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Size polymorphism of chicken major histocompatibility complex-encoded B-G molecules is due to length variation in the cytoplasmic heptad repeat region

(B complex/alternative splicing/coiled coils/nonglycosylated membrane proteins)

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ABSTRACT **B-G antigens are cell-surface molecules encoded by a highly polymorphic multigene family located in the chicken major histocompatibility complex (MHC). Rabbit antisera to B-G molecules immunoprecipitate 3–6 bands from iodinated erythrocytes by sodium dodecyl sulfate (SDS) gels under reducing conditions. These are all B-G molecules because they all map to the *B-G* region of the chicken MHC in congenic and recombinant chickens, most are directly recognized by the antisera, most form disulfide-linked dimers, and none bear N-linked carbohydrate. Both apparent homodimers and heterodimers are found, which bear intrachain disulfide bonds. All 3–6 bands have different mobilities in SDS gels between different haplotypes, ranging from 30 to 55 kDa. This size polymorphism is not affected by glycosidase treatment or addition of protease inhibitors. Partial proteolysis of cell surface-iodinated B-G molecules generates extremely similar patterns of spots, both within and between haplotypes. These surface-iodinated peptides bear either interchain or intrachain disulfide bonds. Additional peptides are generated by proteolysis of B-G molecules iodinated after isolation. Thus, it appears that the extracellular regions of these molecules are very similar and that the length polymorphism is due to variations in the cytoplasmic regions. Inspection of the cDNA-derived protein sequence in this region shows many heptad repeats, which may allow variation in length by step deletion and alternative splicing. The repeats indicate an α-helical coiled-coil structure, which could form an interaction between subunits of the dimer or with the cytoskeleton or both.**

In addition to class I and class II genes, the chicken major histocompatibility complex (MHC) contains another polymorphic multigene family that encodes cell surface molecules called the B-G antigens (1–4). These B-G antigens were originally described as limited to erythrocytes and their precursor cells, but it is now clear that members of this family are found on thrombocytes, lymphocytes, and certain epithelial cells in the bursa, thymus, and intestine as well (ref. 5; J.S., D. Dunon, K.S., D.T., O. Vainio, and J.K., unpublished data).

The biological functions of these molecules are unknown, but the serologically polymorphic ones may have some role in specific recognition of foreign antigens by cells of the immune system (4, 6), based on the high polymorphism, location in the MHC, distinctive tissue distribution, and two interesting phenomena: polymorphic B-G epitopes are recognized by so-called "natural antibodies" in a variety of species (7, 8) and B-G molecules are responsible for the "adjuvant effect" for humoral responses to other molecules on erythrocytes and in liposomes (refs. 9 and 10; J.S., H. Eriksson, K.S., T. Lundgren, M. Simonsen, and J.K., unpublished data). Other B-G molecules may not be polymorphic but nevertheless may be involved in cell–cell interaction.

There is still not much known about the structure of B-G molecules. A B-G molecule isolated from GB-1 chicken strain (*B13* haplotype) with a monoclonal antibody (mAb) was shown to be a disufide-linked multimer that bore no demonstrable N-linked or O-linked carbohydrate and that required detergent for solubilization (11). Using rabbit antisera derived from this purified molecule, we isolated B-G19 cDNA clones with a number of distinctive features, including multiple small repeats and short "introns" that may code for protein when not spliced out (12). In this article, we use these rabbit antisera to show that there is considerable size polymorphism for B-G molecules within and between MHC haplotypes, but that these molecules all have the canonical features found for the B-G13 molecule, along with intrachain disulfide bonds in similar-size extracellular regions of at least 30 kDa. In addition, we show that the intracellular regions of the B-G dimers may be composed of α-helical coiled coils encoded by the multiple small repeats, that the B-G size polymorphism is due to variation in the size of these intracellular regions, and that this variation could be due, at least in part, to alternative splicing of the short "introns" observed in the cDNA clones.

