PCR were: bIgD-5′RACE1, 5′-TCA TCG CTG CTC TCG TCC TG-3′; bIgD-5′RACE2, 5′-GCC GTG GCA AAC TGG GAA GG-3′; bIgD-3′RACE1, CAA TCT GAC CCT CCG CAC TG-3′; and bIgD-3′RACE2, GTG GAG CCA GGA CGA GAG CA-3′; they were designed based on a bovine EST sequence (accession no. AW653692). The primers for the cloning of the sheep Ig δ cDNA were: bIg-JH, 5′-GCC AAG GAC TCC TGG TCA CCG TCT C-3′; bIgD-5′RACE2, 5′-GCC GTG GCA AAC TGG GAA GG-3′; and bIgD-3′S, 5′-GAC ATC CTC CTC ACG TGG CTG-3′. The primers for cloning of the pig IgD cDNA were: swine-JH, 5′-CCA GGC GTT GAA GTC GTC GTG T-3′; and a degenerated primer, IgD-CH3-conas, 5′-CRG AYA CYT CRC ACA GGA GCC A-3′. The RACE PCR amplifications were conducted according to the instructions of the 5′RACE System for Rapid Amplification of cDNA Ends (Life Technologies).

Cloning of cow, sheep, and pig full-length IgD H chain-encoding sequences

All the constant region primers were designed based on the sequences derived in this study, while the variable region primers, all located in the leader sequence of the V exon, were designed based on V_H sequences available in the National Center for Biotechnology Information GenBank. Whereas the sheep IgD H chain cDNA was amplified using primers sheep-IgVHs (5′-ACC CAC TGT GGA CCC TCC TCT T-3′) and sheep-IgDas (5′-GGG AGC AGC AGG CAG CGT GGA G-3′), the cow and pig IgD H chain cDNAs were obtained by employing nested PCR. The primers used for amplification of cow IgD were bovine-IgVHs1 (5′-GCT CCA AGA TGA ACC CAC TGT G-3′), bovine-IgDas1 (5′-CAT GAT GCC CTC CTC TTG GTC T-3′), bovine-IgVHs2 (5′-ACC CTC CTC TTT GTG CTC TCA-3′), and bovine-IgDas2 (5′-GGG CTG GGC TCT GTG ATG GAC-3′). The primers to amplify the pig IgD H chain were pig-IgVHs1 (5′-TTC

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[2] All the sequences reported in this paper have been submitted to National Center for Biotechnology Information GenBank with accession numbers AF411238–AF411247.

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[4] Abbreviations used in this paper: EST, expressed sequence tag; BAC, bacterial artificial chromosome; LINE, long interspersed nuclear element; NED, neutral evolutionary distance; S, switch region; UTR, untranslated region.

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GGC TGA ACT GGG TGG TCT T-3′), pig-IgDas1 (5′-CGG GGC TAC TTC ACC TTG AG-3′), pig-IgVHs2 (5′-CGG CTG AAC TGG GTG GTC TTG T-3′), and pig-IgDas2 (5′-GGG GCT ACT TCA CCT TGA GG-3′). The resulting PCR products were all cloned into a T-vector, and after transformation into *Escherichia coli*, randomly picked recombinants were screened using δCH3-based degenerated primers.

Bacterial artificial chromosome (BAC) clones

A bovine μ, γ3, and γ1 gene-positive BAC clone, BAC66R4C11, was isolated from a previously constructed bovine genomic BAC library (14).

Long PCR amplifications of the bovine and ovine genomic fragments

A long PCR kit (Expand Long Template PCR System Kit, Roche Diagnostics Scandinavia, Bromma, Sweden) was employed to amplify the genomic DNA fragment containing the bovine δ gene. Primers bIgMS (5′-GAC TCC TGT GCG ACC CGA TAG-3′) and bIgD-ESTas (5′-CAG CCA CGT GAG GAG GAT GTC-3′) were used to produce clone bMD, and primers bIgD-3′S (5′-GAC ATC CTC CTC ACG TGG CTG-3′) and bIgD-3′As (5′-ATG ATG CCC TCC TCT TGG TCT-3′) were used to produce clone bDE. The exon-intron boundaries were identified by comparison of the genomic sequences with the cDNA sequence of the bovine Cδ gene. The primers used for cloning of the bovine JH-Cμ intron were bIg-JH (as mentioned above) and bIgM-CH2as (5′-GCG GGA CAA AGA CAC TCA CGA CTG G-3′). To amplify the sheep genomic fragment containing the ovine μTM exon and part of the δCH1 exon, the primers sheep IgM-TMs (5′-ACC TTC ATT GTG CTC TTC CTC CTG-3′) and sheep IgD-CH1as (5′-CGC TGC TGA CCG TGC TGT TGT TGA G-3′) were used.

PCR amplifications of the recombined Sμ-Sδ DNA fragments in cow

A nested PCR was used to amplify the recombined DNA fragments created by class switching using Sμ- and Sδ-specific primers Sμ1 (5′-TCT GAG GGT GGC AAG CGT GTC-3′), Sμ2 (5′-AGG GAA GCT AAA GTC GTC AC-3′), Sδ1 (5′-CCT GAG GTC AGC CCA GTG TTG-3′), and Sδ2 (5′-GTC AAG CCC AGG CAG TTC AT-3′). The DNA samples were purified from the peripheral blood of cows in a herd located in a research facility of the Swedish Agricultural University (Uppsala, Sweden). The cows are of the Swedish red and white breed.

Cloning of PCR products, preparation of plasmids, and DNA sequencing

PCR products were recovered from the agarose gel using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA) and were subsequently cloned into the pGEM-T vector (pGEM-T Vector System I, Promega, Madison, WI) with *E. coli* DH5α as a bacterial host. The plasmids and BAC DNA were prepared using QIAprep Spin Miniprep kits and Qiagen plasmid Maxi kits (Qiagen), respectively. The ABI PRISM BigDye Terminator Ready Reaction kit (PerkinElmer, Foster, CA) was used for sequencing.

Northern and Southern blots and restriction enzyme digestion analysis

All the restriction enzymes were purchased from Promega. The Northern and Southern blots were performed using ExpressHyb hybridization solution (Clontech, Palo Alto, CA) according to the manufacturer's instructions. A bovine δCH3-derived DNA fragment (probe A) was used for both Northern and Southern blottings. Another DNA fragment, spanning the bovine δCH3 and membrane-bound form encoding cDNA 3′ end (probe B), was used to visualize both cow and sheep δ genes. The probes were labeled with an oligolabeling kit (Amersham Pharmacia Biotech, Uppsala, Sweden). To identify the bands obtained in Northern blotting, a DNA fragment from the 3′ untranslated region (UTR) of the bovine IgD membrane-bound form cDNA (probe C) was amplified and used in a second Northern blot.

