Chicken Major Histocompatibility Complex Class I Definition Using Antisera Induced by Cloned Class I Sequences[1]

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ABSTRACT Alloantisera directed against chicken class I MHC (BFIV) antigens were produced by using transfected cell lines expressing cloned *BFIV* sequences. The cloned *BFIV* sequences were from haplotypes **12*, **13*, and **21*. Two laboratory-derived class I mutant sequences (*BFIV13m126* and *BFIV21m78*) were developed to analyze cross-reactive epitopes and to induce specific alloantisera. Antisera were tested in hemagglutination and flow cytometry assays. The antisera produced were highly specific and had minimal cross-reactivity. The antisera induced by the BFIV21m78 mutant confirmed the significance of amino acids 78 and 81 in cross-reactivity between haplotypes *B*21* and *B*5*. The highly specific antisera were tested by hemagglutination on red blood cells of 31 different MHC haplotypes. The consistency of hemagglutination patterns and minimal cross-reactivity demonstrated that these BFIV antisera are extremely valuable in defining *MHC* haplotype in various chicken lines. Because of the extreme low level of recombination between the chicken class I and class II loci, identification of *BFIV* allele can be used to define *MHC* haplotype within a line. Complete identity between the transfected cell line and the chicken used to produce the antiserum is required to ensure the monospecificity.

(*Key words*: antisera, blood-typing, class I, *MHC*, transfected cell)

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INTRODUCTION

The chicken MHC (*B*-complex) is comprised of three classes of highly polymorphic loci, i.e., class I (*B-F*), class II (*B-L*), and class IV (*B-G*). The class I and class II molecules are structurally and functionally equivalent to their mammalian counterparts (Guillemot and Auffray, 1989). However, the highly polymorphic B-G molecules have no mammalian equivalent. They were initially found only on erythrocytes (red blood cells, RBC) and their precursors (Longenecker and Mosmann, 1980; Miller et al., 1984; Salomonsen et al., 1987). B-G-like molecules have since been found on various cells of the immune system including thrombocytes (avian platelets), peripheral B and T lymphocytes, bursal cells, and thymocytes (Salomonsen et al., 1991). At least 18 B-G loci have been identified (Kaufman et al., 1991). The function of these molecules is unknown.

A second cluster of polymorphic class I and class II loci (referred to as *Rfp-Y*) has been identified that segregates independently of the traditional *B*-complex (Briles et al., 1993; Miller et al., 1994). Some of these genes have been shown to be expressed and involved with skin-graft rejection (Pharr et al., 1996), but their role in immune response is uncertain. A significant influence of *Rfp-Y* on Marek's disease resistance has been reported for some genetic stocks (Wakenell et al., 1996) but not others (Bacon et al., 1996; Lakshmanan and Lamont, 1998).

The chicken *MHC* haplotype is defined by using alloantisera in a hemagglutination (HA) assay. Twenty-seven serologically defined haplotypes were identified in an international workshop (Briles et al., 1982). However, haplotype determination can be very complex because alloantisera show extensive cross-reactivity (Briles and Briles, 1982). Even alloantisera produced within *MHC*-congenic birds can result in complex cross-reactions between haplotypes (Fulton et al., 1996). The presence of the immunogenic and polymorphic B-G molecules, the occurrence of either identical alleles or similar antigenic epitopes between *B* haplotypes (Simonsen et al., 1982; Fulton et al., 1995, 1996; Fulton et al., unpublished), and the unknown influence of the independently segregating *Rfp-Y* loci may have all contributed to the complexity of *MHC* serological patterns.

The use of *B-G*/*B-F* recombinants can allow the generation of *B-F* specific alloantisera (Simonsen et al., 1980). However, recombination within the chicken *MHC* is very

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Abbreviation Key: ALV = avian leukosis virus; HA = hemagglutination; ISU = Iowa State University; MLF = mean log fluorescent; PBL = peripheral blood leukocytes; RBC = red blood cells; SBI = specific binding index; UCD = University of California at Davis; WBC = white blood cells.

infrequent, resulting in only a limited set of recombinants (Skjodt et al., 1985; Hala et al., 1988). A limited number of monoclonal antibodies have been produced that either detect monomorphic determinants or react with only certain haplotypes (Longenecker and Mosmann, 1981; Crone et al., 1985; Pink et al., 1985).

