Research Note

Allele-specific expression analysis reveals *CD79B* has a *cis*-acting regulatory element that responds to Marek's disease virus infection in chickens

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ABSTRACT Marek's disease (MD) is a T cell lymphoma disease of domestic chickens induced by the Marek's disease virus (MDV), a highly infectious and naturally oncogenic alphaherpesvirus. Enhancing genetic resistance to MD in poultry is an attractive method to augment MD vaccines, which protect against MD but do not prevent MDV replication and horizontal spread. Previous work integrating QTL scans, transcript profiling, and MDV–chicken protein–protein interaction screens revealed 3 MD resistance genes; however, a major challenge continues to be the identification of the other contributing genes. To aid in this search, we screened for allele-specific expression (ASE) in response to MDV infection, a simple and novel method for identifying polymorphic *cis*-acting regulatory elements, which may contain strong candidate genes with specific alleles that confer MD genetic resistance. In this initial study, we focused on immunoglobulin β (*CD79B*) because it plays a critical role in the immune response and, more important, is transcriptionally coupled with growth hormone (*GH1*), one of the previously identified MD resistance genes. Using a coding SNP in *CD79B* and pyrosequencing to track the relative expression of each allele, we monitored ASE in uninfected and MDV-infected F_1 progeny from reciprocal intermatings of highly inbred chicken lines 6_3 (MD resistant) and 7_2 (MD susceptible). Upon screening 3 tissues (bursa, thymus, and spleen) at 5 time points (1, 4, 7, 11, and 15 d postinfection), we observed that MDV infection alters the *CD79B* allelic ratios in bursa and thymus tissues at 4 and 15 d postinfection in both mating directions. Our results suggest that *CD79B* has a *cis*-acting regulatory element that responds to MDV infection and probably cooperates with *GH1* in conferring genetic resistance to MD. This result helps validates the use of ASE screens to identify specific candidate genes for complex traits such as genetic resistance to MD.

Key words: Marek's disease, genetic resistance, allele-specific expression, *CD79B*, growth hormone

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INTRODUCTION

Marek's disease (**MD**) is a T cell lymphoma disease of domestic chickens induced by the Marek's disease virus (**MDV**), a naturally oncogenic, highly cell-associated alphaherpesvirus that targets lymphoid tissues such as the bursa of Fabricius, thymus, and spleen, where it infects B and T cells (reviewed by Schat and Nair, 2008). Because MDV persists and is ubiquitous in poultry houses, all commercial chickens are exposed at an early age. Marek's disease is characterized by a mononuclear infiltration of the peripheral nerves, gonads, iris, various viscera, muscles, and the skin. Susceptible chickens develop enlarged nerves and lymphomas in visceral tissues, resulting in paralysis, blindness, and eventually death. The economic impact of MD on the worldwide poultry industry is substantial because of losses caused by mortality, reduced egg production, meat condemnations, and immunosuppression (Purchase, 1985). Since the 1970s, MD has been controlled through the use of vaccination and improved animal husbandry. Although vaccination greatly reduces MD incidence and symptoms, it does not prevent MDV infection, replication, and horizontal spread. Furthermore, MD still remains a threat because of unpredictable and sporadic outbreaks of highly virulent strains of MDV combined with the incomplete immunity that is elicited by vaccination (Purchase and Okazaki, 1971). For that reason, research on enhancing MD control has primarily focused on 2 areas: the development of more effective vaccines and increasing genetic resistance to MD.

Genetic resistance to MD is an attractive solution to augment MD vaccines because it is reliable, long

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lasting, and environmentally sound. Genetic resistance to MD is complex and influenced by multiple genes, which is one reason why it is difficult to identify causative genes. Therefore, use of MAS would require genetic tests for specific alleles in multiple MD resistance genes to improve the accuracy of selection in commercial birds. To this end, several loci have been identified that contribute to resistant phenotypes. It is well known that certain major histocompatibility complex haplotypes are associated with MD resistance or susceptibility (Bacon and Witter, 1994). Using an integrated approach of genome-wide QTL scans (Vallejo et. al., 1998; Yonash et al., 1999), transcript profiling (Liu et al., 2001a), and virus–host protein–protein interaction screens (Niikura et al., 2004), we identified 14 QTL (Vallejo et al., 1998; Yonash et al., 1999; Cheng et al., 2007) and 3 genes that confer genetic resistance to MD: growth hormone (***GH1***; Liu et al., 2001b), stem cell antigen 2 (*SCA2*, also known as *LY6E*; Liu et al., 2003), and major histocompatibility complex class II β chain (*BLB*, also known as *CD74*; Niikura et al., 2007).

