A SINGLE PREDOMINANTLY EXPRESSED POLYMORPHIC IMMUNOGLOBULIN V_H GENE FAMILY, RELATED TO MAMMALIAN GROUP, I, CLAN, II, IS IDENTIFIED IN CATTLE*

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Abstract—In order to understand the generation of antibody diversity in cattle, seven cDNAs, from heterohybridomas secreting bovine IgM and IgG1 antibodies, were cloned and structurally analyzed for rearranged bovine VDJ genes. All of the seven bovine V_H genes, together with four available bovine V_H gene sequences, shared a high nucleotide sequence homology (84.2–93.5%). Based upon the criteria of nucleic acid homology >80%, all of the bovine V_H gene sequences isolated from the expressed antibody repertoire constitute a single V_H gene family, which we have designated as bovine V_H1 (Bov V_H1). An analysis of 44 bovine IgM-secreting mouse x cattle heterohybridomas, originating from polyclonally-activated PBLs from bovine leukemia virus-infected cattle, revealed that all of these expressed Bov V_H1 (100%) based upon DNA sequencing and Northern dot blot. The bovine V_H genes showed highest DNA sequence similarity, ranging between 81.5 and 87.6%, with a single sheep V_H gene family related to human V_H4 and are, thus, closest to the V_H genes from another ruminant species. The Bov V_H1 gene family is most homologous to the murine V_H Q-52 (71.8–78%) and human V_H4 (67.4–69.8%) gene families, which belong to mammalian group, I, clan, II, V_H genes. The CDR3 length of rearranged bovine VDJ genes is characteristically long (15–23 amino acids). The bovine J_H gene segments were most homologous to human J_H4 (82.1–87.2%) and J_H5 (84.6–89.7%) genes, suggesting the existence of at least two J_H gene segments. An analysis of CDRs provides evidence that somatic hypermutations contribute significantly to the generation of antibody diversity in cattle. Southern blot analysis of BamH I, EcoR I and Hind III digested genomic DNA from four cattle breeds (Holstein, Jersey, Hereford and Charolais) revealed three RFLP patterns; the genomic complexity of Bov V_H1 ranged between 13 and 15 genes. These observations provide evidence for polymorphism at the bovine Ig-V_H locus, similar to that seen in mice and humans. © 1997 Elsevier Science Ltd.

Key words: bovine V_H genes, CDR3 length, group I V_H genes, human V_H4 genes, murine Q-52 genes.

INTRODUCTION

The antibody diversity in the vertebrate immune system is generated through rearrangement of separated germline gene segments (i.e. variable (V_H), diversity (D) and joining (J_H) for the heavy chain and V_L and J_L for the light chains) and through subsequent heavy and light chain pairings. The variable region antibody diversity is primarily determined by two factors: (a) the number and sequence diversity of germline genes, and (b) somatic diversity, resulting from rearrangements, junctional deletions and additions (N or P nucleotides) and somatic mutations (Tonegawa, 1983).

The process of gene rearrangement is common to all the vertebrates, but the number and sequence diversity of V_H gene segments varies across the species. Based on nucleotide homology of ≥80%, about 100 human V_H genes are grouped into seven V_H gene families (Berman et al., 1988; Van Dijk et al., 1993) while 100–500 murine V_H genes are classified into 15 V_H gene families (Brodeur and Riblet, 1984; Kofler et al., 1992; Mainville et al., 1996). The human and murine V_H genes are further classified into three major groups, group I (human V_H2, V_H4, V_H6, murine V_H 3609, V_H 3660 and V_H Q-52), group II (Human V_H1, V_H5, murine V_H 3558 and V_H VGam 3–8) and group III (humanV_H3, murine V_H1783, V_H S107, V_H...
J606 and V_H X24), according to evolutionary divergence across species (Tutter and Riblet, 1988a, 1989; Kofer et al., 1992). In general, group III V_H genes are conserved during evolution and have been suggested to constitute an obligatory component of heavy chain loci in widely divergent mammalian and non-mammalian lineages (Tutter and Riblet, 1988a, 1989).

In contrast to the mouse and to humans, chickens have approximately 100 V_H genes, but only one V_H gene, proximal to the D genes, is functional: it contributes to antibody diversity via gene conversion in the bursa of Fabricious (Reynaud et al., 1989, 1994). Similarly, the rabbit has a germline pool of at least 100 V_H genes, corresponding to the human V_H3 gene family, but approximately half of these are functional (Becker and Knight, 1990; Knight, 1992). However, a single 3' V_H gene is preferentially rearranged and undergoes gene conversion and somatic hypermutations in the appendix leading to the generation of antibody diversity (Weinstein et al., 1994). The swine V_H genes expressed in the peripheral antibody repertoire also belong to one V_H gene family and, like the rabbit, are closest to human V_H3 genes (Sun et al., 1994). In sheep, a single V_H gene family, a homologue of the human V_H4 gene family, is consistently isolated from the immunoglobulin variable region heavy chain repertoire (Dufour et al., 1996). The light chain repertoire in sheep is diversified via somatic hypermutations in the ileal Peyer's patches during fetal and neonatal life (Reynaud et al., 1991, 1995). Thus, multiple divergent V_H gene families contribute to the generation of antibody diversity in the primary antibody repertoire of mice and humans but few V_H genes appear to generate antibody diversity in other species such as the chicken, rabbit, sheep and pig.

