Corrections

PHYSIOLOGY

The authors note that the author name Jared D. Gorham should have appeared as James D. Gorham. Additionally, the author name Victor G. Bundoc should have appeared as Virgilio G. Bundoc. The corrected author line appears below. The online version has been corrected.

Krisztian Nemetha,b,1, Andrea Keane-Myersc, Jared M. Brownc, Dean D. Metcalfec, James D. Gorhamd, Virgilio G. Bundocc, Marcus G. Hodgesd, Ivett Jelineke, Satish Madalae, Sarolta Karpatib, and Eva Mezeya,1

www.pnas.org/cgi/doi/10.1073/pnas.1003664107

BIOCHEMISTRY

The authors note that due to a printer’s error, several of the Supporting Figures were referenced incorrectly in the main text. All references to Supporting Figure 3 should have instead referred to Supporting Figure 5, and all references to Supporting Figure 5 should have instead referred to Supporting Figure 3. All references to Supporting Figure 4 should have instead referred to Supporting Figure 6, and all references to Supporting Figure 6 should have instead referred to Supporting Figure 4. The online version has been corrected.

www.pnas.org/cgi/doi/10.1073/pnas.1003450107

GENETICS

The authors note the following statement should be added to the Acknowledgments: “This material is based on work supported in part by National Science Foundation Grant MCB-0524167.”

www.pnas.org/cgi/doi/10.1073/pnas.1003235107

BIOCHEMISTRY

The authors note the following statement should be added to the Acknowledgments: “J.B. also acknowledges the support of Czech Grants LC535, MSM0021620806, and AV0Z50110509.”

www.pnas.org/cgi/doi/10.1073/pnas.1003712107
BG1 has a major role in MHC-linked resistance to malignant lymphoma in the chicken

Ronald M. Gotoa,1, Yujun Wanga,1, Robert L. Taylor, Jr.3, Patricia S. Wakenell2, Kazuyoshi Hosomichid, Takashi Shinia, Craig S. Blackmorec, W. Elwood Brilesb, and Marcia M. Millerc

aDepartment of Molecular Biology, Beckman Research Institute, City of Hope, Duarte, CA 91010; bDepartment of Animal and Nutritional Sciences, University of New Hampshire, Durham, NH 03824; cDepartment of Population Health and Reproduction, University of California Davis, Davis, CA 95616; dDepartment of Molecular Life Science, Tokai University School of Medicine, Isehara, Kanagawa 259-1143 Japan; and eDepartment of Biological Sciences, Northern Illinois University, DeKalb, IL 60115

Edited by Richard L. Witter, U.S. Department of Agriculture, East Lansing, MI, and approved August 10, 2009 (received for review June 16, 2009)

Pathogen selection is postulated to drive MHC allelic diversity at loci for antigen presentation. However, readily apparent MHC infectious disease associations are rare in most species. The strong link between MHC-B haplotype and the occurrence of virally induced tumors in the chicken provides a means for defining the relationship between pathogen selection and MHC polymorphism. Here, we verified a significant difference in resistance to gaHV-2-induced lymphomas (Marek’s disease) conferred by two closely-related recombinant MHC-B haplotypes. We mapped the crossover breakpoints that distinguish these haplotypes to the highly polymorphic BG1 locus. BG1 encodes an Ig-superfamily type I transmembrane receptor-like protein that contains an immunoreceptor tyrosine-based inhibition motif (ITIM), which undergoes phosphorylation and is recognized by Src homology 2 domain-containing protein tyrosine phosphatase (SHP-2). The recombinant haplotypes are identical, except for differences within the BG1 3’-untranslated region (3’-UTR). The 3’-UTR of the BG1 allele associated with increased lymphoma contains a 225-bp insert of retroviral origin and showed greater inhibition of luciferase reporter gene translation compared to the other allele. These findings suggest that BG1 could affect the outcome of GaHV-2 infection through modulation of the lymphoid cell responsiveness to infection, a condition that is critical for GaHV-2 replication and in which the MHC-B haplotype has been previously implicated. This work provides a mechanism by which MHC-B region genetics contributes to the incidence of GaHV-2-induced malignant lymphomas in the chicken and invites consideration of the possibility that similar mechanisms might affect the incidence of lymphomas associated with other oncogenic viral infections.

disease resistance gene | GaHV-2 induced lymphoma | Gallus gallus | Marek’s disease