Despite this success, these 3 genes explain only a small fraction of the total genetic variation for MD resistance indicating the involvement of many other genes. Advances in molecular genetics and biotechnology aid in the search for these remaining genes. We (Liu et al., 2001a) and others have employed comparative transcriptional analysis between resistant and susceptible lines, which takes advantage of the growing role found for transcriptional regulation in explaining complex traits, to identify positional candidate genes. A powerful variation of this approach involves allele-specific expression (**ASE**) profiling (reviewed by Pastinen, 2010). In ASE, the absolute or relative expression of each allele is measured for genes of interest. When unequal expression of the 2 alleles (i.e., allelic imbalance) is observed, then a polymorphic *cis*-acting regulatory element must be present at that gene because allelic variation is by definition reflective of *cis*-acting influence (Stamatoyannopoulos, 2004). The primary strength of the ASE approach is that extraneous (*trans*-acting) effects are eliminated because the 2 alleles being compared are present in the same diploid cell. Thus, allelic imbalance immediately identifies a gene with an allele as being under the influence of a polymorphic *cis*-acting regulatory element and, therefore, within the transcriptional regulatory region in which the gene in question resides. This does not mean that *trans*-acting factors do not influence or modulate the expression of a specific allele, but does mean that when unequal expression of alleles is observed, it is sufficient to indicate a *cis*-acting or genetic element. More important, because genetic factors that influence transcriptional regulation in *cis* are generally in close proximity to the gene itself, identification of a *cis*-acting regulatory element essentially identifies a specific gene or locus that contains the polymorphism leading to the allelic expression imbalance.

In this initial study to implement ASE to identify allelic imbalance in response to MDV infection, we screened the key candidate *CD79B*, which encodes for Ig-associated β, is an essential component of the B cell antigen receptor and, together with IgM heavy chain and Igα, is absolutely required for B cell development and function (Hermanson et al., 1988; Benschop and Cambier, 1999). Immunoglobulin α and *CD79B* are disulfide-linked membrane proteins of the Ig superfamily, each of which has a single immunoreceptor tyrosine-based activation motif within its cytosolic tail (Hombach et al., 1990). One of the earliest genes activated in B cell precursors, *CD79B* expression continues through terminally differentiated antibody-secreting plasma cells (Hermanson et al., 1988). Besides its functional interest, *CD79B* expression is transcriptionally coupled with that of *GH1* through a common locus control region (Ho et al., 2006).

MATERIALS AND METHODS

Birds and Viruses

Two inbred lines of chickens that differ greatly in MD incidence (line 6_3 and 7_2 are resistant and susceptible, respectively) were reciprocally crossed to produce F_1 progeny. Half of the F_1 birds in each mating direction were kept as unchallenged controls whereas the other half were challenged with 2,000 pfu of JM strain MDV at 2 wk of age. Six birds from each treatment group were killed by CO_2 inhalation at 1, 4, 7, 11, and 15 d postinfection (**dpi**) and the bursa, thymus, and spleen were recovered immediately into RNAlater (Ambion, Austin, TX). The sampling and handling of the chickens were approved by the Animal Care and Use Committee at the Avian Disease and Oncology Laboratory (East Lansing, MI).

RNA Isolation

Total RNA was extracted from each tissue using the Absolutely RNA Miniprep kit (Stratagene, Santa Clara, CA), and the quantity and quality was evaluated using the Agilent Bioanalyzer 2100 lab-on-a-chip instrument (Agilent Technologies, Santa Clara, CA). Using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA), RNA were converted into cDNA.

Pyrosequencing

Forward, reverse, and sequencing primers were designed by Pyrosequencing Assay Design Software (version 1.0; Biotage, Uppsala, Sweden). Forward (TAGATCTGGGGCCACTGTG) and reverse (CGGTCTTTCCTAAAGTCTCTGG) primers were used to amplify a 144-bp region containing a coding (**c**) SNP (T/C; position 1,329 in GenBank accession no. NM_001006328) of the *CD79B* gene. The resulting amplicon was annealed with the sequencing primer (GTTGGCCAAGCTGGA) and the relative expression

of each allele was determined by pyrosequencing. Data were analyzed using Minitab 15 (Minitab Inc., State College, PA), and determination of significance levels was restricted to within a particular tissue and time point. To determine the accuracy of the pyrosequencing assay, 3 sets of artificial samples were generated by mixing defined amounts of line 6 (T allele) and line 7 (C allele) DNA where the percentage of line 6 DNA varied from 0 to 100% in 10% increments.