MATERIALS AND METHODS

Chicken strains from the Gipf-Oberfrick farm of the Basel Institute for Immunology, mAbs and rabbit antisera to B-G molecules, the isolation and characterization of purified B-G molecules, and B-G cDNA clones have been described in detail (11, 12). Isolation of chicken blood cells, cell surface iodination using lactoperoxidase and glucose oxidase, detergent lysis with phenylmethylsulfonyl fluoride as protease inhibitor, immunoprecipitation using protein A-Sepharose, sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis (with or without 2 mM dithiothreitol as reductant), and fluorography using phosphotungstate screens were performed essentially as described (12, 13). Iodination of purified B-G molecules by the Iodo-Gen method and the protease inhibitor cocktail used in the experiment for Fig. 2 were as described (11).

For deglycosylation, immunoprecipitates were boiled in 30 μl of 0.5% SDS/100 mM Tris chloride, pH 8.8/10 mM EDTA, and the supernatant liquid was transferred to a new tube. Then 5 μl of 10% (vol/vol) Nonidet P-40 and 1 μl of a preparation from *Flavobacterium meningosepticum* (pre-

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Abbreviations: MHC, major histocompatibility complex; nt, nucleotides; mAb, monoclonal antibody.

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pared as in ref. 14 and containing mainly the peptide *N*-glycosidase as defined by ref. 15) were added, and the samples were incubated at 37°C for 18 hr. Then 25 μl of 50% (vol/vol) glycerol/10% (wt/vol) SDS/0.1% bromphenol blue was added, and the samples were boiled before analysis by SDS/gel electrophoresis.

For two-dimensional gels, the appropriate slices from the fixed and dried first-dimension gel were located by reference to the autoradiogram and cut out with a scalpel blade. Such slices were inserted sideways above a flat-topped stacking gel, sample buffer with 1% agarose (with or without 10 mM dithiothreitol as reducing agent) at 65°C was applied all around the slices with a syringe and needle to make a flat-topped sample, and then the samples were allowed to rehydrate for 30 min before electrophoresis. For partial proteolysis (ref. 16), *Staphylococcus* V8 protease (Endoproteinase Glu-C, Boehringer Mannheim) at 0.2 mg/ml in 134 mM glycine/17.5 mM Tris chloride, pH 8.3/0.7% SDS/26% glycerol (i.e., 70 parts of reservoir buffer to 30 parts of 87% glycerol) was layered over the samples embedded in agarose before electrophoresis.

For reduction and alkylation under nondenaturing conditions, immunoprecipitates in wash buffer containing Nonidet P-40 (12, 13) were incubated at room temperature with 2 mM dithiothreitol for 30 min and then with 6 mM iodoacetamide in the dark for 30 min. Then the samples were boiled in sample buffer containing 2% SDS with or without 10 mM dithiothreitol before analysis by SDS/gel electrophoresis.

RESULTS

Each MHC Haplotype Has Different Patterns of B-G Bands, All of Which Map to the *B-G* Region. Erythrocytes from five standard chicken strains were cell surface-iodinated, detergent lysates were prepared, and immunoprecipitates formed by using rabbit antisera to B-G molecules were analyzed by SDS/gel electrophoresis. At least three bands of apparent molecular masses between 30 and 55 kDa can be discerned from each strain by SDS/gel electrophoresis after reduction of the immunoprecipitates (Fig. 1, lanes 6–10). In fact, some of these bands clearly contain several molecules of slightly