In order to understand the development of humoral immunity during evolution, studies of V_H genes of other species are necessary. The bovine immune system develops in the absence of any influence from maternal antibodies due to the presence of a syndesmochorial type of placentation (Tizard, 1992). Additionally, a majority of the peripheral B lymphocytes are CD5 + (Naessens and Williams, 1992), similar to murine B-1 cells, which are suggested to constitute a separate B cell lineage (Herzenberg et al., 1986). Homologues of the murine V_H J558 gene family, which constitute up to 50% of mouse V_H genes, do not exist in the bovine genome (Tutter and Riblet, 1989). The bovine C_J 1 and C_J 2 genes have been cloned and sequenced (Symons et al., 1989; Jackson et al., 1992). The bovine V_J light chain genes have been characterized and grouped into two families, V,J 1 and V,J 2, where V,J 1 predominates in the expressed antibody repertoire (Sinclair et al., 1995). The primary light chain repertoire in cattle has been suggested to be diversified by gene conversion (Parng et al., 1996). Few DNA sequences of bovine V_H genes are available (Jackson et al., 1992; Armour et al., 1994), but further studies are essential to characterize the VDJ gene rearrangements in the bovine peripheral antibody repertoire. This will help to elucidate the mechanisms involved in the generation of antibody diversity in the bovine immune system.

We have analysed seven cDNA sequences from mouse x cattle heterohybridomas secreting bovine IgM and IgG1 antibody. The studies outlined here provide evidence that all the bovine V_H genes isolated so far from different sources constitute a single V_H gene family, which is most homologous to human V_H4 and murine Q-52 gene families and belongs to mammalian group I (Tutter and Riblet, 1988a; Tutter and Riblet, 1989; Kofer et al., 1992) or clan II (Kirkham et al., 1992) V_H genes. We have designated this bovine V_H gene family as Bov V_H1, comprising 13–15 genes. The FR4 of the bovine Ig variable region is encoded by J_H genes, which are most homologous to human J_H4 and J_H5 gene segments. Further, the heavy chain CDR3 length of rearranged bovine V_H genes is unusually long (15–23 amino acids) and somatic hypermutations appear to contribute significantly to the generation of antibody diversity in cattle. Finally, the Bov V_H1 gene family is polymorphic, its genomic complexity varies between 13 and 15 genes, and it is predominantly expressed in the peripheral blood lymphocytes.

**MATERIALS AND METHODS**

**B-cell heterohybridomas**

Two mouse x cattle heterohybridomas, alpha-BL5C2.870005 (HB-9907) and alpha-BL5C2.870009 (HB-9908), secreting anti-bovine herpesvirus 1 IgG1 antibody, were obtained from ATCC (Rockville, MD, U.S.A.). The heterohybridomas were grown in RPMI 1460 medium supplemented with 20% heat inactivated horse serum, 5 mM sodium pyruvate, 1 mM glutamine, 0.5 mM MEM non-essential amino acids, 1% 100 x antibiotic-antimycotic solution and 5 x 10^-5 M 2-mercaptoethanol (all from, Gibco, BRL, Gaithersburg, MD, U.S.A.).

Additional mouse x cattle heterohybridomas were developed by somatically fusing bovine PBLs, stimulated in vitro for 48 hr with 25 pg/ml of lipopolysaccharide from *Escherichia coli* serotype 0127:B8 (Sigma Chemical Co., St. Louis, MO, U.S.A.) and 300 ng/ml of phorbol 12-myristate 13-acetate (Sigma Chemical Co.), with non-immunoglobulin secreting P3x-63.Ag8.653 mouse myeloma cells under limiting dilution conditions as described earlier (Kaushik et al., 1988). The PBLs were obtained from a two year old Holstein heifer suffering from persistent lymphocytosis as a result of BLV infection. Of the heterohybridomas thus developed 44 tested positive for bovine IgM secretion in a sandwich ELISA using unlabelled murine monoclonal anti-bovine IgM antibody (Sigma Chemical Co.), biotin conjugated murine monoclonal anti-bovine IgM antibody (Sigma Chemical Co.) and avidin conjugated with alkaline phosphatase (Pierce, Rockford, IL, U.S.A.). Various methodological and positive and negative controls were included in each test. The optical densities were measured at 405 using an ELISA reader (Ceres UV 900 Hdi, Bio-Tek Instruments Inc., Winooski, VT, U.S.A.).
cDNA synthesis, PCR amplification and cloning of rearranged bovine VDJ genes

