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Immunology

Immunoglobulin variable-region-like domains of diverse sequence within the major histocompatibility complex of the chicken

(*B* system of histocompatibility/blood group antigens/immunoglobulin gene superfamily/heptad repeats/α-helical coiled coil)

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ABSTRACT **The highly polymorphic B-G antigens are considered to be part of the major histocompatibility complex (MHC) of the chicken, the *B* system of histocompatibility, because they are encoded in a family of genes tightly linked with the genes encoding MHC class I and class II antigens. To better understand these unusual MHC antigens, full-length B-G cDNA clones were isolated from a B^{21} embryonic erythroid cell cDNA library, restriction-mapped, and sequenced. Five transcript types were identified. Analysis of the deduced amino acid sequences suggests that the B-G polypeptides are composed of single extracellular domains that resemble immunoglobulin domains of the variable-region (V) type, single membrane-spanning domains typical of integral membrane proteins, and long cytoplasmic tails. Sequence diversity among the five transcript types was found in all domains, notably including the B-G immunoglobulin V-like domains. The cytoplasmic tails of the B-G antigens are made up entirely of units of seven amino acid residues (heptads) that are typical of an α-helical coiled-coil conformation. The heptads vary in number and sequence between the different transcripts. The presence within B-G polypeptides of polymorphic immunoglobulin V-like domains warrants further investigations to determine the degree and nature of variability within this domain in these unusual MHC antigens.**

An unusual class of highly polymorphic cell surface antigens are encoded within the major histocompatibility complex (MHC) of the chicken, the *B* system of histocompatibility. These antigens, the B-G antigens, were first recognized as part of the *B* blood group system (1, 2), a gene region that was later shown to also encode the MHC class I and class II antigens of the chicken (3–5). The presence of a third class of highly polymorphic antigens within the *B* system is an enigmatic departure from the organization of major histocompatibility complexes as known from the study of mammals (6).

Attempts to define a function for the B-G antigens have been limited to experiments focused on their expression on erythrocytes. In this context, B-G antigens have been shown to have an adjuvant activity in humoral responses to the *B* system class I antigens (7) and to antigens of other blood group systems (8). The recent finding that B-G-like antigens are expressed on cells in other tissues opens additional avenues of investigation that may be useful in determining B-G antigen function (9, 40, 41).

The polymorphism of the B-G antigens is evident at the level of polypeptide structure and gene organization, as well as in their alloantigenicity. Not only do B-G polypeptides form a major component of the array of antigens responded to in the alloimmunizations that have defined over 27 *B* haplotypes (10, 11), they also exhibit surprising variability in molecular mass and isoelectric point (9, 12, 13). B-G polypeptides, including those expressed in nonerythroid tissues (9) and erythrocytic B-G in some haplotypes (14), vary widely in size, from 30–70 kDa in SDS/PAGE. Under nonreducing conditions, the molecular mass typically doubles, indicating the presence of disulfide-linked dimers. Variation in polypeptide size is apparently largely due to variation in the length of the cytoplasmic tail (refs. 14 and 15; M.M.M., Q.-Y. Xu, J.C., and R.G., unpublished data), with multiple B-G genes presumably encoding molecules of widely varying lengths. Southern hybridizations between B-G cloned sequences and genomic DNA from birds of different *B* haplotype have provided evidence for the multiplicity and sequence diversity of *B-G* loci (refs. 16–19; M.M.M., unpublished data). The total number of *B-G* genes is not known. Already more than five have been mapped in $B\text{-}G^{21}$ genomic clones (R.G. and M.M.M., unpublished data).