Class I cDNA sequences from several haplotypes have been cloned and sequenced (Kroemer et al., 1990; Kaufman et al., 1992; Hunt et al., 1994; Pharr et al., 1994; Fulton et al., 1995a; Hunt and Fulton, 1998). The sequence from the *BFIV* locus allele *21* has been shown to be expressed and functional (Thacker et al., 1995). We describe here the production of B-F-specific alloantisera by using cloned class I (BFIV) cDNA sequences inserted into an avian retroviral expression vector. An avian B-cell line expressing the transfected class I cDNA gene products was used as the immunogen and resulted in the production of highly specific and very useful BFIV-specific alloantisera. These antisera allow serological definition of BFIV alleles in various lines and types of chickens without the complexity of extensive cross-reactions to B-G antigenic differences. Furthermore, these highly specific antisera detected epitope similarities in BFIV molecules between various defined *MHC* haplotypes. The ability of this method to produce B-F-specific antisera in an otherwise antigenically MHC-identical environment (i.e., B-G and other MHC antigens do not differ) is in apposition with the hypothesis that B-G antigenic differences are required for B-F antibody elicitation (Hala et al., 1981; Salomonsen et al., 1991).

MATERIALS AND METHODS

Chicken Lines and Cell Lines

The development of the 15.*B* congenic lines has been previously described (Shen et al., 1984; Bacon et al., 2000). Seven different *MHC* haplotypes were obtained from various Leghorn sources and introduced into the $15I_5$ inbred White Leghorn line (*MHC* haplotype B**15*) by backcrossing for 10 to 11 generations. Brother × sister matings resulted in the 15.*B* congenic strains used here. The haplotypes used are as follows: *B*2*, *B*5*, *B*12*, *B*13*, *B*15*, *B*19*, and *B*21*. The University of California at Davis (UCD) series of congenic lines used were developed following five backcross generations to inbred White Leghorn line UCD.003 (Abplanalp, 1992). Samples from UCD congenic lines containing the standard haplotypes *B*3*, *B*17*, *B*18*, *B*19*, or *B*21* originally obtained from White Leghorn chicken strains and *B*24* derived from a New Hampshire chicken strain were tested (courtesy of H. Abplanalp). Cells from two recombinants, *B*R4* (*B-G*15:B-F*21*) and *B*R5* (*B-G*21:B-F*15*), also with the UCD.003 congenic background (Mary Delany, 2000, personal communication), were also available for testing (Briles et al., 1982). Several additional UCD congenic lines were tested that contain haplotypes originally derived from unusual sources. The *B*Q* haplotype was obtained from the red Jungle Fowl and the *B*O* and *B*C* haplotypes were obtained from Ceylonese Jungle Fowl (Abplanalp, 1992). Blood samples were also obtained from highly inbred chicken lines at Iowa State University (courtesy of S. J. Lamont). Thirteen *B* homozygous haplotypes in different inbred lines were tested (Chen and Lamont, 1992). The lines tested included line G containing the defined *MHC* haplotypes *B*6* and *B*13*, line GH containing *B*1*, *B*13*, and *B*15.1*, line HN containing the standard *B*12* and *B*15*, line 19 containing *B*15.1* and *B*13*, and line 8 containing *B*15.1*. In addition, two non-White Leghorn inbred lines were also tested. The Spanish line contains haplotype *B*21.1* and the Fayoumi line contains *B*15.2* and *B*5.1*. Those haplotype designations with an additional decimal point plus number, were assigned based on similarities in serological HA reaction patterns with standard haplotypes; thus, the haplotype *B*21.1* found in the Spanish line is similar but not identical, by HA serology, to the standard *B*21*.

A lymphoblastoid B-cell line RP9 (Okazaki et al., 1980) was used for expression of the various BFIV constructs and immunizations for antisera production. The RP9 cell line was originally developed from a bursal tumor from a $15I_5 \times 7_2$ F_1 chicken infected with subgroup B avian leukosis virus (ALV). Cell culture conditions and transfection procedures were described by Fulton et al. (1995). The cells were harvested, washed in PBS, and used in the immunization scheme described. Immunization was performed in $15I_5 \times 7_1$ F_1 (series D) and $15I_5 \times 7_2$ F_1 (series E) chickens. The highly inbred lines $15I_5$, 7_1, and 7_2 have been previously described (Bacon et al., 2000). Line $15I_5$ is homozygous for *MHC* haplotype *B*15* and lines 7_1 and 7_2 (which are both sublines of line 7) are homozygous for *B*2*.

Development of the BFIV Expression Constructs

Cloning of the *BFIV* sequences has been described in detail (Hunt et al., 1994). In brief, cDNA was produced from bursal mRNA obtained from $15I_5$ *B* congenic birds homozygous for either the *B*12*, *B*13*, or *B*21* haplotype. Polymerase chain reaction amplification of the cDNA by using primers specific for *BFIV* resulted in amplification of *BFIV* cDNA (Hunt et al., 1994; Fulton et al., 1995). The 1,274-bp product was initially inserted into the pRC-CMV eukaryotic expression vector.[4]

The avian retroviral vector used for expression in chicken cells was RCASBP(A) (Hughes et al., 1987). Manipulation of the *BFIV* sequences for cloning into RCASBP(A) and optimal expression was described by Fulton et al. (1995; and unpublished). The endogenous poly-A signal of the *BFIV* sequence was removed because this can interfere with subsequent retroviral expression. In addition, a *Cla*I site present in exon 4 (alpha 3 domain) was altered to assist in the transfer of the *BFIV* sequence into the RCASBP(A) vector. This change did not affect the subsequent amino acid sequence. At the junction of exons 1 and 2, a sequence encoding the epitope tag FLAG was inserted (described by Fulton et al., 1995). The following two mutant

[4]Invitrogen, San Diego, CA.