Computational analysis of DNA sequences and construction of phylogenetic trees

A DNA sequence homology search was conducted using the NCBI BLAST program. Sequence alignment and comparison, was conducted using the MegAlign program (DNASTAR, Madison, WI). The dot plot comparison was performed using the same program with the following parameters: percentage, 80; window, 30; min quality, 1. Construction of the phylogenetic trees of IgM and IgD were made using both *dnapars* and *dnaml* (DNA maximum likelihood method) programs from the PHYLIP package (15). A consensus tree was taken from 1000 bootstrapped phylogenetic trees. To estimate the divergence time for different gene sequences, pairwise neutral evolutionary distance (NED) (16) was calculated. NED ($NED = 0.5e^{-kt} + 0.5$; where k is the assumed first order rate constant, and t is the number of years) is an evolutionary distance based upon the rate of 2-fold degenerate synonymous transition and basically represents the proportion of conserved 2-fold degenerate codons (Asp, Cys, Glu, Phe, His, Lys, Asn, Gln, and Tyr) between pairs of nucleic acid sequences, where the differences between each of these codons are represented solely by transitions (16). Except for the bovine, ovine, and porcine δ gene sequences that were generated in this study, all other sequences were obtained from the NCBI GenBank with the following accession numbers: cow IgM, U63637; human IgM, X14940; mouse IgM, J00443; rat IgM, X78895; horse IgM, L49414; pig IgM, U50149; sheep IgM, L04260; rabbit IgM, J00666; hamster IgM, X02804; chicken IgM, X01613; cod IgM, X58870; axolotl IgM, X68700; catfish IgM, M27230; char IgM, X83373; clawed frog IgM, M20484; duck IgM, U27213; trout IgM, X65262; turtle IgM, U53567; human IgD, X57331; mouse IgD, J00447; rat IgD, J00741; catfish IgD, U67437; and salmon IgD, AF141605.

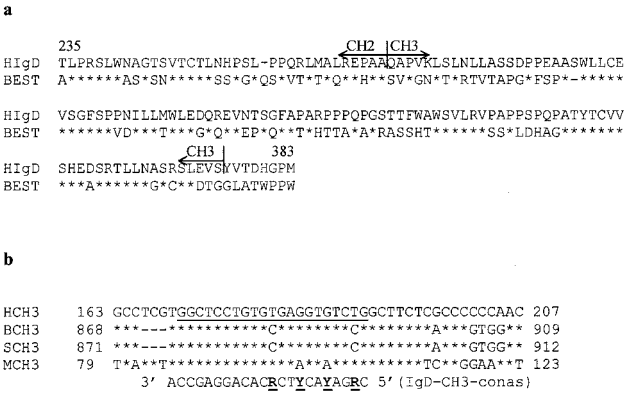

FIGURE 1. Comparison of the deduced peptide sequence from a bovine EST with the human IgD and a degenerated primer design. *a*, Amino acid sequence alignment of the bovine EST with human IgD. Human IgD, human IgD partial sequence; BEST, bovine EST. The numbering of the human IgD sequence is according to a protein data in NCBI GenBank (accession no. P01880). *b*, Design of IgD-CH3-conas-degenerated primer based on homology of IgD CH3 sequences in different species. HCH3, human IgD CH3 (K02879); BCH3, bovine IgD CH3 (AF411240); SCH3, sheep IgD CH3 (AF411238); MCH3, mouse IgD CH3 (J00449). The numbering of sequences is given according to the data in the NCBI GenBank, with accession numbers shown in parentheses. For alignment of sequences, stars indicate the same sequence, and dashes indicate deletions.

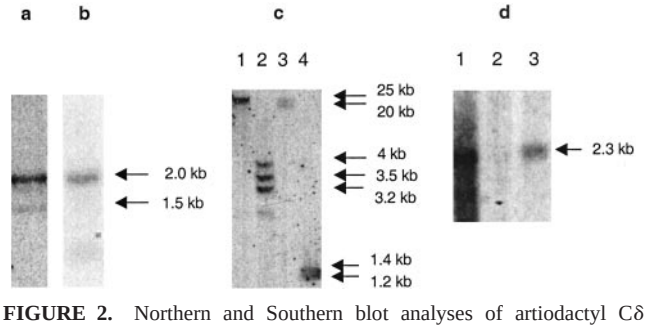

FIGURE 2. Northern and Southern blot analyses of artiodactyl Cδ genes. *a*, Transcriptional pattern of the bovine IgD H chain in spleen detected using probe A. *b*, Transcriptional pattern of the bovine IgD heavy chain in spleen detected using probe C. *c*, Southern blot analysis of ruminant Cδ genes using probe B. 1, *Eco*RI-digested bovine genomic DNA; 2, *Nco*I-digested bovine genomic DNA; 3, *Eco*RI-digested sheep genomic DNA; 4, *Nco*I-digested sheep genomic DNA. *d*, Southern blot analysis of pig Cδ genes using probe A. 1, *Eco*RV-digested pig genomic DNA; 2, *Kpn*I-digested pig genomic DNA; 3, *Pst*I-digested pig genomic DNA.

Results

Molecular cloning of bovine, ovine, and porcine Ig Cδ cDNAs

A homology search of the human Cδ gene sequence using the NCBI BLAST program yielded a bovine EST clone (accession no. AW653692), where the deduced peptide showed a sequence similarity of 53% to the corresponding region of the human δCH2 and δCH3 domains (Fig. 1*a*). This EST clone was derived from a cDNA library made from pooled tissue from lymph node, ovary, fat, hypothalamus, and pituitary (17). The RACE technique was subsequently employed to clone a full-length cDNA from bovine spleen total RNA. Three overlapping clones, bD5E, bDM, and bD3E, containing the 5′ part, the middle part, and the 3′ end of the δ gene, respectively, were obtained. Sequencing of these three clones yielded a typical Ig H chain mRNA transcript containing a rearranged variable region segment and a constant region. The deduced peptide sequence included a typical Ig transmembrane region, indicating that it represented a membrane-bound form of an Ig H chain (accession no. AF411240).

Comparison of the deduced peptide sequence with other bovine Ig H chain constant regions showed sequence similarities of 48.5, 28.3, 28.3, 28.9, 28.3, and 22.4% with the H chain constant regions of IgM (18), IgA (19), IgG1 (20), IgG2 (21), IgG3 (22), and IgE (23), respectively. The first domain is highly homologous to the μCH1 domain with only five amino acid substitutions (4.6%), whereas the hinge region, CH2, and CH3 displayed unique sequences with an overall amino acid homology of only 28% to the last three Cμ domains.