The most polymorphic genes presently known in eukaryotic organisms are those associated with the immune-system functions of antigen presentation and recognition, and so it may be that the B-G antigens serve in one of these roles. The findings in this study that reveal the immunoglobulin-like nature of the B-G extracellular domain and the sequence variability within it support this hypothesis.‖

MATERIALS AND METHODS

Construction and Screening of cDNA Library. Erythroid cells were collected from 13- to 14-day-old B^{21} chicken embryos (UCD line 330). UCD line 330 is highly inbred and homozygous for the B^{21} haplotype. RNA was isolated in RNAzol (Cinna Biotecx Laboratories, Friendswood, TX) by following the manufacturer's protocol. Poly(A)-enriched RNA was prepared (20) and size-fractionated on a sucrose gradient (21) to remove hemoglobin message. B-G mRNA from B^{21} erythroid cells is ≈2 kilobases (kb). λZAP II (Stratagene) cDNA libraries were constructed by the manufacturer's protocol. Approximately 10^5 recombinant clones in an unamplified library (inserts of >1 kb) were screened with a mixture of random-primed bg28 and bg32.1 (22). These are nonoverlapping partial B-G cDNA clones (17, 18) and now known to correspond to 5′ and 3′ portions of full-length clones. Phagemids were excised from 23 clones and the 13 longest (1.2–3.0 kb) were further analyzed.

Nucleotide Sequencing. Purified phagemid DNA (23, 24) was sequenced using Sequenase version 2.0 (United States Biochemical). For five clones, bg14, bg3, bg17, bg11 and bg8, full sequence determinations were made on both strands by using a combination of subcloning and oligonucleotide prim-

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Abbreviation: MHC, major histocompatibility complex.

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‖The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M61860–M61864).

ing (Fig. 1). Conveniently located restriction sites were used to prepare most subclones. Additional subclones were prepared with exonuclease III (Erase-A-Base, Promega). Oligonucleotide primers were used in a few instances to complete the full sequence analyses. Eight clones, bg12, bg6, bg16.1, bg5, bg15, bg1, bg4 and bgI10, were sequenced only at the 5′ and 3′ ends. Clones were considered identical when their restriction maps and the 5′- and 3′-end sequences (generally >250-base overlaps at both ends) matched. One or two base differences were not considered sufficient for separating clones into different transcript types. Nucleotide sequences were assembled and analyzed using DNAstar (Madison, WI) and IntelliGenetics software.

Protein Isolation and Sequence Analysis. B-G21 antigen was purified from erythrocytes of adult UCD line 330 birds (17), precipitated (25) and dissolved in 100 μl of 6 M guanidine hydrochloride/1 mM Tris·HCl/1 mM EDTA, pH 8.5. To effect reduction 1 μl of 10% (vol/vol) 2-mercaptoethanol was added. After 30 min at 37°C, 1 μl of 4-vinylpyridine (Aldrich) was added and the sample was incubated at room temperature for 2–3 hr. Alkylation was stopped with the addition of 5 μl of dithiothreitol and the B-G polypeptides were cleaved with CNBr. Microsequencing was performed on a 475A pulsed-liquid sequencer (Applied Biosystems). Chemicals and programs were also from Applied Biosystems.

RESULTS

Identification of 5 Transcripts Among 13 cDNA Clones. Restriction mapping of the 13 B-G cDNA clones chosen for analysis revealed the presence of internal *Eco*RI sites in only 9 clones. Upon sequence analysis, the 9 clones containing *Eco*RI sites could be further divided into three subgroups. Seven identical clones formed the first of these subgroups (transcript type 1) and are represented by bg14, bg8, and bg14/8 (Figs. 1 and 2). Included in this group is bg8, a clone of 3 kb, which was found to be entirely identical to bg14 except for an additional 28 bases 5′ beyond bg14, a silent nucleotide difference in an asparagine codon, and the presence of nine unprocessed introns (Fig. 1). The 28 nucleotides from bg8 have been appended to bg14 to provide the full sequence designated bg14/8. The second subgroup, transcript type II, is represented by a single clone, bg3 (Figs. 1 and 2). While nearly identical to bg14/8 over nearly its entire length, the most distal portion of the 5′ untranslated region of bg3 is unlike that of bg14/8. The other differences in bg3 are two single base differences (T substituted for C at the positions corresponding to 884 and 1460 in bg14/8), the absence of a codon near the end of the open reading frame (as noted in Fig. 2 in heptad 30), and the presence in bg3 of an additional 3 nucleotides at the end of the 3′ untranslated region just upstream of the poly(A) tail. The third transcript type is also represented by a single clone, bg17. Not a full-length clone, bg17 is only 77.7% similar to bg14/8 (Figs. 1 and 2).