RESULTS

Prior to analyzing actual samples, we first tested the accuracy and precision of our pyrosequencing-based ASE assay for a C/T cSNP in *CD79B* by mixing DNA fixed for alternative *CD79B* alleles in known proportions from 0 to 100% T allele in 10% increments. As shown in Figure 1, whereas the percentage of T was slightly overestimated, which is not unusual, the assay itself was linear and very reproducible.

The results of monitoring ASE of the *CD79B* cSNP are shown in Figure 2. In each reciprocal cross, RNA from the bursa, thymus, and spleen in 6 birds was screened at each of the 5 time points. As expected, the allelic ratios were dependent on the infection status, time after infection, and tissue. At 1 dpi, the earliest time point measured after MDV infection, infection status, or mating direction did not influence *CD79B* ASE. However at 4 dpi, the peak of the early cytolytic phase of MDV replication, the uninfected 6×7 F_1 progeny exhibited significant differences in allelic ratios for the bursa and thymus samples compared with the other samples. Interestingly, it appears that from 1 to 4 dpi, a reduction occurred in relative expression of the line 6 (T) allele in the bursa and thymus [i.e., an increase in relative expression of the line 7 (C) allele] in both infected populations. Surprisingly, this reduction was also observed in uninfected 7×6 progeny but not in the uninfected 6×7 F_1 progeny. The only other significant difference in allelic ratios was observed at 15 dpi, the time of the second cytolytic phase of MDV replication, in both crosses for the bursa and thymus samples. At this time point, there appeared to be an overall general decrease in T allele expression, independent of infection status; additionally, there appeared to be a cross-specific effect. In the 7×6 F_1 progeny, MDV infection resulted in decreased levels of the T-containing allele. In contrast, the reciprocal mating direction had the opposite response to infection, with a significant increase in the percentage of T allele found in the thymus.

DISCUSSION

Identifying the basis for genetic resistance to MD and other complex traits remains one of the greatest challenges facing biology. Despite the existence of genome sequences and powerful genomic tools, most approaches are unable to identify individual genes containing alleles of small effect because they rely on detecting genetic markers that are linked to the causative polymorphisms. Consequently, with most causative genes having allele substitution effects that, individually, may be small compared with the potential influence of environmental factors and stochastic variation, it is extremely difficult even with large-scale studies to identify candidate genes of high confidence. Furthermore, even when linked genetic markers are identified, the extent of linkage disequilibrium in the population being