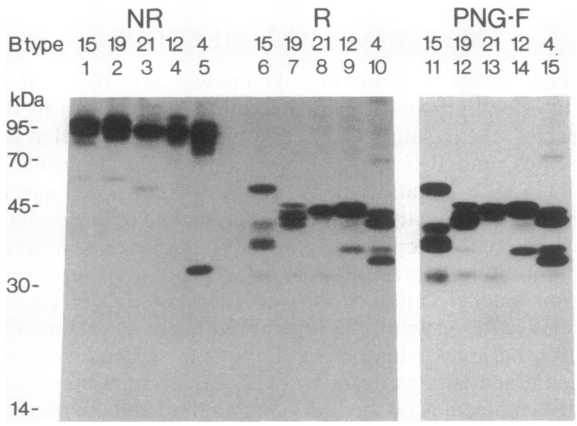

FIG. 1. Immunoprecipitates from detergent-solubilized lysates of cell surface-iodinated blood cells formed by using rabbit antisera to B-G13 molecules (serum 985 for lanes 1–10, serum 981 for lanes 11–15) analyzed by SDS/12.5% polyacrylamide gel electrophoresis. The samples in lanes 1–5 were not reduced prior to electrophoresis (NR), the samples in lanes 6–10 were boiled in reducing agent (R), and the samples in lanes 11–15 were boiled in reducing agent and then treated with a glycosidase preparation (PNG-F). The following chicken strains (and their haplotypes) were used: H.B15 (*B15*) in lanes 1, 6, and 11; H.B19 (*B19*) in lanes 2, 7, and 12; H.B21 (*B21*) in lanes 3, 8, and 13; CB (*B12*) in lanes 4, 9, and 14; and CC (*B4*) in lanes 5, 10, and 15.

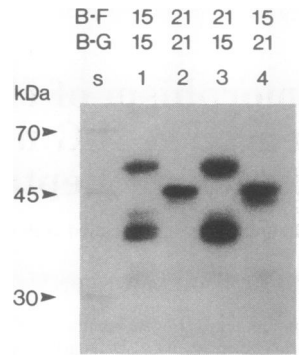

FIG. 2. Immunoprecipitates from detergent-solubilized lysates of cell surface-iodinated blood cells formed by using rabbit antiserum 981 to B-G13 molecules analyzed by SDS/12.5% polyacrylamide gel electrophoresis under reducing conditions. The two strains H.B15 (*B15* haplotype, lane 1) and H.B21 (*B21* haplotype, lane 2) and their apparent reciprocal recombinants H.B21r3 (informally called R4; *B-F/B-L 21*, *B-G15*) and H.B15r1 (informally called R5; *B-F/B-L15*, *B-G21*) were used. s, Standards.

different sizes, which become apparent by using different rabbit antisera (for instance, in Fig. 1, compare lanes 6–10 with 11–15) or with certain mAb (data not shown). None of these patterns are similar and, under close inspection, none of the bands comigrate. All of these bands apparently are directly recognized by the rabbit antisera [as assessed by immunoprecipitation from SDS-denatured lysates and by immunoblots (Western blots); data not shown], except for one band of ≈34 kDa in the CC strain (which is also not disulfide-linked to another chain, see below). All of these bands apparently map to the *B-G* region of the chicken MHC, as defined by congenic (CB and CC; Fig. 1) and recombinant (H.B15, H.B21, H.B21r3, and H.B15r1; Fig. 2) chicken strains.

B-G Molecules Are Disulfide-Linked Homo- and Heterodimers with Intrachain Disulfide Bonds. All of these molecules form disulfide-linked dimers, as assessed by SDS/gel electrophoresis under nonreducing conditions, with the exception of the roughly 34-kDa band from the CC (*B4*) strain (Fig. 1, lanes 1–5). As assessed by two-dimensional gels (Fig. 3*A*), these are mostly apparent homodimers with the same