Among the clones lacking internal *Eco*RI sites, three were found to be identical and are represented by bg11/4 (transcript type IV) in Figs. 1 and 2. Clone bg11/4 signifies the fusion of sequences from two clones. bg11, which contains a 345-base-pair unprocessed intron, was found to lack 153 base pairs at the 3′ end. For convenience in describing the entire transcript type IV, the portion missing from bg11 was fully sequenced in bg4 and appended to bg11 to provide a full sequence, the intron sequence was removed, and hence bg11/4. Evidence for a fifth transcript type was found in a 1.2-kb clone that, although lacking *Eco*RI sites, appears more similar to bg14/8 than to bg11 in the portion of sequence determined. Because this clone was less than full-length and contained an unprocessed intron and a very long poly(A) tail, it was not further analyzed. In summary, a total of five B-G transcripts were identified among the longest cDNA clones obtained in screening an embryonic erythroid cell cDNA library of B^{21} haplotype. Representatives of four of these transcript types, as well as a 3-kb clone of transcript type I, were entirely sequenced in both strands.

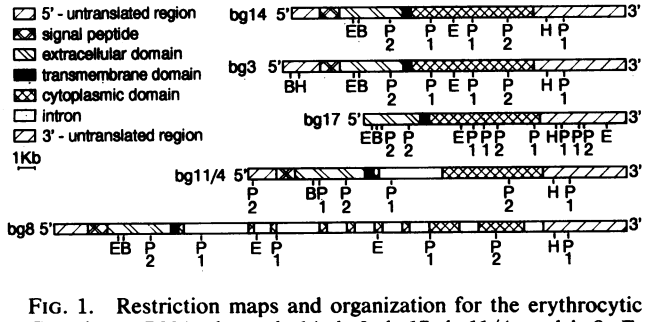

FIG. 1. Restriction maps and organization for the erythrocytic B-G antigen cDNA clones bg14, bg3, bg17, bg11/4, and bg8. E, *Eco*RI; B, *Bam*HI; P1, *Pst* I; P2, *Pvu* II; H, *Hin*dIII.

Comparison of the Sequences of bg14/8, bg3, bg17, and bg11/4. The sequences of bg14/8, bg3, bg17 and bg11/4 have been aligned using the nucleotide sequences only at the 5′ and 3′ untranslated regions and only the amino acid sequences over the entire length of the open reading frames (Fig. 2). Within the deduced sequences are (*i*) an N-terminal signal peptide of 34 amino acids, (*ii*) an extracellular domain of 114 amino acids; (*iii*) a transmembrane domain of 34 amino acids, and (*iv*) cytoplasmic regions varying in length. bg14/8, bg3, and bg11/4 contain full sequences that correspond to polypeptides of M_r 45,298, 45,227, and 41,938, respectively, including the signal peptide (Figs. 1 and 2), sizes in accord with the mature polypeptides expressed on erythrocytes in adult $B\text{-}G^{21}$ birds (17).

The 5′ untranslated region. The 5′ untranslated regions of bg14/8, bg3, and bg11/4 are identical and strongly C-rich in the region immediately upstream from the translation start site. The start site, AGCACAGATGG, is similar, but not identical, to the consensus sequence found by Kozak (26). Farther upstream, the sequences of the three clones diverge. While that of bg11/4 shows only minor differences from bg14/8, the 5′-most portion of bg3 is highly dissimilar.

Signal peptide. The three signal-peptide sequences are identical except for a single residue difference (27).