Total RNA was extracted from two bovine IgG1-secreting and seven IgM-secreting heterohybridomas following the method of Chomczynski and Sacchi (1987). The cDNA was synthesized from 7 µg of total RNA using polydT primer (Pharmacia LKB Biotechnology, Uppsala, Sweden) and amplified with PCR (Perkin Elmer, Branchburg, NJ, U.S.A.) using 5' primer (5'AGCTCGAGATGAAACCCTCTGTG 3') from the leader sequence and 3' primer from either the conserved 5' sequence of CH1 region of bovine Jh gene segments (5' AGACTAGTGAGGAGACGGTGACC 3') or from the 3' sequences of bovine Jh gene segments (5' AGACTAGTGAGGAGACGGTGACC 3'). The 5' and 3' primers have built-in restriction sites for Xho I and Spe I endonucleases, respectively, and were synthesized at the Biotechnology Centre, University of Toronto, Ontario, Canada. The PCR steps involved a hot start at 95°C for 2 min, denaturation at 95°C for 1 min and combined annealing and extension steps at 72°C for 2.5 min, up to a total of 30 cycles. The reaction conditions included 1.5 mM MgCl₂ concentration, 0.8 µM of each primer and 2.5 U of Taq polymerase in 100 µl volume.

The rearranged bovine VDJ gene amplification was confirmed in an agarose gel electrophoresis and Southern blot by hybridization with a [α-³²P]dCTP (Amersham Canada Ltd., Oakville, Ontario, Canada) radiolabeled DNA probe specific for sheep VH gene. The sheep VH gene specific DNA probe was a 255 bp Pst I and Eco52 I fragment from clone 4839 VH2 (Dr W.R. Hein, Basel Institute of Immunology, Basel, Switzerland). The PCR product was digested with Xho I and Spe I, fractionated by gel electrophoresis, purified with Gene Clean II (Bio 101, Vista, CA, U.S.A.) and ligated into the dephosphorylated (calf intestinal alkaline phosphatase from Boehringer Mannheim, Germany) multiple cloning site of Bluescript KS +/− vector (Stratagene, La Jolla, CA, U.S.A.) as described (Reininger et al., 1988; Emara et al., 1995; Lipsanen et al., 1997). High efficiency E. coli, DH10B cells (GIBCO, BRL), were transformed in a Southern blot of restriction enzyme-digested plasmid DNA (Sambrook et al., 1989) by hybridization with a sheep VH gene specific DNA probe radiolabeled with [α-³²P]dCTP by random priming (Boehringer Mannheim). Briefly, the restriction-digested plasmid DNA was transferred onto a nitrocellulose membrane (Schleicher and Schuell Inc.) using an alkaline transfer method (1 M NaOH) by VacuGene (Pharmacia LKB Biotechnology). The membranes were pre-hybridized overnight at 42°C followed by hybridization (50% formamide, 5 × Denhardt’s solution, 5 × SSPE, 0.5% SDS, 100 µg/ml sonicated and denatured salmon sperm DNA and 2.5 × 10⁶ dpm of [α-³²P]dCTP radiolabeled Bov VH₁ gene family-specific DNA probe/ml).

The Bov VH₁ gene family-specific DNA probe was prepared from recombinant plasmid pB7S2 by double restriction digestion. First, a 463 bp fragment containing rearranged VDJ genes was cloned into restriction digestion with Xho I and Xba I enzymes; following purification, it was further digested with Hae III enzyme to obtain a 248 bp VH₁ specific DNA probe spanning nucleotides 23–270. The bovine VH₁ specific DNA probe was radiolabeled by random priming (Boehringer Mannheim). Following overnight hybridization, the membranes were washed three times in 2 × SSC, 0.1% SDS solution at room temperature; once in 1 × SSC, 0.1% SDS for 10–15 min at 65°C; and finally in 0.1 × SSC, 0.1% SDS for 15–20 min at 65°C. The autoradiography was performed using XAR-5 films (Eastman Kodak Company, Rochester, NY, U.S.A.).

DNA sequencing and structural analysis

The recombinant plasmids extracted from at least two clones originating from each heterohybridoma were purified on mini-affinity columns (Qiagen Inc., U.S.A.) and sequenced in both directions with T7 and T3 primers, using an automated DNA sequencing facility at, MOBIX (McMaster University, Hamilton, Ontario, Canada). The clone pH9.1 has a restriction site for SpeI enzyme at the 5' end of CDR3; its nucleotide sequence was thus determined by sequencing with T3 and T7 primers from nucleotide 1 to 291 from the recombinant plasmid. The remaining sequence was obtained by direct sequencing of the PCR product. The nucleotide sequences of all the rearranged VH genes were analysed for the FWs and CDRs, and numbered according to Kabat (Kabat et al., 1991). The DNA sequences were compared with nucleotide sequences from other species using the Gen-bank BLAST program (Altschul et al., 1990), and the amino acid analysis was performed using the GPMAW (version 2.1) program (Kyte and Doolittle, 1982).