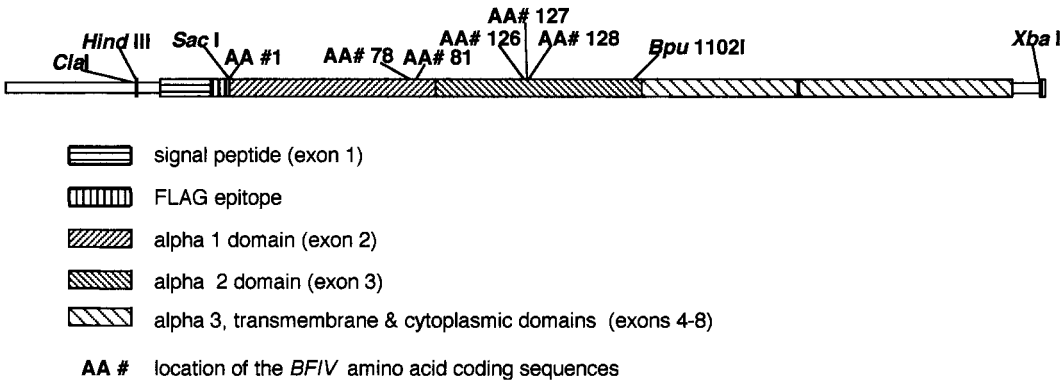

FIGURE 1. The map of the *BFIV* FLAG construct depicting the location of the coding region for FLAG inserted between the coding sequence for the signal peptide and the coding sequence for the first amino acid (AA# 1) of the cell surface-expressed BF glycoprotein is shown. The location of the coding sequence for the mutated amino acids (AA# 78, 81, 126, 127, and 128) is also shown. The restriction enzyme sites (*Sac*I and *Bpu*1102I) used for cloning the site-directed changes in the α-one and two domains along with the sites (*Cla*I, *Hind*III, and *Xba*I) used for insertion into RCASBP are shown. Exons 4 through 8, which encode the α-3, transmembrane, and cytoplasmic domains, are shown as one segment for simplicity.

BFIV sequences previously developed were also used in this study: *BFIV21m78* (Fulton et al., 1995) and *BFIV13m126* (Fulton et al.; unpublished). Expression of the inserted *BFIV* sequences was confirmed by flow cytometry by using the FLAG epitope tag.

Figure 1 shows the components of the *BFIV* constructs within the RCASBP(A) vector. Each construct contains exon 1 from the *BFIV*21* haplotype and the FLAG coding sequence. Exons 2 and 3 (encoding the alpha 1 and alpha 2 domains) are from either the *B*12*, *B*13*, or *B*21* haplotypes or from one of the mutant *BFIV* sequences. Exon 4 (alpha 3 domain) is from *BFIV*21* but with the altered *Cla*I site. Exons 5, 6, 7, and 8 (cytoplasmic and transmembrane domains) are unaltered from the *BFIV*21* sequence.

Five different cell lines were developed by using the *BFIV* constructs and the RP9 cell line. Three cell lines were produced that expressed either *BFIV*12*, *BFIV*13*, or *BFIV*21*. Two mutant *BFIV*-expressing cell lines were produced (Ho et al., 1989) (Figure 2). In the *BFIV13m126* mutant construct, the amino acids in positions 126, 127, and 128 were altered from the D (aspartic acid), M (methionine), and K (lysine) found in *BFIV*13* to G (glycine), T (threonine), and M (methionine) found in *BFIV*12*. The *BFIV21m78* mutant construct has been previously described (Fulton et al., 1995) and has amino acids 78 and 81 altered from the D (aspartic acid) and R (arginine) found in *BFVI*21* and *BFIV*5* to the G (glycine) and E (glutamic acid) found in *BFIV*2* and **15*.

Production of Antisera

Two series of birds consisting of 25 birds each were immunized with the cell lines described above. Series D were birds from a cross of line $15I_5$ and 7_1 and are therefore *B*2/*15* heterozygotes, whereas the series E birds were from a cross of lines $15I_5$ and 7_2 (also *B*2/*15* heterozygotes). Birds were immunized with 1×10^7 cells starting at 7 to 10 wk of age. Immunizations were done twice per week for 15 wk in series D, and in series E for 3 wk followed by eight weekly injections. Within each series, five birds were immunized with each cell line expressing the *BFIV* constructs. Blood was collected weekly starting 4 wk after immunization. Sera were obtained from the clotted blood after 4-h incubation at room temperature. RBC were removed by centrifugation and antiserum was frozen at –20 C.