A Northern blot, using mRNA isolated from bovine spleen, was conducted to analyze the transcriptional pattern of the bovine Cδ gene. Two bands were detected using a Cδ gene-specific probe (Fig. 2*a*, probe A), where the 2-kb mRNA transcripts, corresponding to the membrane-bound form of IgD, gave a strong signal. The 1.5-kb band, corresponding to the secreted form of IgD, was weaker, suggesting that the bovine Cδ gene is mainly transcribed as a membrane-bound encoding form in the spleen. The identities of the two bands were confirmed by a second hybridization using a DNA fragment derived from the 3′ UTR of the membrane-bound form encoding cDNA (Fig. 2*b*, probe C).

To search for the Cδ gene in other ruminants, we performed a Southern blot using the bovine Cδ gene as a probe on sheep and

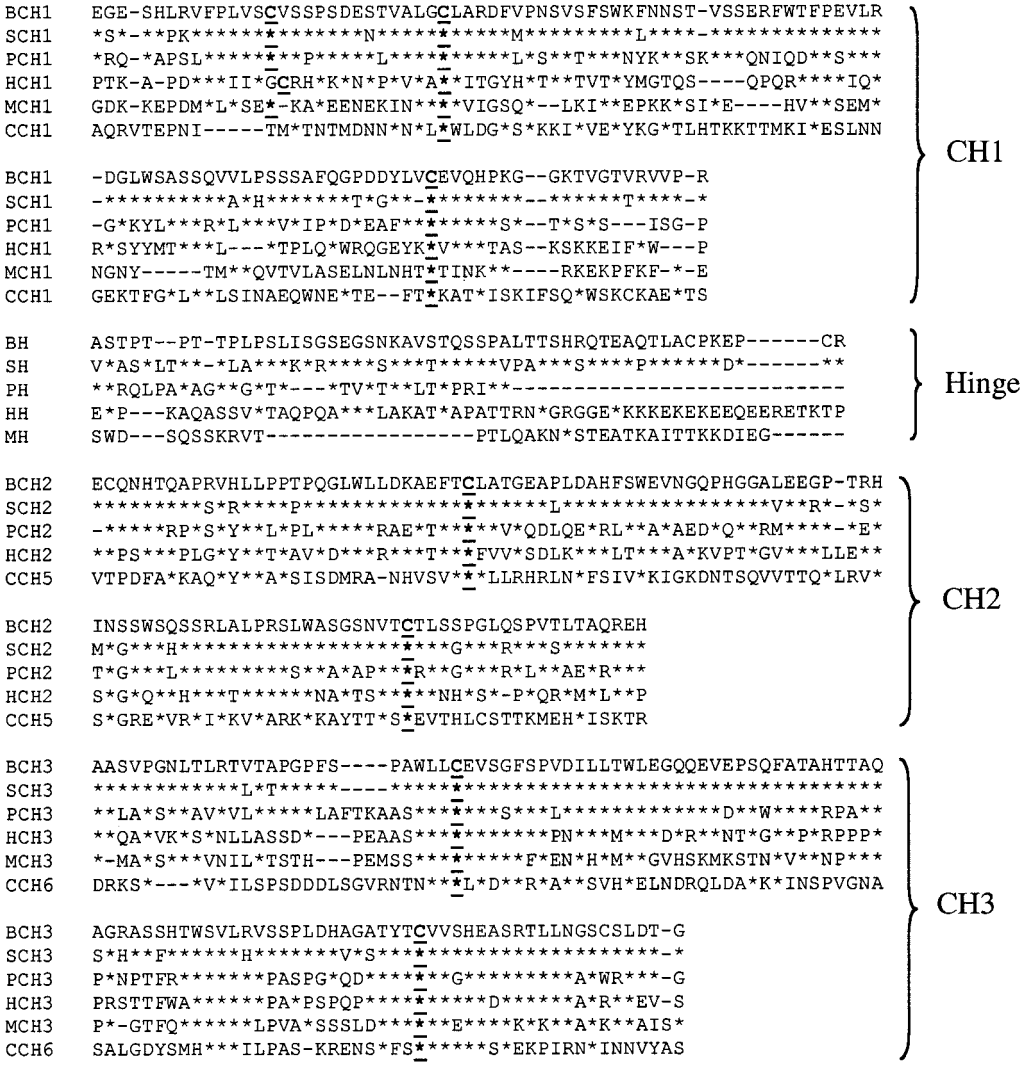

FIGURE 3. A comparison of IgD H chain constant region sequences in different species. B, cow; S, sheep; P, pig; H, human; M, mouse; C, channel catfish. The catfish IgD CH1, CH5, and CH6 domains are aligned with the mammalian IgD domains, showing a maximum homology as previously described (6). While the artiodactyl sequences are derived from this study, the human and mouse sequences are refereed to Refs. 2 and 3. For alignment of sequences, stars indicate the same sequence, and dashes indicate deletions. The conserved cysteines are underlined and in bold.