Southern blot hybridization

Genomic DNA was extracted from the peripheral blood of Holstein Friesian, Jersey, Hereford and Charolais breeds of cattle using a DNA extraction kit (Stratagene, La Jolla, CA, U.S.A.). Approximately 10–15 µg of, D.N.A., digested to completion with BamHI I, EcoR I and Hind III restriction enzymes, was electrophoresed at 18 V for 36 hr in 0.8% agarose gel. The DNA was depurinated in 0.2 N HCl for 30 min and transferred onto maximum strength nitram membrane (Schleicher and Schuell Inc.) using an alkaline transfer method (1 M NaOH) by VacuGene (Pharmacia LKB Biotechnology). The membranes were pre-hybridized overnight at 42°C followed by hybridization (50% formamide, 5 × Denhardt’s solution, 5 × SSPE, 0.5% SDS, 100 µg/ml sonicated and denatured salmon sperm DNA and 2.5–3.0 × 10⁶ dpm of [α-³²P]dCTP radiolabeled Bov VH₁ gene family-specific DNA probe/ml).

The total RNA was extracted from all heterohybridomas using Trizol reagent (GIBCO, BRL).
The specificity of RNA dot-blot assay was confirmed by cloning and sequencing cDNAs prepared from seven randomly selected RNAs which showed nucleotide sequence homology > 80% with pB7S2 clone.

RESULTS

Structural analysis of rearranged bovine Ig variable region heavy chain genes

The cDNAs prepared from seven mouse x cattle heterohybridomas, two secreting bovine IgG1 antibody against bovine herpesvirus 1 (pB7S2 and pBSS1) and five secreting bovine IgM antibody, derived from polyclonally-activated PBLs from BLV-infected cattle (p2C7.5, p4H9.1, p5H4.1, p7B7.2 and p7G1.5), were cloned and structurally analysed for rearranged bovine heavy chain genes.

![Fig. 1. The nucleotide sequences of seven rearranged bovine VDJ genes cloned from *anti-bovine herpesvirus 1 IgG1-secreting heterohybridomas and **bovine IgM-secreting heterohybridomas from polyclonally-activated PBLs. The codons are numbered according to Kabat et al. (1991). The CDRs are shown in bold letters. The sequences presented here are available from GenBank accession numbers: U36823 (pB7S2), U36824 (pBSS1), AF000012 (p2C7.5), AF000013 (p4H9.1), AF000014 (p5H4.1), AF000015 (p7B7.2) and AF000016 (p7G1.5) (a). The deduced amino acid sequences of rearranged bovine VDJ genes are presented in three-letter codes (b). The PI values were determined by GPMAW (version 2.1) programme (Kyte and Doolittle, 1982).](image-url)
VDJ genes (Fig. 1). A high nucleotide sequence homology (84.2–93.5%) was observed among all the cloned bovine $V_H$ genes and four other available bovine $V_H$ genes (Fig. 2). As the nucleotide sequences of all the bovine $V_H$ genes from different sources analysed thus far shared a nucleotide homology of $\geq 80\%$, these correspond to a single bovine $V_H$ gene family which we have designated as Bov $V_{H1}$. In addition, an analysis of 44 bovine IgM-
Fig. 3. RNA dot blot analyses of RNA from bovine IgM-secreting mouse x cattle heterohybridomas probed with a [α-32P]dCTP radiolabeled Bov V₁, gene family specific DNA probe (pB7S2). *RNA from bovine IgG1 secreting heterohybridoma HB-9907 (positive control), **RNA from P3xAg8.653, mouse myeloma fusion partner (negative control) and ***RNA from non-Ig secreting heterohybridomas (negative controls). The nucleotide sequences for five clones (p2C7.5, p4H9.1, p5H4.1, p7B7.2 and p7G1.5) are presented in Fig. 1a.

secretion mouse x cattle heterohybridomas prepared from polyclonally activated PBLs from a BLV infected cattle revealed that Bov V₁, gene family was expressed in 100% of IgM-secreting heterohybridomas (Fig. 3). It should be noted that nucleotide sequences analysed from five cloned cDNAs from randomly selected Bov V₁, RNA (Fig. 3) confirmed their classification in Bov V₁, gene family (Fig. 1a). Further, an analysis of of 22 DNA sequences from Bov V₁, RNAs confirmed that these expressed Bov V₁, gene family (data not shown).

Other V₁, genes may exist in the bovine genome. Some faintly hybridizing restriction fragments of genomic DNA were noted in Southern blots (Fig. 4), which may reflect divergent bovine V₁, gene sequences. An analysis of deduced amino acid sequences revealed extensive amino acid substitutions in the CDR1 and CDR2 of both IgM and IgG1 antibodies (Fig. 1b), which is consistent with the somatic hypermutations which appear to significantly contribute to generation of antibody diversity in cattle. However, a comparison of rearranged bovine V₁, genes with the germline Vg, gene sequences is essential to confirm these observations.

The bovine V₁, genes shared 87.9–86.5% nucleotide homology with the sheep 4839 VH2 gene, and overall nucleotide homology of 81.8–86.6% with other rearranged VDJ genes from sheep (Table 1). Interestingly, a high nucleotide homology (85.6–87.6%) of bovine V₁, genes was noted with the germline sequence of deduced amino acid sequences revealed extensive amino acid substitutions in the CDR1 and CDR2 of both IgM and IgG1 antibodies (Fig. 1b), which is consistent with the somatic hypermutations which appear to significantly contribute to generation of antibody diversity in cattle. However, a comparison of rearranged bovine V₁, genes with the germline Vg, gene sequences is essential to confirm these observations.