There is an astounding breadth of genetic diversity at the MHC, a region found within the genomes of all jawed vertebrates. Despite the seeming likelihood that pathogen selection has an important role in driving allelic diversity at MHC class I and II loci, associations are rarely found between infectious diseases and particular MHC alleles or haplotypes. The presence of multiple polymorphic class I and class II gene family members within MHC haplotypes in many species could contribute to the difficulty in observing MHC disease associations. In the chicken, the MHC-B region has an exceptionally strong role in genetic resistance to Marek’s disease (MD) caused by gallid herpesvirus-2 (GaHV-2) and to Rous sarcoma virus (RSV)-induced tumors (1, 2). Resistance to these diseases maps to the MHC-B subregion marked by a highly expressed classical MHC class I gene (3–6). It has been suggested that strong MHC-B disease associations are apparent as the result of alleles at this single locus for classical class I antigen presentation, BF2, contributing to immune responses either independently or in concert with closely-linked transporter associated with antigen-processing (TAP) genes (6, 7). Antigen presenting molecules encoded by different BF2 alleles bind quite different classes of peptides (7–10) and thereby likely selectively influence adaptive immune responses to antigen, but the gene or genes that provide MHC-linked resistance to virally induced tumors in chickens are not known. Recently, the crossover breakpoint in the recombinant haplotype originally used to map MD resistance to the MHC-B subregion marked by BF2 was localized, which revealed that 27 genes lie within the region between the crossover breakpoint and the BF2 locus (4, 11). Thus, it is now evident that many genes are candidates for providing the long noted MHC-B haplotype-linked resistance to MD. Overall, the MHC region in chicken stands out in contrast to the MHC in mammals. The chicken MHC is compact and segmented into the MHC-B and MHC-Y regions in which the MHC class I and class II loci reside, as well as genes that changed or moved later in evolutionary time in other species (6, 12–15). Meiotic recombination within MHC-B is rare (16), but several recombinant haplotypes are available that are suitable for further investigation of MHC-B-linked disease resistance.

BR2 and BR4 are two recombinant haplotypes within pedigreed matings designed to identify duplicate recombinant haplotypes originating from independent crossover events between the same parent haplotypes (Fig. 1A). Such recombinant haplotypes were sought as a means for defining which genes within the MHC-B region confer resistance to MD (16). When tested in a small challenge trial with GaHV-2 virus at the fourth backcross generation in the development of congenic lines, birds bearing BR2 and BR4 differed significantly in the incidence of MD (17). BR2 and BR4 also apparently contribute differently to the influence of MHC-B on the regression of RSV-induced tumors (18). These observations suggest that, although indistiguishable by the serologically-defined erythroid BG and class I BF antigens used to isolate them, BR2 and BR4 are likely different as the result of meiotic recombination breakpoints having occurred at different locations within the region separating the genes encoding these serological markers. The breakpoints apparently surround a gene that influences disease resistance.

Results

A Larger Challenge Trial Confirmed the Difference Between BR2 and BR4 in Conferring Resistance to Marek’s Lymphoma. We conducted a GaHV-2 challenge trial using the 003.R2 and 003.R4 lines, now available that are suitable for further investigation of MHC-B-linked disease resistance.

The authors declare no conflict of interest.


This article contains supporting information online at www.pnas.org/cgi/content/full/0906776106/DCSupplemental.
more inbred than previously as the result of 10 backcross generations of breeding, to verify the previous observation suggesting that BR2 and BR4 differ in their influence in MD (17). Pedigree-hatched chicks were inoculated with the highly virulent RB1B strain of GaHV-2 and observed for the formation of tumors over 12 weeks. Genotypes were obtained for each bird and combined with disease data at the conclusion of the trial. A difference in MD mortality between the lines was evident during the trial and was consistent with the difference seen in MD incidence upon gross examination of all birds at completion of the trial. MD incidence was significantly lower in line 003.R2 birds compared to line 003.R4 birds (Table 1). Inclusion of the available histopathological findings increased the incidence of MD overall, but the two lines remained significantly different (39% in 003.R2 versus 61% in 003.R4, \( P < 0.0001 \)). Tumors in the 003.R2 and 003.R4 lines were present predominantly in heart, kidney, liver, spleen, and gonad. The difference in tumor incidence between lines 003.R2 and 003.R4 in this trial, after 10 generations of backcrossing, is essentially identical to that reported for the fourth backcross generation (17). Thus, it appears that although BR2 and BR4 are identical for MHC-B serological markers for BG and BF, they differ significantly in the capacity to confer resistance to MD. This observation supports our hypothesis that the BR2 and BR4 haplotypes have different crossover breakpoints bounding a gene conferring disease resistance.