HA Assay

The HA assay has been described in detail by Fulton et al. (1995b). For the titration assay, each antiserum was serially diluted twofold from 1/2 to 1/2,048 in U-shaped bottom, 96-well microtiter plates in PBS containing 0.5% chicken serum. An equal volume of a 0.5% suspension of packed RBC was mixed with the diluted antibody and incubated for 1 to 3 h at room temperature. The cells were

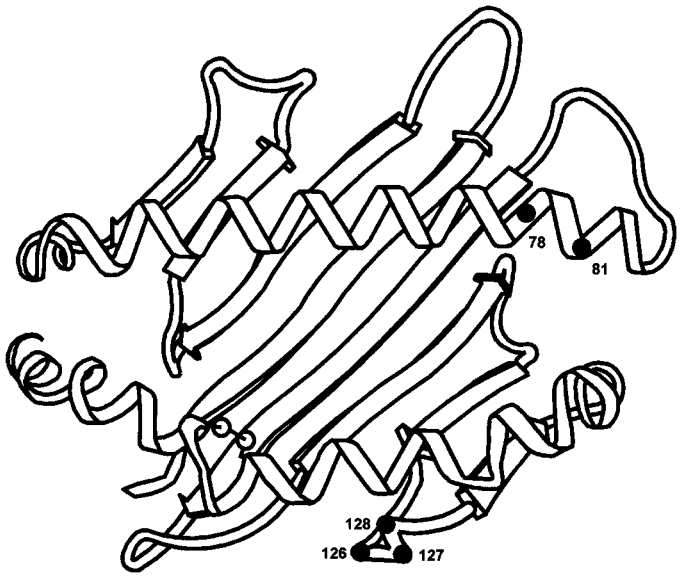

FIGURE 2. The locations of the *BFIV* amino acid mutations are shown on the ribbon diagram for BFIV (α-one and two domains) modeled by using the crystal structure of human leukocyte antigen.

resuspended, incubated overnight at 4 C and then scored. The greatest antibody dilution at which agglutination was visible as a lawn of cells on the well bottom was designated as the HA titer. Titers were expressed as the reciprocal of $\log_2$. For classification of antiserum reactivity with various haplotypes, antisera were serially diluted from 1/8 to 1/512 and scored as described above.

Flow Cytometry

Blood from mature birds of each homozygous haplotype was collected in Alsevier's solution (114 m*M* dextrose, 27 m*M* sodium citrate, 71 m*M* NaCl, pH 6.1). RBC were stored overnight at 4 C. White blood cells (WBC) were isolated over a Histopaque[5] gradient, washed three times in PBS, and stored overnight at 4 C. The flow cytometry procedure has been previously described (Fulton et al., 1996). In brief, antiserum was diluted to 1/100 and incubated with 1×10^6 cells. After washing, the cells were incubated with fluorescein-labeled anti-chicken IgG (with IgM cross-reactivity),[6] washed, and resuspended in 300 μL of PBS + 0.1% NaN_3 with 20 μL of 1/100 dilution of saturated propidium iodide. Dead cells were omitted by propidium iodide exclusion. Five thousand viable cells were analyzed per sample on a FACSort[7] flow cytometer and the mean log fluorescent (MLF) channel number was obtained for each sample. The relative level of antibody binding on each cell type (specific binding index, SBI) was determined by using the following formula:

$$\text{SBI} = \frac{\text{MLF sample}}{(\text{MLF neg1} + \text{MLF neg2})/2}$$

The two negative controls for each sample were from the mean log fluorescence of antisera with cells of lines $15I_5$ (*B*15*) and 7_1 or 7_2 (*B*2*). An alloantiserum directed against *B*21* and produced in a *B*2/*15* heterozygous bird (7283) was used as a positive control for flow cytometry assays (Fulton et al., 1996).