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Table 1. Comparative analysis of nucleotide sequences of the cloned bovine V₁, genes with other species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Clone Number</th>
<th>pB7S2</th>
<th>pB8S1</th>
<th>p2C7.5</th>
<th>p4H9.1</th>
<th>p5H4.1</th>
<th>p7B7.2</th>
<th>p7G1.5</th>
<th>Reference</th>
</tr>
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<tr>
<td>Sheep</td>
<td>Vg, (germline)</td>
<td>86.7</td>
<td>87.3</td>
<td>86.2</td>
<td>85.6</td>
<td>86.6</td>
<td>86.6</td>
<td>87.6</td>
<td>Dufour et al. (1996)</td>
</tr>
<tr>
<td>Sheep</td>
<td>VR-22</td>
<td>83.2</td>
<td>84.9</td>
<td>84.5</td>
<td>83.5</td>
<td>83.2</td>
<td>83.4</td>
<td>85.6</td>
<td>Genbank Z49157</td>
</tr>
<tr>
<td>Sheep</td>
<td>SHg,7</td>
<td>82.8</td>
<td>81.5</td>
<td>82.5</td>
<td>81.8</td>
<td>81.8</td>
<td>83.5</td>
<td>83.2</td>
<td>Patri and Nau (1992)</td>
</tr>
<tr>
<td>Mouse</td>
<td>PCG1-1</td>
<td>78.0</td>
<td>73.2</td>
<td>75.3</td>
<td>74.9</td>
<td>72.9</td>
<td>71.8</td>
<td>74.2</td>
<td>Stenzel-Poore et al. (1987)</td>
</tr>
<tr>
<td>Mouse</td>
<td>N-92</td>
<td>75.6</td>
<td>74.2</td>
<td>76.6</td>
<td>76.6</td>
<td>74.9</td>
<td>73.5</td>
<td>76.3</td>
<td>Czerwinski et al. (1994)</td>
</tr>
<tr>
<td>Human</td>
<td>P2</td>
<td>69.8</td>
<td>67.4</td>
<td>68.4</td>
<td>68.7</td>
<td>69.8</td>
<td>69.8</td>
<td>69.4</td>
<td>Schroeder et al. (1987)</td>
</tr>
</tbody>
</table>
Bovine \( V_\beta \) genes

Fig. 4. Southern blot hybridization of cattle genomic DNA from (1) Holstein Friesian, (2) Jersey, (3) Hereford, and (4) Charolais breeds. Genomic DNA digested with \( \text{BamH} \ I (A) \), \( \text{EcoR} \ I (B) \) and \( \text{Hind} \ III (C) \) restriction enzymes was subjected to electrophoresis, transferred to nylon membrane and hybridized with a \( [\alpha -^32P]dCTP \) radiolabeled Bov \( V_\beta 1 \) gene family specific DNA probe (pB7S2). The arrows indicate polymorphic bands. The size of molecular weight standards is shown in kilobase pairs.

of the sheep \( V_{5\alpha} \) \( V_\lambda \) gene (Dufour \textit{et al.}, 1996). The bovine \( V_\lambda \) genes shared a nucleotide sequence homology of between 71.8 and 78% with the murine \( PCG-1 \) (Stenzel-Poore \textit{et al.}, 1987) and N-92 (Czerwinski \textit{et al.}, 1994) \( V_\lambda \) genes (Table 1), the members of murine \( Q-52 \) gene family and protein sub-group \( 1B \) (Kabat \textit{et al.}, 1991), and 67.4-69.8% nucleotide homology (Table 1) with human \( 58-P2 \) \( V_\lambda \) gene (Schroeder \textit{et al.}, 1987), a member of the human \( V_{\lambda 4} \) gene family. Thus, the predominantly expressed single Bov \( V_\lambda 1 \) gene family described here is a homologue of the murine \( V_\lambda 4 \) and human \( V_{\lambda 4} \) gene families, which belong to the consensus mammalian group I (Tutter and Riblet, 1988a, 1989) and clan II (Kirkham \textit{et al.}, 1992) \( V_\lambda \) genes.

The bovine \( V_\lambda \) genes showed a conserved CDR1 length of five amino acids, similar to murine \( V_\lambda \) genes. However, the CDR2 length comprised 16 amino acid residues, longer than that of murine \( Q-52 \) genes, with a CDR2 length of 11 amino acids, but similar to the CDR2 length of the human \( V_{\lambda 4} \) gene family (Kabat \textit{et al.}, 1991). In contrast to the murine CDR3 length distribution of 7-12 amino acid residues (Kabat \textit{et al.}, 1991), the bovine CDR3 length of rearranged VDJ genes ranged between 15 (p5H4.1) and 23 amino acids (pB8S1). The published CDR3 length of other rearranged bovine VDJ genes ranged between 14 and 25 amino acids (Jackson \textit{et al.}, 1992; Armour \textit{et al.}, 1994), which is consistent with our observation. The exceptionally long CDR3 length (similar to that of humans) of rearranged bovine \( V_\lambda \) genes reflects unique characteristics of rearranged bovine Ig variable region heavy chain genes; because of the limited germine diversity in cattle, these appear to be important in antigen recognition and in the development of antibody combining sites. The contribution of heavy chain D gene segments to the CDR3 length must await the identification of bovine D genes as both germine and rearranged nucleotide sequences become available.