The Difference Between MHC-BR2 and MHC-BR4 Maps to BG1. To define the crossover breakpoints, we initially assayed BR2, BR4, and the parental haplotypes B2 and BR1 (a recombinant haplotype derived earlier from B23 and B24, Fig. 1A) for BG restriction fragment patterns, LEI0258 microsatellite PCR-product lengths, and MHC class I and class II single strand conformation polymorphism patterns. Alleles in BR2 and BR4 were assigned based on matches with alleles in the parental haplotypes (Fig. 1B). Consistent with earlier serological typing, BR2 and BR4 were found to share MHC class I and class II alleles with the B2 parent haplotype (Fig. 1B). The restriction fragment patterns of the BG genes for BR2 and BR4 resembled those of the B23 portion of the BR1 parent haplotype, confirming earlier serological identification that the BG gene cluster originated from the BR1 parent haplotype. BR2 and BR4 were identical at the microsatellite marker LEI0258, and matched the BR1 parent haplotype. Thus, the crossover breakpoints producing BR2 and BR4 occurred downstream of LEI0258, narrowing further investigation to seven genes—B-BTN1, B-BTN2, RNA-1eu, BG1 [a structurally distinct member of the BG gene family, located about 100-kb distant from the cluster of BG genes encoding BG antigens (11, 19)], and Blec2, Blec2c, and Blec1 (Fig. 1C).

We resolved the BR2 and BR4 crossover breakpoints through sequencing. Single nucleotide polymorphism (SNP) differences revealed closely positioned, but distinct, crossover breakpoints in the vicinity of the BG1 gene (encoded on the complementary strand) (Fig. 1D). In the formation of the BR4 haplotype, crossover between the BR1 and B2 parent haplotypes occurred somewhere within a 338-bp interval defined by SNPs 16900 and 16741.

Table 1. Congenic lines 003.R2 and 003.R4 birds differ in the incidence of Marek’s disease tumors following infection with GaHV-2

<table>
<thead>
<tr>
<th>Line</th>
<th>MD mortality during trial</th>
<th>Total MD incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>003.R2</td>
<td>10% (14/140) b, c</td>
<td>19% (27/140) b, c</td>
</tr>
<tr>
<td>003.R4</td>
<td>39% (51/130) b, c</td>
<td>47% (61/130) b</td>
</tr>
</tbody>
</table>

Specific pathogen free (SPF) birds, infected secondarily by inhalation of shed virus, served as controls to gauge the success of the initial infection. \( b = P < 0.0001 \) vs SPF; \( c = P < 0.0001 \) vs 003.R4.

Fig. 1. Derivation of the BR2 and BR4 MHC-B recombinant haplotypes and the genetic differences between them. (A) BR2 and BR4 haplotypes were recovered in crosses made between animals bearing BR1 (a recombinant haplotype derived from B23 and B24) and B2 haplotypes when atypical segregation of antigens encoded by the BG gene cluster and BF (MHC class I) genes were detected in serological typing of progeny. (B) The crossover breakpoint regions in BR2 and BR4 were narrowed to a region between LEI0258 and the BLB loci. The BG gene cluster, about 100 kb upstream from LEI0258, was typed by restriction fragment patterns; the LEI0258 by PCR product length; and the BF and BL loci by single strand conformation polymorphism. Images for each locus were obtained from single gels and are duplicated and rearranged as needed here to aid comparisons. (C) Diagram (not to scale) of the MHC-B, illustrating the genes located within the 27-kb crossover breakpoint region (arrows indicate direction of transcription). (D) Map of SNPs within the 27-kb region that define the crossover (CO) breakpoints in BR2 and BR4 as distinct, with the BR2 crossover occurring within the 3’-end of BG1, which is encoded on the complementary strand, as indicated by the arrow. Positions are numbered based on GenBank AB266858. E) The BG1 gene sequence is presented 5’ to 3’, illustrating the consequences of crossover breakpoints on the structure of the BG1*R2 and BG1*R4 alleles and the presence of a 225-bp insert in BG1*R4 that provides an alternate polyadenylation signal sequence (PAS, indicated by ‘*’).
The 3'-UTR Difference Between BG1*R2 and BG1*R4 Had Little Influence on Transcription but Produced Consistent Differences in Assays for Translation. To determine whether the 3'-UTR differences found in BG1*R2 and BG1*R4 affect gene expression, we tested the relative transcription efficiency of the BG1*R2 and BG1*R4 alleles in quantitative PCR assays. Although BG1 was more highly expressed in some tissues than others, we found no significant differences in BG1 ΔCt values between paired tissue samples across the 003.R2 and 003.R4 lines (Table S2). These data indicate that the 3'-UTR difference has little effect on BG1 transcription. We then tested the 3'-UTRs for their effect on translation in dual luciferase reporter assays. Translation of firefly luciferase in LMH cells in conjunction with the shorter and longer BG1*R4 3'-UTRs was consistently repressed as compared to expression with the BG1*R2 3'-UTR (Fig. 2B), suggesting that differences in BG1 translation in vivo could be the basis for the differences in MD between the 003.R2 and 003.R4 lines.