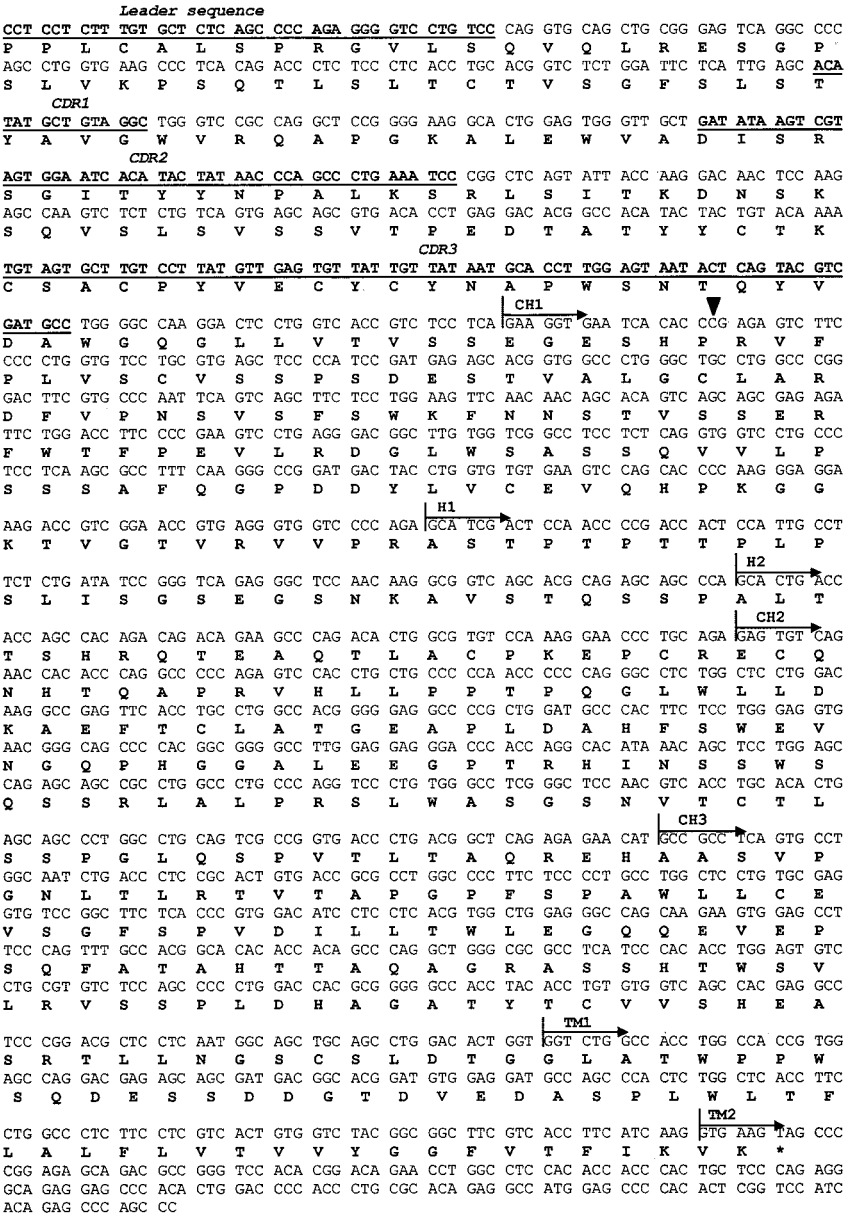

FIGURE 4. Sequence alignment of a bovine IgD H chain encoding cDNA and the deduced amino acid sequence. The exon distinction is based on comparison of cDNA and genomic DNA sequences obtained in this study. ▲, Polymorphism found in different clones where C is replaced by T.

pig genomic DNA. Under stringent hybridizing conditions, positive bands were visualized in restriction enzyme-digested sheep (Fig. 2*c*, probe B) and pig DNA (Fig. 2*d*, probe A), suggesting that both genomes contain a gene homologous to the bovine Cδ. The multiple bands obtained in the bovine *Nco*I-digested sample were probably due to the presence of at least two *Nco*I sites in the probe-spanning genomic region. To clone the sheep δ cDNA, a primer, bIg-JH, designed based on the bovine JH sequence that is known to be highly homologous to the sheep JH, and primer bIgD5′RACE2 were used to amplify the 5′ portion of the sheep Cδ gene from sheep blood total RNA. The 3′ end of a membrane-bound encoding form of cDNA was obtained using 3′RACE PCR with the primer bIgD3′s and an anchored primer.

The deduced sheep Cδ amino acid sequence from the cDNA (accession no. AF411238) shows 87.5 and 44% homology to cow and human IgD, respectively. As in the cow, the first domain is also highly homologous to its μCH1 (24), showing a similarity of 96.6% (Fig. 6*b*) and 93.5% at the DNA and protein levels, respectively, whereas the CH2 and CH3 domains only show an overall amino acid homology of 27.6% to the last two Cμ domains.

A degenerate primer, IgD-CH3-conas (Fig. 1*b*), was designed based on the conserved sequence of the human, sheep, and cow

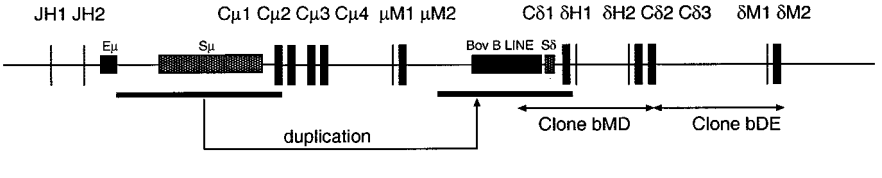

FIGURE 5. Genomic structure of the bovine Cδ gene. The organization of the bovine Ig Cμ gene is as previously described (18). ■, Coding exons; lower thick lines, DNA fragments involved in the putative duplication; Eμ, 5′ intronic enhancer; Sμ, switch μ; Sδ, switch δ.