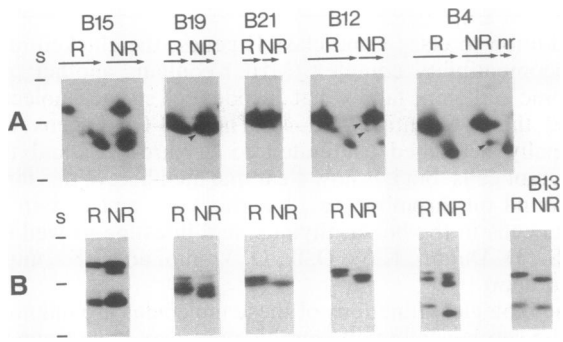

FIG. 3. Immunoprecipitates from detergent-solubilized lysates of cell surface-iodinated blood cells formed by using rabbit antiserum 981 to B-G13 molecules analyzed by SDS/12.5% polyacrylamide gel electrophoresis. (*A*) Samples in the first dimension were either reduced (R) or not reduced (NR, nr) before electrophoresis in the first dimension (in direction of arrows) and were reduced before electrophoresis in the second dimension. (nr indicates the 34-kDa *B4* band that fails to dimerize, which was excised separately.) Small arrowheads indicate spots representing apparent heterodimers. (*B*) Samples were reduced and alkylated under nondenaturing conditions and then were either reduced (R) or not reduced (NR) prior to electrophoresis. Chicken strains are as in Fig. 1, except GB-1 (*B13*). Standards (s) were approximately 70, 45, and 30 kDa.

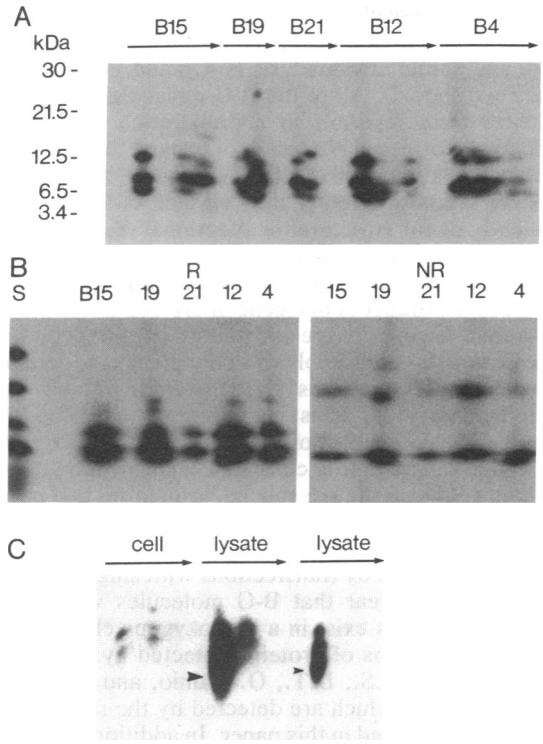

FIG. 4. *Staphylococcus* V8 protease partial peptides of iodinated B-G molecules analyzed by SDS/12.5–20% polyacrylamide gel electrophoresis. (*A*) Immunoprecipitates from detergent-solubilized lysates of cell surface-iodinated blood cells formed by using rabbit antiserum 985 to B-G13 molecules were run on SDS gels under reducing conditions, and the dried gel strips were turned sideways above the second-dimension gel before proteolysis. Arrows show the direction of electrophoresis in the first dimension. Chicken strains were as in Fig. 1. (*B*) As in *A*, but the first dimension was under nonreducing conditions and the second dimension was either under reducing (R) or nonreducing (NR) conditions. (*C*) As in *A*, but with rabbit antiserum 493Y to immunoprecipitate B-G peptides from surface-iodinated H.B15 erythrocytes (cell in *C* left) and purified detergent-solubilized B-G15 molecules (lysate in the center and with shorter film exposure in *C right*). Arrowheads indicate additional partial peptides.

mobility on both reducing and nonreducing gels, though apparent heterodimers (small arrowheads) are present. In addition, nearly all of these bands represent molecules with intrachain disulfides as well, as assessed by the mobility of molecules that were reduced and alkylated under nondenaturing conditions (to reduce interchain disulfide bonds), followed by denaturation and electrophoresis either with reduction (to reduce the intrachain disulfide bonds) or without reduction (Fig. 3*B*).