Extracellular domain. The limits of the B-G extracellular domain are defined by the signal peptide and the putative transmembrane domain. Within this region clones bg14/8 and bg3 are identical; however, multiple amino acid substitutions are present within the sequences of bg11/4 and bg17. In database searches (GenBank, EMBL, September 1990) for sequence similarities, statistically significant matches were found between this portion of the predicted B-G sequence and the immunoglobulin domains of members of the immunoglobulin superfamily. In Table 1 are examples of the ALIGN scores obtained in comparisons between the B-G extracellular domain (that of bg14/8 is illustrated) and the immunoglobulin domains of immunoglobulin superfamily members of the three homology unit types, V, C, and H (33). The basis of these highly significant alignment scores is shown in Fig. 3. The majority of the features that define immunoglobulin V homology units (33) are found within the B-G extracellular domain sequence. Included are appropriately spaced cysteine residues (residues 57 and 129) and a tryptophan (residue 72) 11–15 residues proximal to the distal cysteine, features common to all immunoglobulin homology units. The features that further define the B-G extracellular domain to be of the V-unit type are the wide spacing of the cysteine residues, the Tyr-Xaa-Cys configuration at the proximal cysteine, and the presence of amino acid residues with the appropriate physical properties that further define the V unit. Additional cysteine residues are found near the distal end of the extracellular domains, one in both bg14/8 and bg3, and two in bg11/4 (Fig. 2).