RESULTS

Specificity of the BFIV Alloantisera

Table 1 summarizes the HA titers obtained by using the BF antisera produced by birds immunized with RP9 cells expressing a transfected *BFIV* gene product and tested on RBC of defined *MHC* haplotype. The mean HA titer obtained by using series D sera from five birds immunized with the same BFIV-expressing cell line and RBC from each of seven *B* haplotypes existing in the 15.*B* congenic lines, as well as RBC from line 7_2 (*B*2* haplotype), are given. Sera from birds immunized with the BFIV*12-expressing cell line agglutinated *B*12* cells and also had a lower level of reactivity with RBC from the *B*13* haplotype. All other haplotypes showed no HA with the BFIV*12-induced sera. Similar results were seen with the BFIV*13-induced alloantisera; RBC of the B*13 haplotype were agglutinated and a lower reactivity was seen with RBC of the *B*12* haplotype. Antisera induced by the *B*13m126* mutant also agglutinated RBC from both *B*12* and *B*13* haplotypes but with a lower mean titer. The BFIV*21-induced antisera agglutinated RBC from the *B*21* haplotype and showed strong cross-reactivity with the B*5 haplotype RBC. The BFIV*21m78-induced antiserum was also positive for *B*21* RBC and showed negligible cross-reactivity with *B*5* RBC. Occasional low-level cross-reactivity (<1.0) was seen, reflecting reactivity of one of the five antisera at the highest concentration of a 1:2 dilution. Mean HA titer values below 1.0 were considered to be negative. The D series antisera all reacted very strongly to RBC from the line 7_2, with HA titers ranging from 5.6 to 8.2, but were negative on RBC from all other chicken lines maintained at the Avian Disease and Oncology Laboratory (data not shown). The E series antisera were also tested and showed the same patterns of reactivity with similar titers on the various RBC haplotypes tested. One exception was that RBC of the line 7_2 were negative for all E series antisera (data not shown).

Samples of the D series antisera, and RBC from line 7_2, were analyzed extensively by W. E. Briles (Northern Illinois University). The results suggested that the antigenic differences between line 7_1 (used for D series antisera) and 7_2 (used for E series antisera and as a parent for the RP9 cell line) and other lines at the Avian Disease and Oncology Laboratory were due to differences in alleles at the *J* blood locus (W. E. Briles, 1995, personal communication).

To further examine the specificity of the antisera, one serum from each immunization of the D and E series was tested by flow cytometry on RBC and WBC of the 15.*B* congenic haplotypes and from line 7_2 (Table 2). The SBI of one D series antiserum induced by each of the five *BFIV* constructs on cells of each homozygous haplotype is shown. With RBC, the patterns of specific binding and cross-reactivity are similar to those seen with HA. Even the low-level agglutination seen with the D series BFIV*21m78 antisera on *B*5* and *B*19* cells is reflected in the flow cytometry data. Each of the D series antisera also had a high SBI on RBC from line 7_2, reflecting the HA of RBC by this antisera. Absorption of the BFIV*13-induced sera with the RP9-*BFIV*12*-expressing cell line resulted in elimination of most of the antibodies specific for B*12 cells but left B*13-specific antibodies. This absorption also removed antibodies specific for the line 7_2 RBC.

The flow cytometry data with WBC also shows the same cross-reactivity patterns as observed in the HA data within the 15.*B* congenic lines. However, reactivity of the D series antisera to WBC from line 7_2 was seen with only one antiserum (BFIV*12 specific) and at a very low level compared with the RBC flow cytometry data and the HA titer. The other D series antisera had nominal SBI values on line 7_2 WBC. All of the E series antisera showed similar

[5]Sigma Chemical Company, St. Louis, MO 63178.
[6]Bethyl Laboratory, Inc., Montgomery, TX 77356.
[7]Becton-Dickinson, Immunocytochemistry Systems, Mansfield, MA 02048-1145.

TABLE 1. Mean log_2 hemagglutination titers of BFIV-specific antisera from D series on red blood cells (RBC) from seven 15.*B* congenic homozygous haplotypes and line 7_2

Antibody to transfected haplotype[1]	Haplotype of RBC							
	*B*2*	*B*5*	*B*12*	*B*13*	*B*15*	*B*19*	*B*21*	Line 7_2
BFIV-12	0	0.2	7.4	4.4	0	0	0	nd[2]
BFIV-13	0	0	3.4	4.8	0	0.2	0	4.8
BFIV-13m126	0	0.8	3.2	3.2	0	0.2	0	6.2
BFIV-21	0	2.6	0	0	0	0	4.8	5.6
BFIV21m78	0	0.2	0	0	0	0.2	2.6	5

[1]Haplotype of *BFIV* gene product expressed by transfected RP9 cells used to produce antisera.
[2]Titer is given as the mean of the reciprocal of the highest reacting log_2 dilution of five antisera; nd = no data.

reactivity patterns in the flow cytometry assay to the D series antisera except that there was no reactivity with cells from the line 7_2 (data not shown).