Size Heterogeneity Is Due to Differences in the Size of Cytoplasmic Tails, Rather than to N-Linked Glycosylation or Apparent Proteolysis. In contrast to chicken class I α chain (11), none of the B-G bands changed mobility on SDS gels after treatment of the denatured immunoprecipitates with a preparation of glycosidases from *F. menigosepticum* (Fig. 1, lanes 11–17; control samples without glycosidase treatment are not shown), indicating a lack of detectable N-linked glycans. Inclusion of various protease inhibitors (EDTA, *o*-phenanthrene, and iodoacetamide, as in ref. 11) in addition to phenylmethylsulfonyl fluoride during and after lysis affected only the intensity of the bands (compare Fig. 1 with Fig. 2; other data not shown).

In an attempt to determine which bands in one haplotype corresponded to bands in other haplotypes, strips of dried gel containing the appropriate bands after a first dimension of

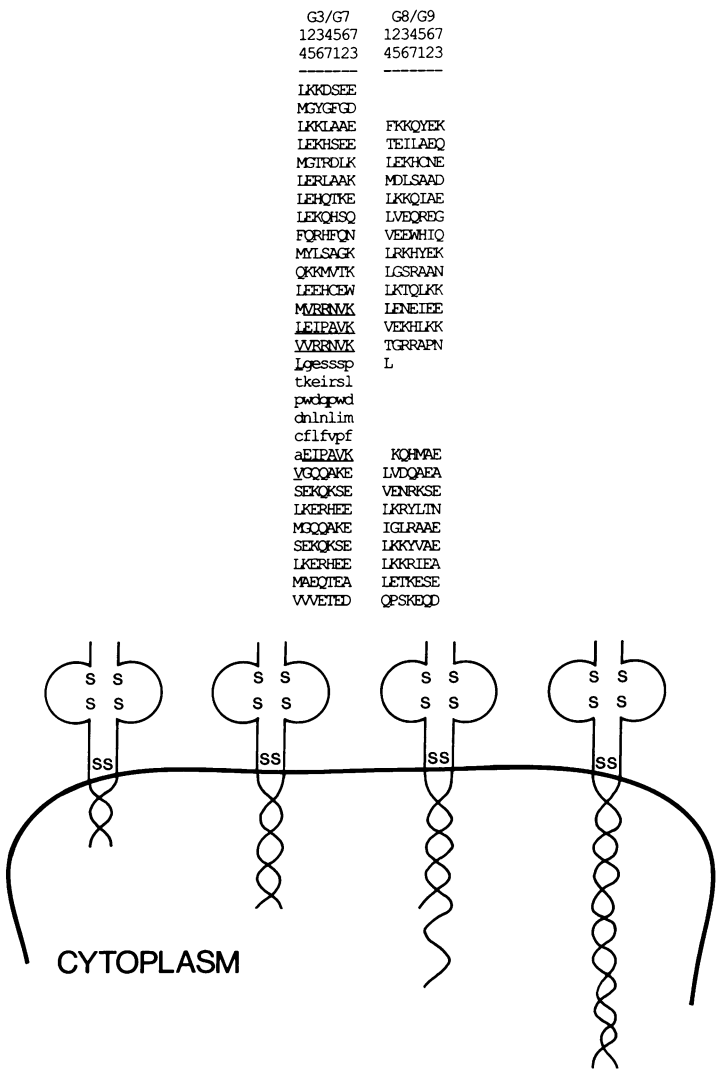

FIG. 5. Biochemical structure of B-G molecules. (*Upper*) Amino acid sequences derived from the cDNA clones G3, G7, G8, and G9 representing the final C-terminal cytoplasmic regions of the encoded proteins and organized to show heptad repeats in two different registers. The 42-nt direct repeats in G3 and G7 are underlined; the 105-nt "intron" found in G3 but not G7 is shown in small letters. Amino acids are represented by using the single-letter code: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. (*Lower*) Model of B-G molecules based on the data in this paper. SS indicates intra- and interdomain disulfide bonds; twisted lines within the cytoplasm indicate the heptad repeat regions, presumably forming α-helical coiled coils.