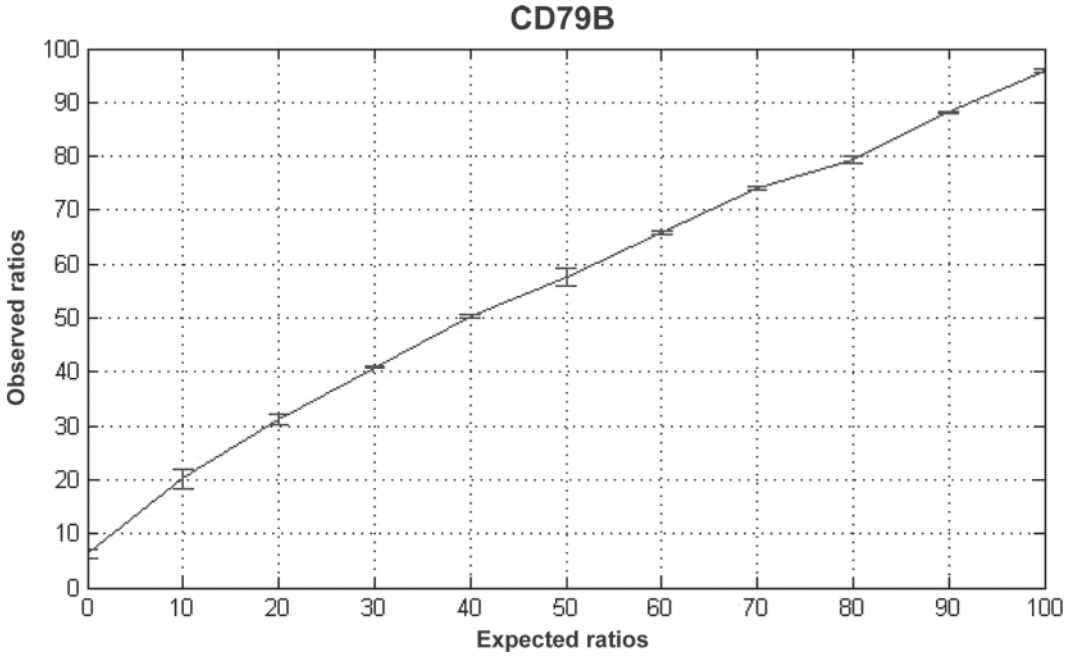

Figure 1. Observed ratios of the T allele for *CD79B* when measuring defined DNA mixtures that vary in the percentage of T in 10% increments. The error bars represent the SD from 3 replicate measurements.