Specificity of BFIV Antisera for Additional B-haplotypes

The use of BFIV-specific antisera in HA of RBC from chickens of various lines and *MHC* haplotypes is summarized in Table 3. The standard Avian Disease and Oncology Laboratory line *B*-haplotypes were tested at the same time for comparisons and as internal controls. For this assay, the strength of the HA reaction is indicated af follows: ++ = very strong, + = strong, ± = weak, and − = negative, to give an indication of similar agglutination reactions between different haplotypes. Within the UCD line, 003.*B* congenic haplotypes, RBC from the *B*21*, *B*O*, *B*Q*, and *B*R4* haplotypes had agglutination reactions identical to RBC from the 15.*B*21* haplotype. RBC from three haplotypes (*B*3*, *B*19*, and *B*R5*) were negative with all of the antisera. RBC from the remaining UCD line 003.*B* congenic haplotypes were agglutinated by BFIV*13 and BFIV*13m126 antisera either strongly (*B*24*, *B*C*) or weakly (*B*17* and *B*18*). Both of the *B*13* haplotypes from the Iowa State University (ISU) lines G and GH reacted identically to the 15.*B*13* haplotype. The *B*12* from ISU line HN was serologically identical to 15.*B*12*. HN *B*15* was negative with all antisera and comparable with the 15.*B*15* haplotype. In contrast to the *B*15* haplotype from ISU, the haplotype *B*15.1* from lines 8, 19, and GH, which had been previously classified as similar to but not identical to *B*15*, reacted with BF*13 or both BFIV*13 and BFIV*21 antisera. The Fayoumi *B*15.2* also reacted with the BFIV*12 antisera. The haplotype *B*6* from line G showed weak reactivity with the BFIV*13 antisera. The GH haplotype *B*1* (a reference line for B*1; Briles et al., 1982) showed a unique pattern, reacting with only the BFIV*21 antisera. The Fayoumi *B*5.1* showed an unusual pattern of reactivity, being weakly positive with the BFIV12, BFIV13, and BFIVm126 sera and strongly positive with the BFIV21 and BFIV21m78 sera. None of the *B*-haplotypes found in the ISU lines showed HA patterns identical with 15.*B*21* haplotype, although the ISU Spanish haplotype *B*21.1* was very similar.

TABLE 2. Specific binding index (SBI) of antisera from D series immunizations on red blood cells (RBC) and white blood cells (WBC) of different 15.*B* congenic haplotypes and line 7_2

Antibody to transfected haplotype[1]	Haplotype of RBC							
	*B*2*	*B*5*	*B*12*	*B*13*	*B*15*	*B*19*	*B*21*	Line 7_2
BFIV*12	1	1	355	196	1	1	1	164
BFIV*13	1	1	214	232	1	1	1	136
BFIV*13m126	1	2	246	184	1	1	1	164
BFIV*21	1	165	1	1	1	1	53	148
BFIV*21m78	1	6	1	1	1	4	31	80
BFIV*13 abs with BFIV[2]	1	1	8	202	1	1	1	1
B*21 alloantisera	1	110	1	1	1	1	63	1
	Haplotype of WBC							
	*B*2*	*B*5*	*B*12*	*B*13*	*B*15*	*B*19*	*B*21*	Line 7_2
BFIV*12	1	1	184	196	1	2	1	15
BFIV*13	1	1	72	163	1	1	1	2
BFIV*13m126	1	1	125	152	1	2	1	3
BFIV*21	1	155	1	1	1	2	81	2
BFIV*21m78	1	3	1	1	1	3	84	2
BFIV*13 abs with BFIV[2]	1	1	3	109	1	1	2	1
B*21 alloantisera	1	56	1	1	1	1	73	8

[1]Haplotype of *BFIV* gene product expressed by transfected RP9 cells used to produce antisera.
[2]Antisera produced by immunization with BFIV*13-expressing cells then absorbed with *BFIV*12-expressing cells.

TABLE 3. Reactivity of RBC from various inbred chicken lines with the BFIV-specific reagents[1]

BFIV antiserum[2,3]	ADOL *B* congenic White Leghorn lines							UCD *B* congenic White Leghorn lines										
	B = 2*	*5*	*12*	*13*	*15*	*19*	*21*	*3*	*17*	*18*	*19*	*21*	*24*	*C*	*O*	*Q*	R4	R5
BFIV-12	–	–	++	+	–	–	–	–	–	–	–	–	–	±	–	–	–	–
BFIV-13	–	–	++	++	–	–	–	–	+	±	–	–	++	++	–	–	–	–
BFIV-13m126	–	–	++	++	–	–	–	–	±	±	–	–	++	++	–	–	–	–
BFIV-21	–	±	–	–	–	–	++	–	–	–	–	++	–	–	++	++	++	–
BFIV21m78	–	–	–	–	–	–	±	–	–	–	–	±	–	–	±	±	±	–

	ISU inbred lines												
	Line = G	G	GH	GH	GH	HN	HN	19	19	SP	8	FAY	FAY
	B = 6*	*13*	*1*	*13*	*15*	*12*	*15*	*15*	*13*	*21*	*15*	*15.2*	*5*
BFIV-12	–	+	–	+	–	++	–	–	±	–	–	±	±
BFIV-13	±	++	–	++	+	++	–	+	++	–	+	–	±
BFIV-13m126	+	++	–	++	++	++	–	+	++	–	+	±	±
BFIV-21	–	–	++	–	±	–	–	–	–	++	±	++	++
BFIV21m78	–	–	–	–	–	–	–	–	–	++	–	±	++

[1]RBC = red blood cells; ADOL = Avian Disease and Oncology Laboratory; UCD = University of California at Davis; ISU = Iowa State University.