SDS/gel electrophoresis under reducing conditions were subjected to partial proteolysis with *Staphylococcus* V8 protease during a second dimension. Unexpectedly, all of the bands from all of the haplotypes generated very similar partial proteolysis patterns, with three bands of around 5, 6.5, and 10 kDa (Fig. 4*A*). The largest of these partial peptides contains at least one interchain disulfide bond, while the other two contain intrachain disulfide bonds, as determined by comparison of the patterns after electrophoresis with and without reduction (Fig. 4*B* and data not shown). The presence of disulfide bonds is consistent with the derivation of these cell surface-iodinated peptides from the extracellular regions of the B-G molecules and indicates that all of B-G size variants probably have extracellular regions of similar size and structure. Similar results were found for chymotrypsin partial peptides (data not shown).

Direct evidence was sought for size variation of the intracellular regions by comparison of the partial proteolysis patterns of cell surface-iodinated B-G preparations and B-G preparations iodinated after detergent solubilization (Fig. 4*C*). The presence of additional partial peptides derived from the largest band of the material iodinated after solubilization, compared with both the smaller bands and the cell surface-iodinated molecules, is consistent with the presence of iodinated intracellular peptides. This data can be considered direct biochemical evidence for transmembrane orientation of the B-G15 protein (17).

Intracellular Cytoplasmic Regions of the B-G Molecules Are Composed of Heptad Repeats with the Potential for Variable Lengths. Recent isolation of partial cDNA clones for two homologous B-G molecules (represented by the clones G3/G7 and G8/G9) revealed the presence of long regions of 21-nucleotide (nt) repeats in the 3′ coding region (12). While the nucleotide and amino acid sequences in these regions are poorly conserved, the first (and fourth or fifth, depending on the register) residue of the seven-amino acid repeat is almost always hydrophobic, and the others are often highly charged (Fig. 5 *Upper*). A great variety of proteins, including fibrous proteins like tropomyosin and intermediate filaments, contain long stretches of heptad repeats in which the first and fourth residues are usually (but not always) hydrophobic, and the rest may be highly charged, often with a particular pattern. These heptad repeats give rise to coiled coils of α-helices (18, 19), and it seems likely that the two cytoplasmic tails of the B-G dimer interact in some related structure.

Two of these cDNA clones were completely identical (G8 and G9) and homologous to the other two clones (G3 and G7). These latter two clones were identical except for an apparent 105-nt "intron" with all the canonical splicing signals embedded in two 42-nt repeats. This "intron," if not spliced out, would still code for protein in-frame with the rest of the sequence (Fig. 5 *Upper*; see also ref. 12). Thus, some of the size variation could be due to alternative splicing. In the preliminary sequence of a *B-G* gene, five pairs of exons with an embedded 105-nt "intron" are present (J.K., K.S., C. Auffray, and F. Guillemot; unpublished data) and, if used, would give ample scope for variation in the size of the cytoplasmic tail.

DISCUSSION

In this article, we make three points about B-G molecules. First, all B-G molecules have a characteristic structure: transmembrane disulfide-linked dimeric proteins with intrachain disulfide bonds and no N-linked carbohydrate. Second, the two chains in the B-G dimer have cytoplasmic regions composed of heptad repeats, which probably interact as α-helical coiled coils. Third, there is substantial size heterogeneity in the B-G molecules, both within and between chicken MHC haplotypes, which is apparently due to variations in the length of the cytoplasmic tails. Consistent with the biochemical characterization, the preliminary structure of a B-G protein deduced from cDNA and genomic clones has an apparent variable immunoglobulin domain-like region with cysteine residues in the normal positions, followed by a presumed hydrophobic transmembrane region, which is followed by a large C-terminal cytoplasmic region composed of multiple small repeats subject to apparent alternative splicing (ref. 12; J.K., K.S., C. Auffray, and F. Guillemot, unpublished data). There remain a number of areas of interest.