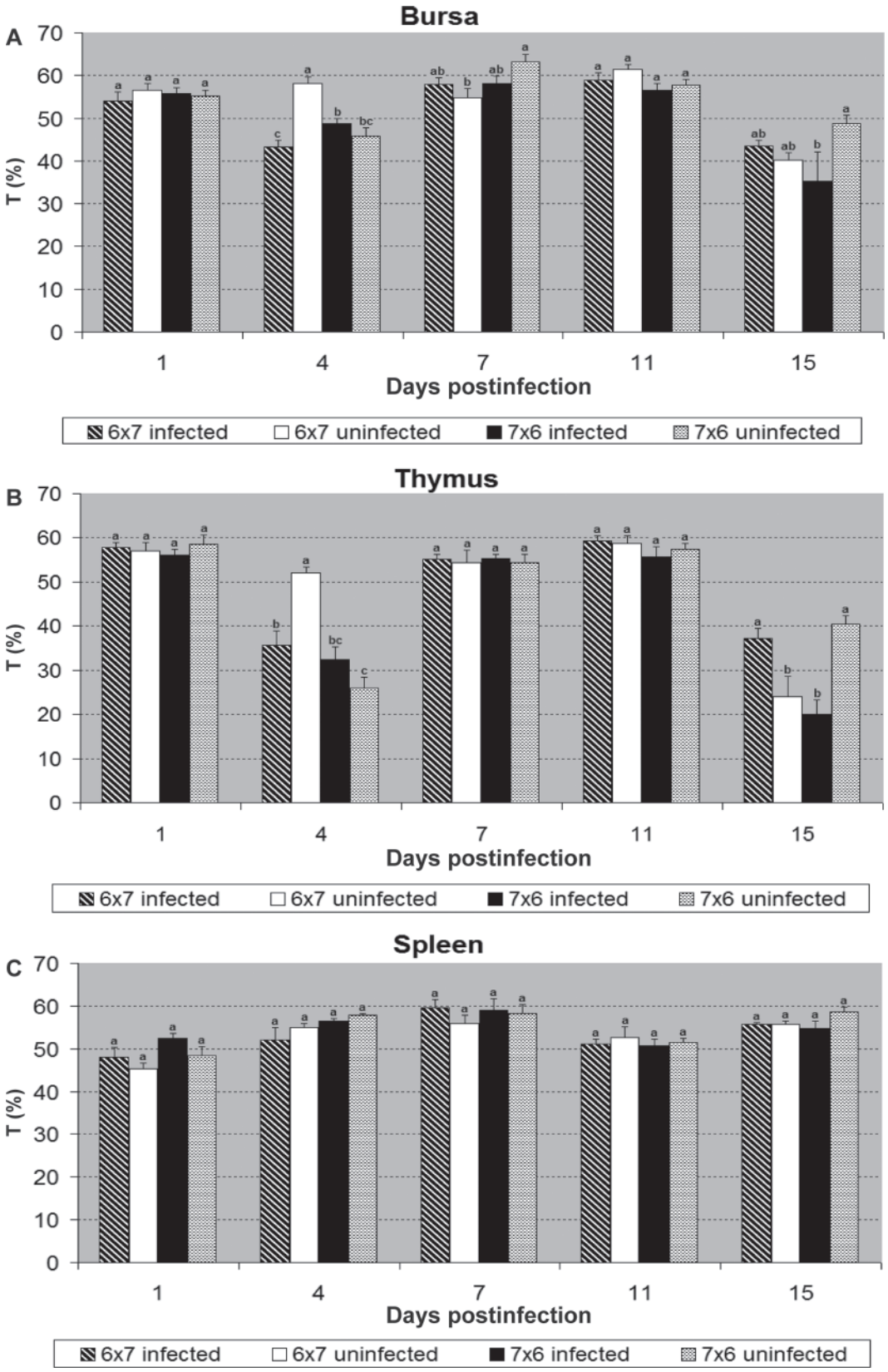

Figure 2. Expression level of the *CD79B* T allele in the bursa, thymus, and spleen at various time points in uninfected and Marek's disease virus-infected line 6×7 F_1 and line 7×6 F_1 progeny. Letters (a–c) above columns indicate values that are significantly different within each tissue and time point grouping at $P < 0.05$. The error bars reflect the SE of each mean.

tested is such that the locus in question encompasses multiple genes.

In this preliminary experiment, we tested the novel method of ASE, which has been primarily used in the biomedical field (e.g., Serre et al., 2008; Lee et al., 2009; Cheung et al., 2010; Pickrell et al., 2010), to examine a candidate gene for genetic resistance to MD, a problem of agricultural importance. The main advantage for this approach is that because the relative expression of each allele is being monitored in a common, heterozygous environment, only *cis*-acting alleles at the candidate gene locus can have an effect. Thus, in our situation, if a difference exists in the allelic ratio between uninfected and MDV-infected birds, this must indicate a specific gene for which there are 2 polymorphic alleles that respond differently to MDV infection.

We selected *CD79B* as a candidate gene to evaluate because of its biological importance in lymphocyte activation and because it is transcriptionally coupled with *GH1*, a previously identified MD resistance gene. As shown in Figure 2, *CD79B* shows allelic imbalance in response to MDV infection in RNA from the bursa and thymus at 4 and 15 dpi. No difference was detected in the spleen at the same time points, which likely reflects the fact that the bursa and thymus should have a higher percentage of B and T cells, respectively. Days 4 and 15 during the MDV infection process are critical times for the early and second round of cytolytic infection. Thus, our results reinforce the conclusion that these are the critical time periods for evaluating genetic responses to MDV infection in these tissues. Surprisingly, ASE was also observed in uninfected 7×6 F_1 birds on d 4 and 6×7 F_1 birds on d 15 (especially in the thymus). Although we don't have an obvious explanation for this observation, it may reflect differences in response of the 2 *CD79B* alleles during normal B and T cell development as well as in the infected state. We also observed differences in ASE response between the 2 mating directions. Using the same 2 inbred lines, Stone (1975) found mating direction significantly influenced MD incidence. Specifically, when the dam was line 6 (MD resistant), MD incidence ranged from 7 to 13% in inoculated birds for 3 trials. On the other hand, progeny from the line 7 (MD susceptible) dam exhibited 44 to 70% incidence. These results are consistent with our observations and suggest maternal or epigenetic influences, which are being identified more frequently in other organisms with the increasing use of epigenetic analyses (e.g., Gregg et al., 2010).

In conclusion, by intermating inbred chicken lines to make F_1 progeny with the maximum number of heterozygous genes and using the novel method of ASE, we find MDV affects the allelic ratio of *CD79B* in the bursa and thymus at 4 and 15 dpi. Thus, the *CD79B* region appears to contain one or more polymorphic *cis*-acting regulatory element(s) that responds to MDV infection and may contribute to genetic resistance to MD in conjunction with *GH1*. Because *CD79B* and *GH1* expression are coupled (Ho et al., 2006) and because *GH1* expression is already known to differ between the 6 and 7 lines (Liu et al., 2001b), it is possible that ASE is attributable to changes in their common locus control region. If so, our results confirm that the effect of that polymorphism acts on *CD79B* (and probably *GH1*) in *cis*. Finally, our results suggest that ASE screens are simple and powerful approaches to identifying genetic elements and specific alleles for genetic resistance to MD and other complex traits, especially those that involve 2-state situations in disease challenge experiments. Use of the cSNP as a segregating marker can also be applied to determine whether the expression differences are linked to measurable phenotypic changes (e.g., disease incidence) and, if so, it might be used in genomic selection programs.

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