[2]Haplotype of *BFIV* gene product expressed by transfected RP9 cells used to produce antisera.

[3]Each *BFIV* antiserum was diluted from 1:8 to 1:512 and mixed with RBC. The agglutination score is summarized as ++ = very strong, + = strong, ± = weak, and – = negative.

DISCUSSION

The BFIV antisera produced in series E immunizations appear to specifically identify *BFIV* haplotypes. Specific antisera had minimal cross-reactivity with RBC or peripheral blood leukocytes (PBL) from other *B*-haplotypes as shown by both HA and flow cytometric analysis. This high level of specificity was due to the identical match of transfected immunizing cell line (RP9, originally derived from a $15I_5 \times 7_2$ bird) and the host animal (from a cross of $15I_5 \times 7_2$). The only immunogenic difference between the immunizing (donor) cells and the antisera-producing host was the transfected and expressed MHC antigen. The importance of this identical match between the immunizing cell and the serum producer was dramatically shown in the antigenic differences between the series D and series E immunizations. Hosts for the D series antisera were a hybrid F_1 derived from a cross of $15I_5 \times 7_1$, whereas the donor cells were derived from a cross of $15I_5 \times 7_2$. There was an antigenic difference between lines 7_2 and 7_1 because line 7_2 had a J blood group different from that found in line 7_1. Antibodies directed against this antigenic difference were seen in D series antisera, which was produced by $15I_5 \times 7_1$ birds. This reactivity was removable by adsorption. When $15I_5 \times 7_2$ birds were used as the host animals for antisera production (series E), no J blood group antibodies were induced. The RP9 cell line was developed more than 20 yr ago from an F_1 chicken from a cross of $15I_5 \times 7_2$. The high specificity of the E series antisera indicates that no changes have occurred in antigens of these highly inbred lines in the last two decades. Lines 7_1 and 7_2 are derived from the same parental line 7, and the observation of antigenic differences between them stresses the importance of complete matching between the origin of the cells used for immunization and the line used to produce the antiserum. The flow cytometric results of D series antisera with RBC and PBL suggests that the J blood group antigens are expressed on RBC but not on PBL.

Historically, it has been nearly impossible to produce BF specific antisera. It had been hypothesized that BG differences are also required in order to elicit antibodies directed against BF antigens (Hala et al., 1981; Salomonsen et al., 1991). This study clearly shows that BG differences are not essential for BF antibody production. There are, however, two additional antigens in the immunizing system described here. The immunizing cells (RP9) were originally transformed by using ALV subgroup B virus, and the transfecting vector used produces infectious ALV subgroup A virus. Thus, the immunizing cells contain ALV virus of both subgroups A and B, and the induced antiserum contains antibodies directed against these viruses. In addition, the FLAG epitope is also expressed on the end of the transfected BFIV sequences. The influence of the viral immunogens and the FLAG epitope on the production of BF-specific antibodies is unknown.

The two laboratory-developed mutant forms of *BFIV*, *BFIV*21m78*, and *BFIV*13m126* were used to induce antibodies. The *BFIV*21m78* mutant is identical to *BFIV*21* except for amino acids 78 and 81, which were modified from the aspartic acid and arginine found in *BFIV*21* to the glycine and glutamic acid found in *BFIV*B2* (Fulton et al., 1995). These amino acids are believed to be involved with antisera cross-reactivity between *B*21* and *B*5* (Fulton et al., 1996) due to their exposed location on the alpha helix and their presence in both *BFIV*21* and *BFIV*5* sequences (Hunt and Fulton, 1998). It was hypothesized that by replacing this epitope with that found in the *B*2* haplotype, the cross-reactivity commonly found in B*21 antisera would be eliminated. Use of the *BFIV*21m78* mutation clearly eliminated the B*5 cross-reactivity, which confirms the involvement of this epitope in B*21 and B*5 antiserum cross-reactivity.