The BG1 Transmembrane Protein Is Phosphorylated on Tyrosine in Pervanadate-Treated Cells and Co-Precipitates SHP-2. BG1 encodes a dimerizing 32-kDa type I transmembrane protein bearing an IgV-like ectodomain and a cytoplasmic tail made up of a coiled-coil region and a C-terminal domain containing a single immunoreceptor tyrosine-based inhibition motif (ITIM) (Fig. 3A). Therefore, BG1 is similar to receptors that bear single IgV-like ectodomains known to attenuate lymphoid cell activation via cytoplasmic ITIM signaling (20). To determine whether BG1 is capable of signaling, we expressed a full-length FLAG epitope-tagged BG1 cDNA clone in LMH cells. At least a portion of the BG1 expressed reached the cell surface (Fig. 3B). As suggested by its structure, FLAG-BG1 underwent tyrosine phosphorylation in the presence of pervanadate, as illustrated in BG1 immunoblots developed with 4G10, a monoclonal antibody specific for phosphotyrosine (Fig. 3C). Further, the protein tyrosine phosphatase SHP-2 (Src homology 2 domain phosphatase-1) co-precipitated with phosphorylated BG1 (Fig. 3D). Although the BG1 protein remains to be fully analyzed, the data presented here indicate that BG1 reaches the cell surface, can be phosphorylated on tyrosine, and that, in turn, it associates with the signaling phosphatase SHP-2. These are all features consistent with BG1 functioning as a surface receptor that limits cell activation.

Discussion

The selection of rare recombinants originating from the same parent haplotypes has provided two MHC-B haplotypes, BR2 and BR4, which differ in their contribution to genetic resistance to lymphomas induced by infection with GaHV-2. When tested at the fourth backcross generation in the production of congenic lines, the difference in tumor incidence between BR2 and BR4 line birds was 22% (17). As reported here, when tested in a substantially larger trial conducted after six additional genera-
were used to develop the blots. Clonal anti-SHP-2 C-18 (9% gel) antibodies. IRDye 800CW secondary antibodies gel, and then immunoblotted either with monoclonal M2 (12% gel) or poly-

---

**Fig. 3.** BG1 has the structure of a tyrosine-based inhibitory receptor. (A) Depiction of the predicted structure of BG1 based on the sequence in Fig. S3. (B) Flow cytometry of LHM cells stably expressing FLAG-BG1 shows that BG1 reaches the cell surface. Cells were stained with the anti-FLAG monoclonal antibody M2 and anti-mouse IgG–allophycocyanin (APC) (striped histogram) and with anti-mouse IgG-APC alone (open histogram) and analyzed by flow cytometry gating on the DAPI-negative cell population. (C) BG1 is phosphorylated on tyrosine. LHM cells stably expressing FLAG-BG1 were lysed following 0-, 5-, and 15-min treatments with pervanadate, a tyrosine phosphatase inhibitor. BG1 was immunoprecipitated (IP) from total cell lysates using the anti-FLAG antibody M2 and then immunoblotted (IB) from a 12% polyacryl-
amide gel and probed with either M2 or the monoclonal anti-phosphotyrosine antibody 4G10. (D) The tyrosine phosphatase SHP-2 co-precipitates with BG1. FLAG-BG1 was immunoprecipitated (IP) from LHM cells stably expressing FLAG-BG1 with M2 using cultures without (+) or with treatment with pervana-
date for 15 min (+), electrophoresed, transferred from a 12% gel, and then immunoblotted with monoclonal M2 (12% gel) or poly-
clonal anti-SHP-2-C-18 (9% gel) antibodies. IRDye 800CW secondary antibodies were used to develop the blots.