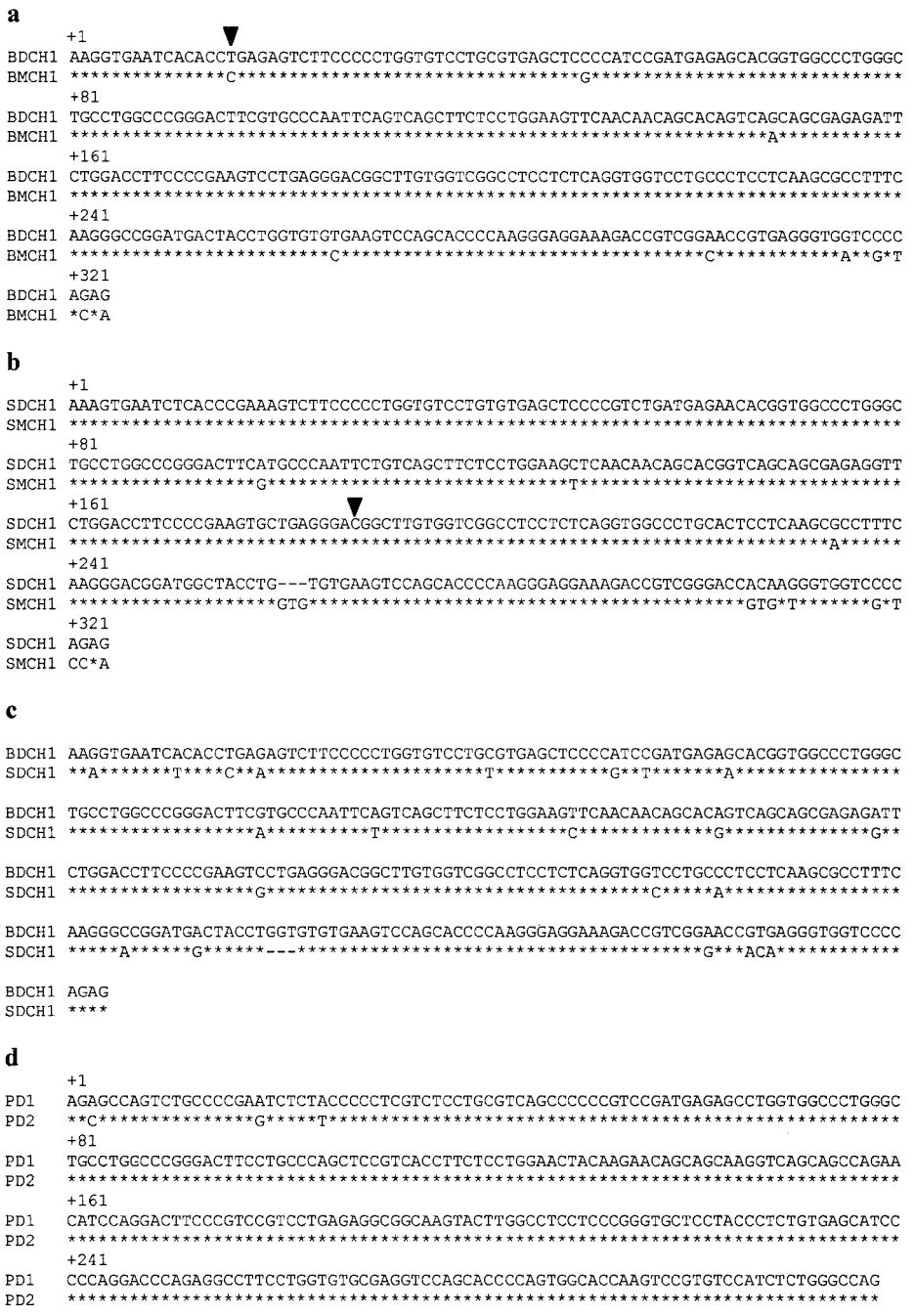

FIGURE 6. Comparison of the IgM and IgD CH1 domains in sheep, cows, and pigs. *a*, Comparison of the bovine IgD and IgM CH1 sequences. BDCH1, bovine IgD CH1; BMCH1, bovine IgM CH1. BDCH1 represents the IgD CH1 sequence from both the bovine IgD H chain cDNA and genomic δCH1 sequence. *b*, Comparison of the sheep IgD and IgM CH1 sequences. SDCH1, sheep IgD CH1; SMCH1, sheep IgM CH1. *c*, Comparison of the bovine and sheep IgD CH1 sequences. *d*, Comparison of CH1-encoding sequences of pig IgD H chain derived from two animals, PD1, First obtained pig IgD CH1-encoding sequence; PD2, pig IgD CH1-encoding sequence derived from the full-length clone from a second animal. ▼, Nucleotides that are polymorphic in different cows and sheep. The indicated T in the bovine δCH1 is replaced by a C in some animals, and the C in the sheep δCH1 is sometimes replaced by a T. For alignment of sequences, stars indicate the same sequence, and dashes mean deletions.

δCH3 domain-encoding sequences to clone the porcine Cδ cDNA. We first cloned the 5′ portion from blood lymphocyte total RNA using the primers swine-JH and IgD-CH3-conas, the former being based on the published porcine JH sequence (11). Furthermore, the 3′ end of a secreted form of δ-encoding cDNA was amplified using RACE PCR, employing the primers, pig-IgD-3′RACE, and an anchored primer.

The porcine IgD (accession no. AF411239) is more similar to cow (60.6%) and sheep (60.9%) than human (46.8%) and mouse (38.4%) when comparing the amino acid sequences of the whole IgD H chain constant region. Sequence data from four independent cDNA clones suggest that, like teleost fish, the pig may use the μCH1, which is spliced onto a short hinge segment and unique CH2- and CH3-encoding exons to produce IgD, since all the sequenced cDNA contained sequences that were identical with the μCH1 (25).

A sequence comparison of the deduced peptides of cow, sheep, and pig δ-chains with those of human, mouse, and channel catfish is shown in Fig. 3.

Cloning of the cow, sheep, and pig full length of IgD H chains

All the above IgD constant region sequences were obtained and compiled using RACE PCR. To prove functionality and integrity of the IgD H chains in these three species, we directly amplified and cloned the IgD H chain cDNAs encompassing both the variable region and the constant region sequences (accession no. AF515672–AF515674). Sequence analysis showed that these cDNAs were functional and encoded normal Ig H chains, as no stop codon resulting in premature termination or other sequence abnormalities were found. Compared with our previously sequenced clones, a single nucleotide polymorphism was found in both cow and sheep δCH1 exons (Fig. 4 and Fig. 6, *a* and *b*). The CH1

Table I. *The exon-intron boundaries of the bovine Ig Cδ gene*

Ex/In	Intron Size (kb)	In/Ex
Ex1-In1-Ex2 TCCCCAGAG/gtgagccag	0.149	ctggtttag/CATCGACTC
Ex2-In2-Ex3 GCAGCCCAG/gtgagcagc	1.4	tctccacag/CACTGACCA
Ex3-In3-Ex4 CCTGCAGAG/gtcagtccc	0.247	tccccgcag/AGTGTCAGA
Ex4-In4-Ex5 GAGAACATG/gttgagggc	0.104	tccccgcag/CCGCCTCAG
Ex5-In5-Ex6 ACACTGGTG/gtgagtcac	3.7	cccccacag/GTCTGGCCA
Ex6-In6-Ex7 TTCATCAAG/gcaggtggc	0.216	ttgctgcag/GTGAAGTAG

domain-encoding sequence of the pig IgD clone showed a 3-bp difference from the first sequenced pig cDNA (Fig. 6*d*), again suggesting allotypic polymorphism.

Genomic organization of the bovine Cδ gene

We have previously isolated a cow Cμ and Cγ3 gene-positive BAC clone, termed BAC66R4C11, from a bovine library constructed using the pBeloBAC11 vector (14). Based on the δ gene cDNA and the sequences downstream of the Cμ gene, two overlapping genomic clones, bMD and bDE (Fig. 5), were obtained by cloning the long PCR products, amplified using BAC66R4C11 DNA as a template. The insert of the clone bMD spans ~4 kb DNA in size, containing a 1-kb sequence upstream of the Cδ gene, δCH1, δH1, δH2, δCH2, and part of δCH3, while the clone bDE contains part of δCH3, δTM1, δTM2, and part of the 3′ UTR (Fig. 5). Since the sense primer generating the clone bMD was based on the sequence 4 kb downstream of the bovine Cμ gene, it can be deduced that the ~7.4 kb long bovine Cδ gene is located 5.1 kb downstream of the bovine Cμ gene. Bovine genomic DNA was used as a control to ensure that the BAC clone used had not been rearranged during the cloning process and yielded the same results (data not shown).

The sequence data (accession no. AF411244–AF411246) obtained for the genomic bovine Cδ gene excluded the possibility that the first domain-encoding sequence of the cDNA was spliced from the Cμ gene. However, there is a striking similarity between the two, and comparing the 324-bp DNA sequences of the δCH1 and μCH1 exons, only a 10-bp difference was observed (Fig. 6*a*).