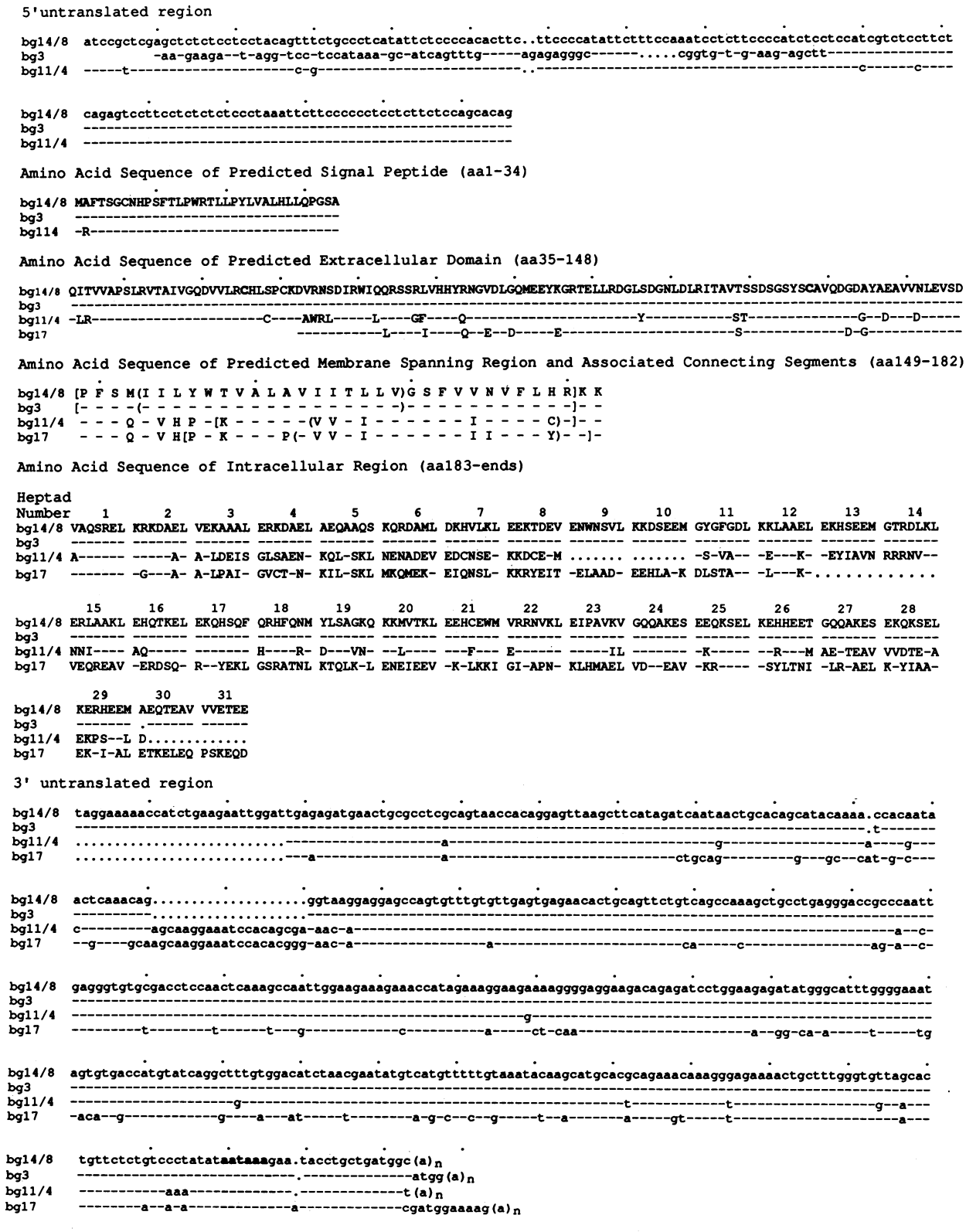

FIG. 2. Alignment of the nucleotide sequences of untranslated regions and the deduced amino acid sequences of the coding regions of B-G cDNA clones bg14/8, bg3, bg11/4, and bg17. The amino acid sequences are tentatively assigned to domains. Dashes (- - -) indicate identity. Gaps (. . .) were introduced to optimize alignments. The inner and outer limits of the predicted transmembrane domains are indicated by parentheses and brackets, respectively. The predicted intracellular-region sequences are presented in units of 7 amino acids to highlight the repeating heptad units that nearly always end with a hydrophobic residue. Polyadenylylation signal is indicated in bold.

Membrane-spanning region and associated connecting segments. A single membrane-spanning domain is predicted in the sequence of each clone (34).

Cytoplasmic region. The four clones diverge greatly in the cytoplasmic region both in sequence and in the total number of predicted residues. The predicted amino acid sequences are presented in units of 7 amino acids (heptads) to emphasize the pattern of this portion of the predicted sequences (Fig. 2). There is a hydrophobic residue, often leucine, at nearly every seventh position. The perception that heptad units are the

Table 1. Alignment scores of comparisons between the predicted amino acid sequence of the bg14/8 extracellular domain and members of the immunoglobulin (Ig) gene superfamily

Sequence compared	Score
V set	
Link protein, rat (LKRT2)	9.01
Igλ1 V region, mouse (L1MS4E)	7.20
Myelin Po protein, rat (MPRTO)	6.81
OX-2 membrane glycoprotein, rat (TDRTOX)	6.68
T-cell receptor β-chain V region, human (RWHUVY)	6.17
Poly(Ig) receptor, rabbit (QRRB)	5.95
Ig light-chain V region, chicken (28)	4.15
H set	
CEA-B3 (29)	3.96
NCAM-N (30)	3.33
C set	
IgG1 C region, human (GHHU)	3.82
MHC class Iα3, chicken (31)	3.82
MHC class II β chain, human DR (HLHU3D)	3.61
β_2-Microglobulin, human (MGHUB2)	2.63
T-cell receptor β-chain C region, human (RWHUCY)	2.40
Ig light-chain C region, chicken (28)	2.31

Domains were defined as described (32). Sequences were taken from original publications as indicated by reference notation or from the Protein Identification Resource (National Biomedical Research Foundation) using the name provided in parentheses. Alignment (SD unit) scores above 3.00 indicate significant homology. Scores were determined using PCOMPARE (IntelliGenetics) with bias and gap penalties set at 60 and 100 random permutations.

basic unit of this region is supported by the sequence of bg8, where three of the nine unprocessed introns were found to delineate two 21-nucleotide exons (Fig. 1). The heptad motif is typical of proteins that form α-helical coiled-coil structures (35, 36). Both perfect and imperfect repeats can be found within a single clone, as illustrated by a portion of the bg14/8 sequence where GQQAKES EEQKSEL KEHHEET is followed by GQQAKES EKQKSEL KERHEEM. Identical heptad units may occur in the different clones. These may be "in register" as occurs near the distal end of the intracellular domain or offset as seen in the necessity of introducing a 14-residue gap in the bg11/4 and bg17 sequences in Fig. 2 to maintain optimal alignment of the four sequences. Similar patterns are evident in comparisons of these sequences with B-G sequences described earlier in B^{19} (15).