The second mutant, *BFIV*13m126*, was identical to *BFIV*13* except for the region encoding amino acids 126 to 128. There is a polymorphism unique to *B*13* on this exposed antigenic loop (Hunt and Fulton, 1998). The *BFIV*13* sequence codes for aspartic acid, methionine, and lysine, in contrast to glycine, threonine, and methionine for *BFIV*12*. The *BFIV*13* and *BFIV*12* sequences have a shared sequence of amino acids from position 147 to 156. This region is on the alpha helix of the MHC molecule and the altered sequence involving 7 of 10 amino acids is unique to *BFIV*12* and *BFIV*13*. It was hypothesized that this region is responsible for the extensive cross-reactivity seen between sera developed for the *B*12* and *B*13* haplotypes (Fulton et al., 1996; and unpublished). Altering of the B*13 unique amino acid sequence at amino acid 126 to 129 was expected to result in antisera that were directed against primarily the antigenic region of amino acids 147 to 156 that was shared between BF*12 and BF*13. Adsorption of the BFIV*13-induced antisera with RP9 cells containing BFIV*12 did remove all B*12-specific activity, but considerable BF*13 activity remained. The logical extension of this analysis would be to develop another mutation in which amino acids 126 to 128 are altered in the BFIV*12 sequence to that of the BFIV*13 sequence. Immunization with this mutation followed by subsequent adsorption with BFIV*12 should result in BFIV*13-specific antisera. This would confirm the importance of the amino acids 147 to 156 in B*13-specific antisera.

The utility of this method of antiserum production is further shown by the results of testing the antiserum with RBC of numerous haplotypes from other birds. The antisera gave relatively simple patterns when tested against RBC of various defined haplotypes. The *B*24* and *B*C* haplotypes from the 003.*B* congenics reacted to both of the BFIV*13-induced sera, which suggests commonality in *BF* region between the *B*13*, *B*24*, and *B*C* haplotypes. The RBC from the line 003.*B*21* birds reacted identically to the 15.*B*21* cells. The *B*O* and *B*Q* haplotypes both reacted in an identical pattern to that of the 15.*B*21* haplotype, which suggests similarity in their *BF* regions. The *BFIV* region of the *B*Q* haplotype has been cloned and sequenced and found to be identical to the *BFIV* region of haplotype *B*21* (Hunt and Fulton, unpublished data). The antisera results confirmed the sequence information. Testing of the *B*R4* and *B*R5* recombinants was particularly informative. The *B*R4* recombinant contains the *BG*15:BF*21* combination (Briles et al., 1982). RBC from *B*R4* reacted only with *BFIV*21-* and *BFIV*21m78*-induced antisera, as expected, because of the presence of *BF*21* in the recombinant. The *B*R5* recombinant has the reverse situation, containing the *BG*21:BF*15* alleles. There was no reactivity of this recombinant haplotype to either of the *BFIV*21*-induced sera. The sera results are in complete agreement with the known allelic constitution of both recombinants. Further testing would need to use a *BFIV*15*-induced alloantisera, which has not yet been produced.

The HA data from the 13 inbred lines from ISU show more antisera cross-reactivity, consistent with the wider variety of haplotypes tested. The defined haplotypes of *B*12*, *B*13*, and *B*15*, regardless of the line in which the haplotype exists, react as expected. All three haplotypes from the two exotic breeds (Spanish and Fyoumi) reacted with both of the BFIV*21 antisera. The two Fayoumi haplotypes also reacted with the BFIV*12 and *13 antisera, which suggests some common epitopes. Thus, although we do not know the *BFIV* types in the ISU lines, most were differentiated with the *BFIV*-specific antisera. It is tempting to use the serological information to hypothesize the allelic constitution of these other haplotypes. The *B*1* haplotype of the GH line appears to be *BF*21*, the *B*6* haplotype from line G is *BF*13-like*, as is *BF*15.1* from line 19. The *B*15.1* from lines 8 and GH also have some BF*21-like epitopes. Obtaining the *BFIV* sequences from these *B*-haplotypes and using them in the immunization system described here would be extremely useful to better define these *B*-haplotypes and develop reagents specific for *BFIV* typing of these lines, several of which are designated as reference lines for *B*-haplotypes (Briles et al., 1982).

One limitation of this method is the haplotype of the birds used for immunizations. Due to the source of the RP9 cell line (from *B*2/*15*) and the required identity of the birds used for producing antisera (also *B*2/*15*), antisera directed against either the *B*2* or *B*15* haplotypes cannot be produced. These two haplotypes are very commonly found in both laboratory and commercial chickens. Use of a different cell line and its corresponding parental bird for immunizations would be required for production of antisera against these two haplotypes. However, the system described here is highly effective in producing antisera directed against all other *B*-haplotypes. As more *BFIV* sequences are cloned (Li et al., 1999), they could be moved into this vector and used for developing *BFIV*-specific antisera. These sera could then be used to quickly and easily assay potential *BFIV* alleles within the numerous *MHC* haplotypes found in both layer- and broiler-type chickens and, thus, assay commercial chicken strains for Marek's disease-resistant alleles.

Recently, this method was used successfully to produce sera directed against *Rfp-Y* sequences (Miller et al., 2001). Sera specific to numerous non-immune function-related epitopes could also be produced by using cloned sequences transferred into the vector and expressed in cell lines.

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