tions of backcrossing, the difference in GaHV-2 tumor incidence between the 003. R2 and 003. R4 lines remained the same. Thus, the results indicate that genetic variability within the lines in regions outside MHC-B, which was no doubt greater at the fourth backcross generation than at the tenth, had little influence on the difference in the incidence of lymphomas between the lines in the two challenge trials. The findings in this study map the basis for the difference observed between 003. R2 and 003. R4 chickens in GaHV-2 lymphomas to BG1.

As noted in a recent comprehensive review by Calnek (21), the pathology that ensues following infection with GaHV-2 is complex, with a variety of cells responding in different ways at different times to the presence of the virus. In natural infections, GaHV-2 is transmitted by the inhalation of poultry dander laden with enveloped GaHV-2. The virus is transported from the respiratory tract to lymphoid organs. Infection of lymphocytes, viral proliferation, and subsequent necrosis in the spleen, bursa and thymus trigger the migration of additional lymphocytes, granulocytes, and macrophages to these organs and acute inflammatory responses ensue. Cytolytic infection occurs mostly in B cells, but also appears in some T cells and in some epithelial tissues as infected lymphoid cells disseminate. There is a fairly abrupt shift from cytolytic to latent infection, with latency developing mostly in T cells. The period of latent infection is short in genetically susceptible birds, and the virus soon emerges to begin a second cytolytic phase of infection. Transformation of activated CD4 T cells soon occurs. Tumors and death typically follow within weeks. There are a number of points in the course of infection at which an inhibitory molecule, the role suggested for BG1, could influence the outcome of GaHV-2 infection.

Numerous investigations into the difference between MHC-

B-resistant (B21-resistant lines N or N2a) and -susceptible (B19-resistant lines P or P2a) lines provide intriguing data to consider in light of identification of BG1 as a candidate gene affecting MD. There is little evidence that MHC-B genotypes-associated differences exist during the initial phase of infection, aside from the slightly delayed appearance of splenomegaly in MHC-B-resistant birds and occasional subtle differences in cytokine profiles [reviewed by (21); see also (22–24)]. However, by the end of the first 7–10 days post inoculation (dpi), substantial differences in MD pathogenesis become clearly evident. There is an abrupt decrease in the viral load in the white cells in resistant birds (23) that is accompanied by lower levels of infected T cells (25), relatively higher levels of virus-neutralizing antibodies (26), fewer AV37+ cells (22), and the absence of induction of the proinflammatory cytokines IL-6 and IL-18, which in contrast occurs in susceptible birds during the same interval (23). In the absence of complicating stress or infection, the genetically resistant birds remain healthy, with the only sign of active infection being the shedding of virus from the feather follice.