The 3′ untranslated region. The 3′ ends of the five clones (Fig. 2) are highly similar except in discrete regions where the clones appear to form two different groups. Clones bg14/8 and bg3 contain sequences immediately adjacent to the stop codon that are not present in bg11/4 or bg17. Then, at a short interval more 3′, the bg11/4 and bg17 clones contain segments of 20 bases not present in bg14/8 and bg3. Each clone terminates at a slightly different point with a poly(A) tail following a single polyadenylylation signal.

Correspondence Between B-G Predicted Sequences and CNBr Peptides. In order to determine whether cDNA clones isolated in this study correspond to the B-G antigens expressed on erythrocytes, CNBr peptides were prepared from the B-G antigens expressed on the surfaces of erythrocytes of adult birds of B^{21} haplotype and subjected to microsequencing. Earlier attempts to sequence intact polypeptides failed because the N terminus is blocked in the mature proteins. Because the B-G polypeptides are difficult to handle once purified (ref. 37; M.M.M., unpublished data), obtaining protein sequence data has been difficult even though the protein can be fairly readily purified (17, 37). Even so it was possible to obtain some sequence data. One assignable sequence was obtained in the analysis of CNBr peptides purified by reverse-phase chromatography, N(S)GVADLKELA(S), peptide 1. The sequence of peptide 2, PAV(K,I)(L,V)GQQAKES(G)KQK(S), was obtained by employing the following strategy (38): a portion of the crude mixture of CNBr peptides was sequenced for 20 cycles. The chromatograms indicated several sequences were present, as expected, and also that a proline residue was present at cycle 10. A second portion of the same mixture was then resequenced, but after the ninth round of degradation (when the cycle 10 proline was N-terminal), the sequencer was paused and the filter was exposed to *o*-phthalaldehyde, blocking the primary amino groups. Normal sequencing was then continued until the sequence could no longer be assigned (about 17 cycles).

The sequences of the CNBr peptides that could be produced by the deduced polypeptide sequences of the four sequenced cDNA clones were compared with the sequences of peptides 1 and 2. The inherent similarities in the B-G polypeptides are such that CNBr peptides are not ideal for distinguishing among different gene products. In spite of this, the sequence of peptide 1 agrees well with the expected sequence of a region of bg11/4 (see heptads 11 and 12 in Fig. 2). Peptide 2 aligns well with both bg14/8 (or bg3) and bg11/4 (see heptads 23–25), since both lysine and isoleucine are found at residue 4 and valine and leucine at residue 5. Thus it is clear that the cDNA clones encode polypeptides highly similar to the B-G antigens expressed at the erythrocyte surface. Whether the same genes are expressed in both embryonic and adult animals remains to be determined, since there are 4 mismatches among the 29 residues, only 2 of which are at positions corresponding to tentatively assigned residues in the peptide sequences. In bg17, the equivalent CNBr site, a methionine in heptad 21, is missing and so bg17 sequence would not contribute to the sequence obtained by the *o*-phthalaldehyde method and peptides originating from bg17 transcripts could not be verified. With these data it is possible to conclude that the B-G polypeptides expressed at the surfaces of erythrocytes of adult birds are either highly similar or identical to the transcripts most commonly expressed in the embryonic erythroid cells, i.e., types I (bg14/8) or II (bg3) and IV (bg11/4).