The genomic organization of the bovine Cδ gene was constructed based on a comparison of the cDNA and genomic sequences (Fig. 5). The bovine Cδ gene resembles the human Cδ gene in the number of exons, but differs slightly in the length of the introns. The boundaries for the exon-intron junctions are shown in Table I. It is worth noting that a noncanonical splicing site, GC-AG, is used in the intron between the δTM1- and δTM2-encoding exons.

Duplication of the bovine Cμ-Cδ and JH-Cμ introns

To look for the origin of the δCH1, we determined the complete sequence of the 5.1-kb intron between the bovine Cμ and Cδ genes. A detailed sequence analysis of the Cμ-Cδ intron (accession no. AF411241) shows the presence of a bovine non-long terminal repeat retro-element, Bov B-long interspersed nuclear element (B-LINE) (26), with a truncated 5′ end, in the Cμ-Cδ intron (Fig. 5). This retro-element contains a reverse transcriptase-encoding region that is thought to be responsible for DNA transposition (26).

A BLAST search using the whole intron sequence showed that, except for the Bov B-LINE, the bovine Cμ-Cδ intron was highly homologous to the sheep JH-Cμ intronic DNA, indicating that the bovine Cμ-Cδ intron may have been duplicated from its JH-Cμ intron. To address this question, we cloned and sequenced the bovine JH-Cμ intron (accession no. AF411242 and AF211243). The ~7-kb region was PCR-amplified from the BAC66R4C11 DNA using primers bIg-JH-S and bIgM-As and cloned into pGEM-T for sequencing. The sequence data revealed that the fragment contained two functional JH segments, JH1 and JH2, the bovine intronic enhancer region, and the Sμ (switch μ) region.

A dot plot analysis of the Cμ-Cδ and JH-Cμ introns showed long homologous DNA stretches in the two introns (Fig. 7), strongly suggesting that the δCH1 exon together with close to 4 kb upstream DNA originated from the 3′-flanking region of the bovine intronic enhancer down to the μCH1 exon (Fig. 5). The duplicated sequence was later interrupted by introduction of the retro-element, Bov B-LINE.

Long PCR amplifications were also performed to roughly determine the distances between the Cμ and Cδ genes in sheep and pig, where the results show that the sheep Cδ gene is located ~6.5 kb downstream of the Cμ gene, while the pig Cδ gene is located roughly 4 kb downstream of the Cμ gene (data not shown).

A short Sδ region mediates IgD class switching in cow

Consistent with the Southern blot results reported by Knight et al. (27), a short, ~280-bp Sμ-like region, abundant in switch μ motifs, CTGGG (15 repeats) and CTGAG (12 repeats), was identified immediately upstream of the Cδ gene (Fig. 5). The Sδ may theoretically be used to mediate class switch recombination. To test this hypothesis, a nested PCR, shown in Fig. 8*a*, was conducted to amplify recombined DNA fragments. While the Sμ-specific primers Sμ1 and Sμ2 are located in the 5′-flanking region of the switch μ region, the Sδ-specific primers Sδ1 and Sδ2 are located in the 3′-flanking region of the switch δ. Several fragments ranging from 400–600 bp in size were generated using genomic DNA from

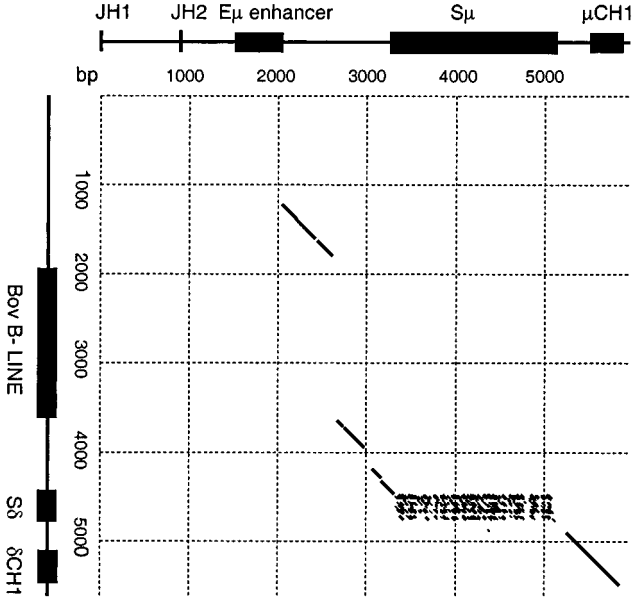

FIGURE 7. Dot plot analysis of the bovine JH-Cμ and Cμ-Cδ introns. An ~1.6-kb sequence in the middle of Sμ is still unsequenced.