Part of the change in MD pathogenesis in genetically resistant birds that occurs at 7–10 dpi could be due to differential expression of specific cytotoxic T lymphocytes (CTLs) directed against viral antigens. Specific CTL responses in MD-resistant B21 homologous birds to the GaHV-2 transcription regulatory protein ICP4 may be a factor in genetic resistance, particularly as immunity develops following vaccination (27). At the same time, there are clearly other genetic factors contribut-
ing in responses to MD. The generally low immune responsiveness of MHC-B-resistant birds lead Calnek and colleagues to suggest that progression of MD tumors is, contrary to logic, associated with greater immune activation or reactivity (21, 28). This proposal is especially interesting in light of the apparent role of BG1 in inhibitory signaling. Many studies provide data showing greater immune responsiveness in MHC-B genetically susceptible birds. For example, lymphocytes from MD-susceptible Line P (MHC-B19) are more responsive to mitogens (ConA and PHA) than birds from MHC-B-resistant Line N birds (MHC-B21) (29, 30). When infected in vitro lymphocytes from MHC-B-susceptible lines produce significantly more infected T cells compared to lymphocytes from MHC-B-resistant lines (31). Elevated tumor formation in the presence of allogeneic immune responses suggests, in another way, that immune activation promotes tumor formation (32). The striking difference in transcription of the proinflammatory IL-6 and IL-18 cytokine genes between MHC-B genetically resistant and susceptible lines suggests these cytokines drive the enhanced immune responses contributing to increased tumors (23). Although it remains to be tested, allelic differences at BG1 might contribute to these differences in activation noted between MHC-B-resistant (B21/
with the differences being either more modest or greater than those of others. Hence, expression in vivo could vary between tissues, BG1*R4 binding of sequence-specific small RNAs or proteins. Such regulators of retroviral expression that might occur through affects translation. The presence of a 3\'-UTR of the ITIM-coding sequence (19). While it has not yet been deter-
mined whether the latter structural variation contributes to a few synonymous and non-synonymous nucleotide substitu-
tions result in different subgroups of BG1 alleles. In addition to a few synonymous and non-synonymous nucleotide substitu-
tions, prominent variations in coding sequences appear as result of duplications of a set of four exons and adjacent introns. These variations result in different subgroups of BG1 alleles that possess one, two or four copies of this exon/intron “quartet” encoding a portion of the BG1 cytoplasmic tail. Another group of BG1 alleles lacks the penultimate BG1 exon that contains the ITIM-coding sequence (19). While it has not yet been determined whether the latter structural variation contributes to disease incidence, it is notable that two haplotypes (B5 and B15) associated with MD susceptibility and with RSV tumor progres-
sion lack this ITIM encoding exon (34). Additional study of BG1 structural variants is needed to determine whether they contrib-
ute to the disease associations observed among the different MH-C alleles. The coding region in which they are found and whether they reflect the results of pathogen selection.

Even though the BG1*R2 and BG1*R4 alleles have different 3\'-UTRs, we found little evidence that these alleles were differentially transcribed. In contrast, the dual luciferase assays indicate that the 3\'-UTR differences affect posttranscriptional regulation (Fig. 2B). Investigations are underway to identify the mechanism by which the 225-bp insert in the 3\'-UTR of BG1*R4 affects translation. The presence of a 3\'-UTR derived from a retroviral sequence could put BG1*R4 under the control of regulators of retroviral expression that might occur through binding of sequence-specific small RNAs or proteins. Such regulators, particularly if they are microRNAs, could result in BG1*R4 expression being more greatly affected in some tissues than others. Hence, expression in vivo could vary between tissues, with the differences being either more modest or greater than those observed in the LMH cell luciferase assays illustrated in Fig. 2B. Studies are ongoing to define the mechanism influencing translation of the BG1*R4 allele, including investigation of several miRNA targets sequences harbored within the 225-bp insert.

Other polymorphic loci within MH-C, in addition to BG1, may also contribute to the differences in the incidence of GaHV-2 lymphoma observed between MH-C-B haplotypes. Dif-
ferent MH-C class I and class II alleles, some better expressed than others, likely contribute to the effectiveness of adaptive immune responses to GaHV-2. An important series of experi-
ments with MH-C-B congeneric lines has shown that MH-C-B haplotypes differentially influence the effectiveness of vaccinal (adaptive) immunity (35–37). The MHC-B haplotypes providing the greatest protective immunity in GaHV-2 challenge trials following vaccination differ between vaccine serotypes, suggest-
ing that allelic differences in antigen presentation have a critical role in the development of long-lasting immunity. Importantly, from the perspective of understanding the contribution of BG1 haplotypes providing the best protection following vaccination in this series of experiments were not always the same as those providing protection in the absence of immunization. Perhaps allelic differences at BG1 affect activation early in immune re-
sponses, while class I and class II allelic differences contribute primarily to the effectiveness of memory-based adaptive immunity.

Materials and Methods

Origin of BR2 and BR4 Haplotypes and the Production of Congenic Lines. The BR2 and BR4 recombinant haplotypes were derived from a lineage of birds at Northern Illinois University originating from a mating between a male of B23/B24 genotype (derived from a strain of New Hampshire chickens) and a female of BR4 genotype (as revealed by serological typing reagents) (Fig. 1A). The BR2 and BR4 recombi-
ant haplotypes were recovered in subsequent matings between BR1/B2 males and line 7 (BR2/B2 females). Congenic line birds carrying BR2 and BR4 were produced at the University of California, Davis, by introduction of New Hampshire males into lines BR2 and BR4 haplotypes into the genetic background of the highly inbred line UCDO003 and selecting progeny bearing BR2 and BR4 by serological typing over ten backcross generations. To ensure that the lines would not be lost, several individuals were selected at each generation for the next backcross. Hence, alleles at some loci across the genome continue to segregate at random in lines 003.R2 and 003.R4. The congenic lines were established by matings among the recombinant progeny 