DISCUSSION

This study has provided evidence that part of the polymorphism observed in the B-G antigens residues in the B-G extracellular domain, a domain that resembles immunoglobulin domains of the V-region type and by virtue of its extracellular position must bear the allogeneic epitopes of the B-G antigens. The B-G antigens are exceptional immunoglobulin superfamily members in that sequence variability occurs within the immunoglobulin domain. While the full extent of variability within the immunoglobulin-like domain of different B-G antigens (and, for that matter, the rest of the

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            *              *  * * *              ****              *       ***          ** * *      *** * **
V Motif   f-Q--------------G--h-f-C--------------Whpp--------------L-------sRh------- --ph-L-I------ass-YhCs------------oG-GTpf
            |              |  | | |              ||||              |       |||          || | |      ||| | ||
bg14/8      QITVVAPSLRVTAIVGQDVVLRCHLSPCKDVRNSDIRWIQQRSSRLVHHYRNGVDLGQMEEYKGRTELLRDGLSDGNLDLRITAVTSSDSGSYSCAVQDGDAYAEAVVNLEVSD
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FIG. 3. Alignment of the predicted extracellular domain of bg14/8 with the immunoglobulin V motif as defined by Hunkapiller and Hood (33). Asterisks and vertical bars mark the positions of matches between the bg14/8 sequence and the V motif. Uppercase letters in the motif represent the single-letter amino acid code. Lowercase letters identify the positions of amino acids with functional or physical properties as follows: a, acidic (D, E); f, aliphatic (L, I, V); h, hydrophobic (L, I, V, M, Y, F); o, aromatic (Y, F, W); p, polar (K, R, H, D, E, Q, N, T, S); s, small (A, G, S, T, V, N, D).

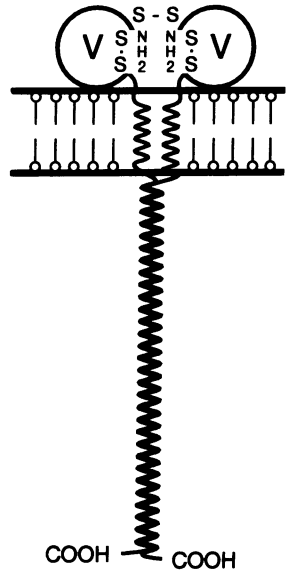

FIG. 4. Schematic model of a B-G dimer.

polypeptide) remains to be determined, in this study already three different variants are represented in four transcript types found within the embryonic erythroid cells of a single haplotype. Three additional B-G clones isolated from the intestinal epithelia, presumably only a small portion of the total transcript types in these cells, show additional sequence variation within the immunoglobulin-like domain (M.M.M., Q.-Y. Xu, J.C., and R.G., unpublished data). The implication of high variability in the immunoglobulin-like domains is a possible function in anticipatory immune defense.

Variability among the B-G antigens that is evident in gel analyses of the B-G polypeptides rests largely in the highly variable intracellular domains. It is now evident that the entire intracellular portion of the B-G polypeptides is made up of heptad repeats typical of an α-helical coiled-coil motif. The total number of heptads varies between clones. The variation from 27 to 31 heptads is as would be expected for the B-G polypeptides expressed on erythrocytes in this genotype. Other clones from the intestinal epithelium show a greater variability in the total number of heptads in the cytoplasmic tails (M.M.M., Q.-Y. Xu, J.C., and R.G., unpublished data), in agreement with their correspondingly greater variation in size (9). It will be some time before it is known whether the entire range of variability in the cytoplasmic tails of the B-G antigens is directly encoded in the multiple genes within the B-G subregion or whether other mechanisms such as alternative splicing or more complex events in gene expression introduce the variability observed in cDNA clones and in the B-G polypeptides. It is clear, however, that the *B-G* genes are numerous and in sufficient number to encode the distinct B-G messages encountered so far in the study of the $B\text{-}G^{21}$ haplotype.

Studies with various gel electrophoretic methods have suggested that the B-G antigens exist in the native state as disulfide-linked dimers, most often homodimers (14, 39). The cysteine residues in the extracellular domain, in addition to the two that presumably form the immunoglobulin-domain bridge, apparently participate in crosslinking the individual polypeptides in dimers. Given the α-helical coiled-coil motif of the cytoplasmic tail sequences, it is likely that the polypeptides coil together to form a stable fibrous cytoplasmic tail. With this information it is possible to compose a model of the B-G dimers that illustrates the immunoglobulin domain structure of the antigens and the fibrous cytoplasmic tails (Fig. 4).

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