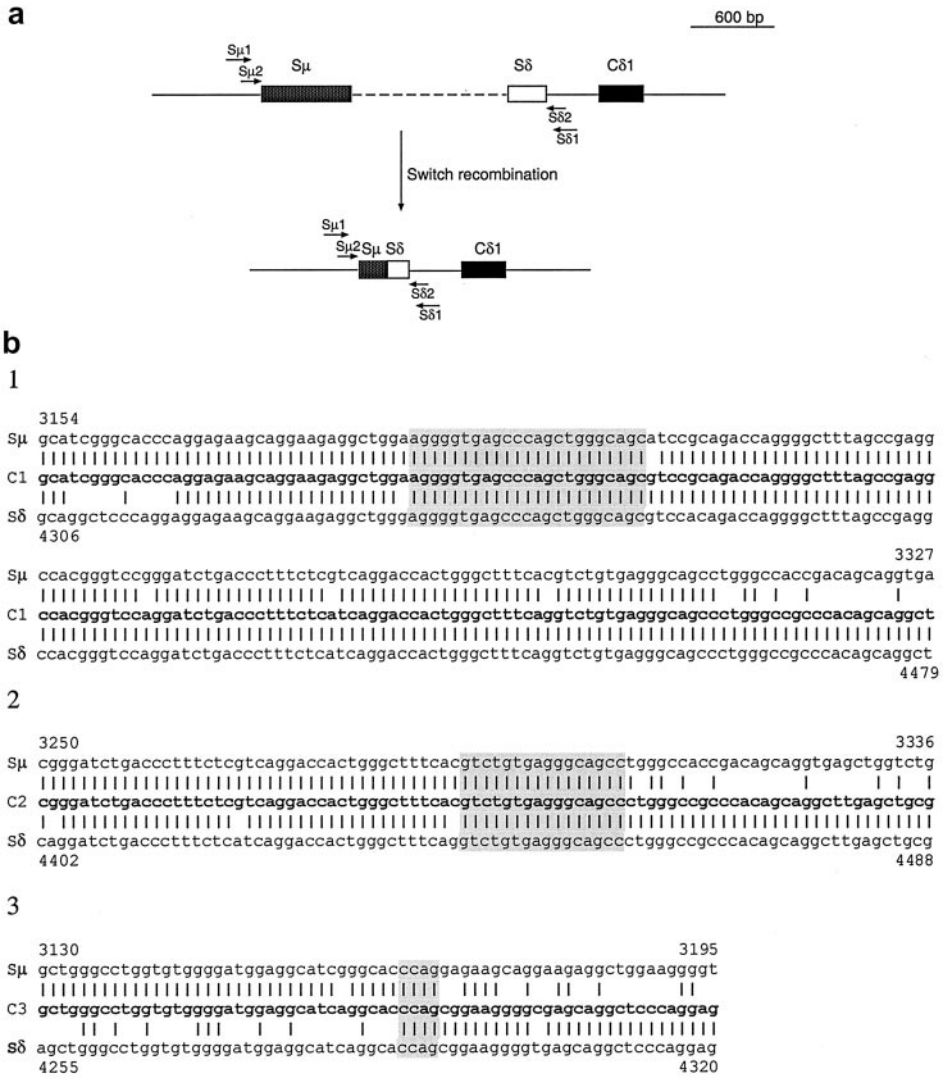

FIGURE 8. Class switch recombination to the Cδ gene is mediated by the Sδ. *a*, The DNA between the Sμ and Sδ is indicated by a dashed line. The positions of the primers Sμ1, Sμ2, Sδ1, and Sδ2 are shown by arrows. Sμ, switch μ; Sδ, switch δ. *b*, DNA sequences of the recombined Sμ-Sδ junction regions. The upper string (Sμ) represents the bovine germline Sμ region (accession no. AF411242); the lower string indicates the bovine germline Sδ (accession no. AF411241). The central sequences are the cloned PCR products. Identical nucleotides are shown by a vertical line. The sequences shared by both the Sμ and Sδ around the recombination sites are shaded.

different cows and were subsequently cloned and sequenced. Although there is a very high sequence homology between the very 5′ part of the Sμ and Sδ regions, we could identify the recombination breakpoints using the mismatches between the two regions as markers (Fig. 8*b*). Our data clearly show that switch recombination, involving the Sμ and Sδ regions, occurs in the cow.

Phylogenetic analysis of the Cδ and Cμ genes in vertebrates

One striking feature of the IgD in cows and sheep is that their CH1 domains share almost the same sequence as their IgM CH1. An examination of the bovine JH-Cμ and Cμ-Cδ intron sequences supports the idea that the bovine δCH1 exon, together with its 5′-flanking sequence were recently duplicated from the DNA-spanning, 3′-flanking region of the intronic enhancer to the μCH1 exon. Since the sheep Cδ gene also has a μCH1-like δCH1, the duplication would be expected to have occurred before the speciation of cows and sheep. To estimate when the bovine and ovine δCH1 exons were duplicated from their respective Cμ genes, unrooted phylogenetic trees were constructed using the first domain of both IgD and IgM from a number of species (Fig. 9, *a* and *b*). Unexpectedly, the results suggested that the duplication event creating the present δCH1 exon occurred independently in cows and sheep after the evolutionary divergence of these two species ~20 million yr ago (28). However, it is widely appreciated that gene conversion and other nonrandom processes act on immune system genes, which, in turn, are well documented to misrepresent phylogenetic relationships (29). To analyze the pairwise distances in more detail, NED values were therefore calculated (16). The results indicated that all bovidae sequence pairs were approximately equally related ($NED_{\text{sheep IgD:cow IgD}} = 0.11$; $NED_{\text{sheep IgD:sheep IgM}} = 0$; $NED_{\text{cow IgD:cow IgM}} = 0.10$), with the exception of those involving cow IgM and sheep IgM/IgD (NED = 0.22). This provides support for a gene conversion event occurring after duplication in the common ancestor of cows and sheep. Even more convincing data, supporting a gene conversion model, can be deduced from a comparison of the μ and δ CH1 exon sequences in both cows and sheep, where alignments of sheep and cow δCH1 with their respective μCH1 sequences show that the differences are clustered in their 3′ ends (Fig. 6, *a* and *b*). However the most 3′ 16-bp DNA in both sheep and cow δCH1 are identical (Fig. 6*c*), indicating that a gene conversion event between the μCH1 and δCH1, following the second duplication event that either replaced a pre-existing δCH1 or introduced a missing exon, may have occurred after speciation in both sheep and cows.

The structural similarity between the μ and δ genes suggest that the latter was initially duplicated from the former. The recent identification of a Cδ like gene in teleost fish suggests that the Cδ gene appeared in primitive vertebrates, ancestral to birds, reptiles, and mammals, since the lineage leading to teleost fish diverged from other vertebrates ~450 million yr ago (28, 30). This is consistent