Viral Challenge Trials of Birds in BR2 and BR4 Congenic Lines. BR2 and BR4 con-
genic line chicks were pedigreed at University of California, Davis, double wing-banded, and challenged intra-abdominally with 500 plaque-
forming units (pfu) of the very virulent RB1B strain of GaHV-2 (a serotype 1 virus also called MDV1) (40) at two days-of-age. MD virus is classified into three serotypes. Only Serotype I is oncogenic. Serotype I strains are divided into four pathotypes based on their capacity to cause MD in vaccinated birds. The “very virulent” category is the next to the most virulent category in this scale (41). Intra-abdominal inoculation broadly mimics natural infection by inhalation (42), and is the preferred inoculation route to achieve greater uniformity in GaHV-2-viral challenge trials. The RB1B inocula were propagated through not more than two passages on chick kidney culture cells. Five replicate trials were conducted at UC Davis under the supervision of a board-certified avian pathologist. In all trials, specific pathogen free (SPF) chicks (Charles River Laboratories) of a similar age were housed with the inoculated birds to serve as controls for GaHV-2 infectivity. The chicks were checked at least once per day for clinical signs of disease (paralysis, depression or anorexia) and euthan-
aized if unable to stand and feed. Carcasses of any animals that died in advance of trial termination were collected and frozen for necropsy at the end of the trial at 12 weeks when all remaining birds were euthanized. At necropsy, all 270 birds were examined for signs of tumors. Skin, oral cavity, eyes, thymus, sciatic nerve, vagus nerve, brachial plexus, and all visceral organs were examined for gross evidence of tumors. Tissue were collected from nearly all birds found negative upon gross examination for microscopic ex-
amination; however, 37 carcasses (23 from 003.R2 and 14 from 003.R4) were unavailable for histopathological examination. Histological sections were examined for evidence of pleomorphic nodules containing lymphocytes with increased nuclear to cytoplasmic ratios and for the appearance of infiltration or invasion of adjacent tissues. All animal experiments were carried out under institutionally approved protocols. All BR2 and BR4 birds in the challenge trial (and their parents) were MHC-B haplotyped by PCR (See SI Materials and Methods). Disease incidence (number of birds positive versus number of birds negative) for 003.R2, 003.R4 and SPF controls was compared using the $\chi^2$ test.

Mapping BR2 and BR4 Crossover Breakpoints and Sequence Differences. A combination of methods was used narrow the region in BR2 and BR4 con-
taining the crossover breakpoints (Table S3). Birds were typed for the BG gene family using Southern hybridizations, as previously described (43). BF and BL types were determined using single strand conformation polymorphism (44). Microsatellite LEI0258 types were revealed by PCR, as previously published (45). Sequencing of BR1, BR2, BR4, and BR4 DNA was carried out to identify the crossover breakpoints. Once the crossover breakpoints were localized, the MHC-B regions in BR2 and BR4 were fully sequenced across a 61-kb region (See SI Materials and Methods).

Assays. Standard methods were used for 3’-RACE, reverse-transcriptase real-time quantitative PCR, and firefly/Renilla dual luciferase reporter assays. These and assays for the expression of FLAG-BG1, tyrosine phosphorylation and phosphatase association are described in detail in SI Materials and Methods.

ACKNOWLEDGMENTS. We thank Lei Zhang for help with flow cytometry, Garrett Larson for helpful discussions on SNP typing, Roshni Patel for assistance with the genome-wide SNP assay, Kathy Resinger for excellent support with figures, and Amanda Baillis and Kewlly Walker for critical reading of this manuscript. This work was supported in part by National Cancer Institute Grant R21 CA105426 and U.S. Department of Agriculture National Research Initiative Competitive Grants Program Grants 2004-35205-14203 and 2006-35205-16678.

13. Salomonsen J, et al. (2005) Two CD1 genes map to the chicken MHC, indicating that CD1 genes are ancient and likely to have been present in the primordial MHC. Proc Natl Acad Sci USA 102:8668–8673.