The bovine \( J_\lambda \) gene sequence showed highest nucleotide similarity with the human \( J_{\lambda 4} \) (82.1-87.8%), murine \( J_{\lambda 5} \) (84.6-89.7%), murine \( J_{\lambda 1} \) (74.4-79.5%) and murine \( J_{\lambda 4} \) (74.3-76.9%) genes (Table 2). When compared with the sheep \( J_\lambda \) gene sequence, the bovine \( J_\lambda \) genes shared nucleotide homology ranging between 82.1 and 87.2%. All the bovine \( J_\lambda \) gene segments shared a relatively low level of nucleotide homology with the single swine \( J_\lambda \) gene segment (66.7-69.2%). These observations suggest that the FR4 of rearranged bovine VDJ sequences is probably encoded by at least two \( J_\lambda \) gene segments, which are closest to human \( J_{\lambda 4} \) and \( J_{\lambda 5} \) gene segments.

Amino acid analysis of all of the antibodies revealed
that serine, leucine, threonine and glycine contributed most to the Ig heavy chain variable regions of bovine antibodies. The amino acids methionine and histidine were rarely used. Four of the seven antibodies, both IgM and IgG1, had arginine (pB7S2, pC7.5, pB7.2 and pG1.5), while six of these had cysteine (pB7S2, pB7.2, pG1.5) while six of these had cysteine (pB7S2, p2C7.5, p4H9.1, p7B7.2 and pG1.5) in their CDR3 (Fig. 1b). The clone pB7S2 had two cysteine residues at positions 95 and 100, intervened by threonine, glycine, alanine and tyrosine. This feature, similar to humans, appears to contribute to the rigidity of CDR3. A higher degree of hydrophilicity, especially in FR3, was clearly evident in the protein sequences of all the antibodies. The PI values of these antibodies ranged between 7.92 and 9.04. The PI values noted in pB7S2 (Fig. 1b). The clone pB7S2 had two cysteine residues at positions 95 and 100, intervened by threonine, glycine, alanine and tyrosine. This feature, similar to humans, appears to contribute to the rigidity of CDR3. A higher degree of hydrophilicity, especially in FR3, was clearly evident in the protein sequences of all the antibodies. The PI values of these antibodies ranged between 7.92 and 9.04. The PI values noted in pB7S2 (Fig. 1b). The clone pB7S2 had two cysteine residues at positions 95 and 100, intervened by threonine, glycine, alanine and tyrosine. This feature, similar to humans, appears to contribute to the rigidity of CDR3. A higher degree of hydrophilicity, especially in FR3, was clearly evident in the protein sequences of all the antibodies. The PI values of these antibodies ranged between 7.92 and 9.04. The PI values noted in pB7S2 (Fig. 1b). The clone pB7S2 had two cysteine residues at positions 95 and 100, intervened by threonine, glycine, alanine and tyrosine. This feature, similar to humans, appears to contribute to the rigidity of CDR3. A higher degree of hydrophilicity, especially in FR3, was clearly evident in the protein sequences of all the antibodies. The PI values of these antibodies ranged between 7.92 and 9.04. The PI values noted in pB7S2 (Fig. 1b).

**Genomic complexity and polymorphism in the Bov Vn1 gene family**

The genomic complexity of cloned Bov Vn1 gene (pB7S2) was determined in a Southern blot using BstNI, EcoR I and Hind III digested genomic DNA from cattle. A maximum number of Hind III digested DNA fragments, ranging from 13 to 15, hybridized with the Bov Vn1 gene family specific DNA probe (Fig. 4). In order to determine whether bovine Vn1 genes are polymorphic, the genomic DNA from four different breeds of cattle (Holstein, Jersey, Hereford and Charolais) was analysed. Three polymorphic RFLP patterns were evident in all the four cattle breeds studied that involved either deletion or addition of Vn1 gene segments. Such polymorphism may be associated with inter- or intra-breed recombination as a result of cattle breeding practices. These observations provide evidence that bovine Vn1 genes, similar to murine and human Vn1 genes, are polymorphic and the genomic complexity of the Bov Vn1 gene family varies between 13 and 15 genes.