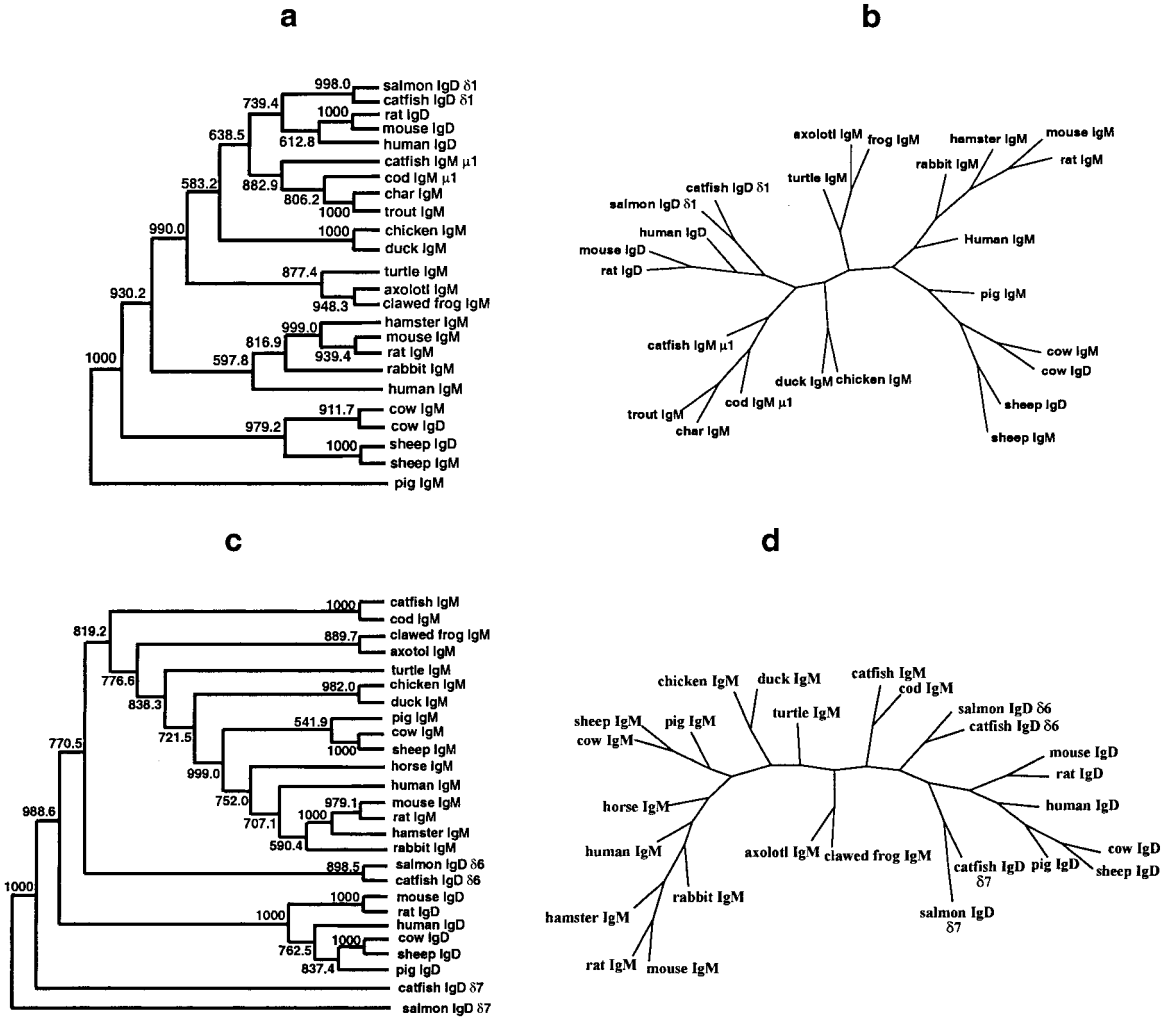

FIGURE 9. Phylogeny of IgM and IgD. The values supporting each node are derived from 1000 bootstrapped phylogenetic trees. *a* and *b*, The trees were constructed using the CH1 domain of both the IgD and IgM. *c* and *d*, The trees were constructed using IgD CH3 and IgM CH4 domains (including the δ6 and δ7 exons of both catfish and salmon). *a* and *b* were both unrooted and derived from the same tree file, but differ in format, as were *c* and *d*. All e trees presented here were constructed using the *dnapars* program (15).

with the phylogenetic tree constructed using the IgD CH3 and IgM CH4 domains (Fig. 9, *c* and *d*), where the calculated NED values ($NED_{\text{mammalian IgM:bird IgM}} = 0.45$, $NED_{\text{mammalian IgM:mammalian IgD}} = 0.91$, $NED_{\text{mammalian IgD:bird IgM}} = 1.43$) suggest that the first duplication of the Cμ gene, generating the Cδ gene, is much more ancient than the divergence between birds and mammals, ~310 million years ago (28).

Discussion

The present study conclusively shows the existence of a Cδ gene in mammals other than primates and rodents and sheds significant light on the evolution of the mammalian IgD H chain constant region gene. Apparently, the mammalian IgD constant regions have developed structural diversity with regard to both the hinge region and the CH2 domain (9, 10, 31). Structurally, the ruminant IgD H chain constant region is more similar to that of humans than rodents, since they all share three CH domains and a relatively long hinge segment, which is encoded by two separate exons. However, the hinge regions of these molecules differ from each other not only in length, but also with regard to their peptide sequences (Fig. 3).

Unlike their counterparts in human and rodents, the CH1 domain of IgD constant regions in cows and sheep share an extremely high homology with their respective μCH1 domains (Fig. 6, *a* and *b*). In the cow we have demonstrated that the δCH1 exon is used to produce the IgD H chain mRNA transcripts. In pigs, however, sequence data derived from two animals yielded inconsistent results, necessitating further research. One possibility is that the genomic δCH1-encoding sequence is indeed present in pigs, but in some animals these exon sequences are not used and, as in teleost fish, the μCH1 is spliced onto the δCH2 and CH3 sequences. The deduced pig IgD H chain is characterized by a shorter hinge region compared with those of humans, cows, and sheep, and although the hinge segment is similar to that of mice and rats in length, the homology between them is quite low (Fig. 3).

The finding of the Cδ gene in artiodactyls raises the question of whether the gene is widely distributed in vertebrates and not present only in some selected mammals and teleost fish. The results derived from the phylogenetic analysis indicate that the Cδ gene might be present in birds. However, in the recently sequenced duck Ig H chain constant region locus (32), the ~4-kb intron between the Cμ and Cα genes does not contain any Ig-like sequences, questioning the existence of a Cδ gene in birds. We have previously mapped the chicken Ig H chain constant region gene

locus (33) and shown that, as in the duck, an inverted Cα gene is located between the Cμ and Cυ genes. Our own unpublished observations also show that there is no Cδ gene in the intron between the μTM- and αTM-encoding exons, indicating that the insertion of the Cα gene may have deleted or displaced the avian Cδ gene.

In human and mouse B cells, coexpression of IgM and IgD depends on alternative splicing of a long primary transcript. In teleost fish, post-transcriptional RNA splicing is involved, resulting in a chimeric form of IgD H chain with the μCH1 domain being fused to unique Cδ sequences (6–8). Due to the absence of an authentic Sδ region, B cells expressing exclusively IgD (IgM^-IgD^+) are extremely rare in humans and are almost absent in mice (34). Homologous recombination mediated by two 442-bp repeats localized upstream of the Sμ and within the Cμ-Cδ intron, or nonhomologous recombination between Sμ and $\sigma\delta$ regions has been suggested as the molecular basis for generating these rare IgM^-IgD^+ cells (35, 36). To date, the cow is the only species in which a true Sδ region has been demonstrated. The identification of Sμ-Sδ recombination junctions, suggests that the expression of bovine IgD might depend on a deletional event, class switch recombination, which is a dominant mechanism for IgG, IgA, and IgE switching in most species. The bovine Sδ region is, however, rather short compared with other switch regions (typically 2–10 kb), which may reflect a gradual deletion of the Sδ sequences during evolution. This process might also, assuming that these sequences were involved in the first duplication event, have led to a complete loss of Sδ sequences in humans and mice.

It has previously been shown that IgD may replace IgM in B cell ontogeny (37), and the presence of Cδ genes in artiodactyls, possibly in most mammals, suggests that IgD may have some as yet unknown biological properties distinct from those of IgM, conferring a survival advantage.

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