(*i*) Virtually all membrane proteins with a large extracellular region acquire N- or O-linked carbohydrate or both during biosynthesis (20, 21). B-G molecules isolated with particular mAb bear neither detectable N- and O-linked carbohydrate, as assessed by inhibition of glycosylation during biosynthesis (B-G19), pulse–chase analysis (B-G19), enzymatic deglycosylation (B-G6 and B-G13), chemical deglycosylation (B-G6), lectin binding (B-G6, B-G13, and B-G19), and amino acid analysis (B-G6 and B-G13) (ref. 11 and data not shown). All of the B-G molecules examined in this article were resistant to a preparation including the peptide *N*-glycosidase. We have not proven that all of these B-G molecules are devoid of all carbohydrate, but it would be interesting to know whether they all lack N-linked glycans coincidently or for structural or functional reasons.

(*ii*) It is not yet clear which of the multiple B-G proteins are due to proteolysis, biosynthetic modification, or alternative splicing of the product of one gene or are due to the products of different *B-G* genes or are due to both. It is difficult to rule out the possibility of proteolysis in the present experiments, although many precautions were taken to inhibit known proteolytic activities. It is also difficult to categorically eliminate the possibility of uncharacterized biosynthetic modifications, though it is clear that glycosylation plays no significant role. Alternative splicing is an attractive possibility, especially in view of the apparent use of a short "intron" in two otherwise identical cDNA clones (12), but the issue can only be resolved by transfections with single *B-G* genes.

However, it is clear that B-G molecules with different extracellular regions exist in a homozygous chicken strain, based on the patterns of proteins detected by various mAb (J.S., D. Dunon, K.S., D.T., O. Vainio, and J.K., unpublished data), all of which are detected by the rabbit antisera to B-G molecules used in this paper. In addition, two distinct cDNAs have been isolated from a homozygous chicken (12), and the genes determining two B-G proteins detected by two-dimensional gels have been separated in an MHC recombinant chicken strain (22). Finally, many fragments hybridizing with B-G cDNA probes are observed in Southern blots of genomic DNA and of cosmids, although it is not yet clear how many are pseudogenes (refs. 12 and 23; J.K., K.S., C. Auffray, F. Guillemot, unpublished data). The variation in size of different gene products could be caused by mutations (affecting splicing or translation) or by deletion, insertion, and recombination (facilitated by the multiple small repeats in the cytoplasmic regions).

(*iii*) The presumed alternative splicing is interesting for two reasons. First, while the protein sequence of the "intron" has five of these heptad repeats with some features of a coiled coil, there are a number of prolines present, which should disrupt the secondary structure. The length of the cytoplasmic region may not really matter, but a more attractive possibility is that alternative splicing controls the association of B-G chains, either with each other or with some other cytoplasmic protein. Second, while alternative splicing in eukaryotes includes a few examples of "retained introns" (24), we are not aware of an example of such an in-frame "intron" being read completely through in a eukaryotic gene. This use of an "intron," like the presence of small introns in genes of the chicken MHC (4, 6), may reflect poorly understood pressures derived from the production of microchromosomes in chicken (25).

(*iv*) It is not clear whether the apparent homo- and heterodimers are homo- or heterodimeric in the extracellular regions. If a band contains proteins of different cytoplasmic size that are truly the product of a single gene, then from a cell–cell recognition standpoint they may be considered homodimers. However, it is also possible that some of the apparent homodimers actually have different extracellular regions and thus are really heterodimers. Transfections with isolated *B-G* genes should clarify this issue as well as whether multiple protein products can arise from single genes by alternative splicing. Along with the question of the number of extracellular domains and the location of the serologically polymorphic residues, these questions have a bearing on any proposed theory of B-G function.

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