**DISCUSSION**

The human and murine variable region genes of both heavy and light chains are grouped into a number of multi-gene families based on the criteria of >80% nucleotide homology among the members of a gene family (Brodeur and Riblet, 1984; Berman et al., 1988; Kofler et al., 1992; Van Dijk et al., 1993; Mainville et al., 1996). However, certain other vertebrate species such as the chicken (Reynaud et al., 1989, 1994), rabbit (Becker and Knight, 1990; Knight, 1992), sheep (Dufour et al., 1996) and pig (Sun et al., 1994) predominantly utilize a single Vn gene family in the expressed antibody repertoire. For these reasons, elucidation of the structure and organization of Ig loci from phylogenetically distant species is essential to understand the development of antibody diversity and the evolution of the complex multigene variable region gene families. Due to the presence of placental barriers the bovine immune system develops in the absence of influences from maternal antibodies, and a majority of the bovine peripheral B lymphocytes express CD5 glycoprotein (Naessens and Williams, 1992), a marker related to B-1 lymphocytes (Herzenberg et al., 1986). We have, therefore, analysed the structure and VDJ rearrangements of bovine Ig-Vn genes from two anti-bovine herpesvirus1 IgG1-secreting hybridomas and five bovine IgM-secreting heterohybridomas from polyclonally activated PBLs. Because of nucleotide homology of >80%, all the cloned Vn genes, as well as four other cattle Vn genes isolated from different sources (Jackson et al., 1992; Armour et al., 1994), were found to constitute a single Vn gene family. We have designated this bovine Vn1 gene family as Bov Vn1, a homologue of the human Vn4 and murine Q-52 gene families which belong to mammalian group I (Tutter and Riblet, 1988a; Tutter and Riblet, 1989; Kofler et al., 1992) and clan II (Kirkham et al., 1992) Vn genes. In addition, the Bov Vn1 gene family was observed to be expressed at 100% frequency among 44 mouse x cattle heterohybridomas originating from polyclonally-activated PBLs. The nucleotide sequence of two cDNAs (five of these sequences are shown in Fig. 1) prepared from randomly selected RNAs which tested positive for Bov Vn1 gene in the dot-blot assay showed them to belong to Bov Vn1 gene family. These observations suggest that a single Bov Vn1 gene family is predominantly expressed in the peripheral antibody repertoire of cattle.

We noted an unusually long CDR3 length in rearranged bovine VDJ gene sequences, a finding also noted by other authors (Jackson et al., 1992; Armour et al., 1994) and in recent sequencing data from our laboratory, where CDR3 length of up to 56 amino acids

<table>
<thead>
<tr>
<th>Species</th>
<th>Clone Number</th>
<th>pB7S2</th>
<th>pB8S1</th>
<th>pC7.5</th>
<th>pH9.1</th>
<th>P5H4.1</th>
<th>pB7.2</th>
<th>pG1.5</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep Vn1</td>
<td>Vr 15</td>
<td>87.2</td>
<td>82.1</td>
<td>84.6</td>
<td>84.6</td>
<td>82.1</td>
<td>84.6</td>
<td>84.6</td>
<td>Dufour et al. (1996)</td>
</tr>
<tr>
<td>Pig Vn1</td>
<td>Consensus</td>
<td>66.7</td>
<td>66.7</td>
<td>69.2</td>
<td>69.2</td>
<td>69.2</td>
<td>69.2</td>
<td>69.2</td>
<td>Sun et al. (1994)</td>
</tr>
<tr>
<td>Mouse Vn1,4</td>
<td>MEP 203</td>
<td>74.4</td>
<td>79.5</td>
<td>76.9</td>
<td>76.9</td>
<td>76.9</td>
<td>76.9</td>
<td>76.9</td>
<td>Czerwinski et al. (1994)</td>
</tr>
<tr>
<td>Human Vn1,5</td>
<td>—</td>
<td>84.6</td>
<td>84.6</td>
<td>89.7</td>
<td>89.7</td>
<td>84.6</td>
<td>87.8</td>
<td>89.7</td>
<td>Schroeder et al. (1987)</td>
</tr>
<tr>
<td>Human Vn1,4</td>
<td>—</td>
<td>87.2</td>
<td>82.1</td>
<td>87.2</td>
<td>87.2</td>
<td>84.6</td>
<td>87.8</td>
<td>87.2</td>
<td>Schroeder et al. (1987)</td>
</tr>
</tbody>
</table>
is noted (unpublished). The identification of bovine D genes is required to ascertain their contribution to this unusually long CDR3 length, possibly via D-D fusions. This is especially important as the CDR3 is located in the middle of the antibody combining site and its length is important in fitting the contour of the antigen molecule.

The amino acid substitutions in the CDR1 and CDR2 of the cloned bovine \( V_H \) genes of both IgM and IgG1 antibodies suggest that somatic hypermutations significantly contribute to the generation of antibody diversity in cattle. However, comparison with germline \( V_H \) gene sequences is essential to confirm the contribution of somatic hypermutations, and to determine if other mechanisms such as gene conversion also contribute to antibody diversity in cattle. The nucleotide sequences encoding FR4 of seven rearranged VDJ genes are most homologous to human \( J_H \)-4 and \( J_H \)-5 gene segments. Based on FR4 analysis, at least two \( J_H \) gene segments exist in cattle (unpublished).

The genomic complexity of the designated Bov \( V_H \), gene family ranges from 13 to 15 members. Despite the fact that the genomic DNA analysed from PBLs may include some rearranged VDJ fragments, three polymorphic RFLP patterns were noted among the four cattle breeds analysed. The faintly hybridizing restriction fragments seen most likely reflect either rearranged VDJ segments or differences in the copy number of a gene. Further investigations conducted with sperm or liver genomic DNA would be required in order to confirm the observed polymorphism. Nevertheless, the presence or absence of strongly hybridizing restriction fragments (Fig. 4) suggest polymorphism at the Ig-\( V_H \) locus. The existence of polymorphism at the Ig-\( V_H \) locus of cattle, similar to that seen in other species such as mouse (Tutter and Riblet, 1988b) and human (Pincus, 1987), is of interest as ongoing inbreeding in cattle would be expected to reduce the polymorphism. Nevertheless, recombination in natural populations of cattle may be responsible for sustaining the observed polymorphism at the Ig-\( V_H \) locus.

In contrast to our previous observation, where homologues of the murine \( V_H \)-11 gene were detected in a Southern blot of bovine genomic DNA (Saini et al., 1996), and to the observation that the homologues of group III \( V_H \) genes (murine \( V_H \)-7183, \( V_H \)-S107, \( V_H \)-J606 and \( V_H \)-X24 and human \( V_H \)-3 gene families) are conserved during evolution (Tutter and Riblet, 1988a, 1989), a single bovine \( V_H \) gene family has been consistently isolated from the expressed antibody repertoire from different sources (Jackson et al., 1992; Armour et al., 1994). It is unknown at present whether the homologues of \( V_H \)-11 and other group III \( V_H \) genes, which appear by Southern blotting to be present in bovine genome, are actually expressed.

The single bovine \( V_H \) gene family (Bov \( V_H \)-1) defined here is closest to the single sheep \( V_H \) gene family, a homologue of the human \( V_H \)-4 gene family, which predominates in the Ig variable region heavy chain repertoire of sheep (Dufour et al., 1996). Swine also express a single \( V_H \) gene family, related to the human \( V_H \)-3 gene family (Sun et al., 1994). Similarly, a 3' gene from a single \( V_H \) gene family, also related to human \( V_H \)-3 gene family, preferentially rearranges in the rabbit Ig repertoire (Becker and Knight, 1990; Knight, 1992; Weinstein et al., 1994). Chicken B cells also utilize the single available functional \( V_H \) gene (and one functional \( V_J \) gene) to achieve antibody diversity via gene conversion (Reynaud et al., 1989, 1994). Similar to both the horse (Home et al., 1992) and to sheep (Reynaud et al., 1991, 1995), the \( \lambda \)-light chain predominates in the primary antibody repertoire of cattle (Butler, 1983). Further, of the two \( V_\lambda \) light chain gene families, the \( V_\lambda \)-1 gene family is predominantly expressed in the light chain repertoire of cattle (Sinclair et al., 1995).

The diversity of the bovine \( \lambda \)-light chain repertoire has been suggested to be generated through gene conversion (Parng et al., 1996). It has also been suggested that the bovine \( V_\lambda \) light chain repertoire contributes relatively little to the recognition of antigens (Sinclair et al., 1995) and, therefore, bovine \( V_\lambda \) genes are expected to contribute most to antigen recognition. It thus appears that the single \( V_\lambda \) gene family (Bov \( V_\lambda \)-1) and the single \( V_\lambda \) gene family contribute most to the development of humoral immunity in cattle. This situation is in contrast to both mice and humans where multigene families, representing divergent germline sequences, contribute most to both heavy and light chain diversity. The ruminant immune system also differs from that of rabbits and swine, where a single \( V_H \) gene family, related to the human \( V_H \)-3 gene family (mammalian group III), is consistently expressed (Knight, 1992; Sun et al., 1994). Both the bovine and ovine \( V_H \) genes are homologues of human \( V_H \)-4 and murine Q-52 gene families, corresponding to mammalian group I \( V_H \) genes which, unlike group III genes, are not highly conserved during evolution (Tutter and Riblet, 1988a, 1989).

The conservation of mammalian group III \( V_H \) gene families throughout the vertebrate immune system has led to the suggestion that members of this group might be closest to the ancestral primordial genes. They are probably endowed with an important non-coding function related to the nucleotide sequences in the FR1 and FR3 regions, which target a series of recombinogenic events (Tutter and Riblet, 1989). Our observations from the ruminant immune system do not support such a hypothesis, as the expressed bovine and ovine \( V_H \) genes were found to belong to relatively less conserved group I \( V_H \) genes. Further studies on other mammalian and non-mammalian Ig-\( V_H \) loci are essential to better understand the origin of the \( V_H \) genes during phylogeny.

To conclude, the studies outlined here demonstrate that (i) a single bovine \( V_H \) gene family (Bov \( V_H \)-1), a homologue of human \( V_H \)-4 and murine Q-52 gene families and corresponding to mammalian group I and clan II genes, is consistently isolated from different sources and is predominantly expressed among antigen-induced and polyclonally-activated PBLs from BLV infected cattle, (ii) the CDR3 length of the rearranged bovine VDJ genes is unusually long (15–23 amino acids), (iii) the FR4 of rearranged bovine VDJ sequences appears to be encoded by two \( J_H \) gene segments which are most homologous to human \( J_H \)-4 and \( J_H \)-5 genes, (iv) the genomic complexity...
of the Bov V_H gene family ranges between 13 and 15 genes, and (v) polymorphism exists at the Ig-V_H locus of cattle.

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