Utility of Three Restriction Fragment Length Polymorphism Probes for Genotyping of the Chicken Major Histocompatibility Complex Class IV Region

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ABSTRACT Three chicken *B-G* cDNA probes (*gene 8.5*, *bg28*, and *bg32.1*) were used to detect restriction fragment length polymorphisms (RFLP) in the chicken MHC class IV (*B-G*). By using inbred and selected chicken lines with different *B* haplotypes identified by hemagglutination, we identified *B* haplotypes (B^2, B^9, B^{11}, B^{12}, B^{15}, B^{19}, B^{21}, B^{31}, and B^{32}) by RFLP using the three probes following digestion of genomic DNA with four restriction endonucleases (*Bgl*II, *Eco*RI, *Hae*III, and *Pvu*II). The GSP inbred line, previously shown to contain $B\text{-}F^{21}$ by the use of a monoclonal antibody, did not contain $B\text{-}G^{21}$, based on RFLP tests, whereas line N had $B\text{-}F^{21}$ and $B\text{-}G^{21}$. Consequently, the RFLP typing with the clone of *B-G* cDNA was able to determine the *B* haplotype in more detail than typing by hemagglutination. In inbred and selected lines, three *B-G* cDNA are useful DNA probes for RFLP to identify *B* genotypes. Two families of chickens with segregating *B* haplotypes were analyzed by RFLP using these probes; however, identification of the *B* genotype by this method was difficult in the randomly bred population. Genotypic comparisons of RFLP with *gene 8.5* and *Bgl*II and *bg 28* as probes and digestion by the endonucleases *Eco*RI, *Hae*III, and *Pvu*II between the parents and their offspring were generally compatible within the expectations of Mendelian inheritance.

(*Key words*: *B* genotype, chicken, mendelian inheritance, major histocompatibility complex class IV, restriction fragment length polymorphism)

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INTRODUCTION

Genotyping of the chicken MHC by restriction fragment length polymorphism (RFLP) using class IV (*B-G*) probes has been reported (Miller et al., 1988; Chaussé et al., 1989; Kuragaki et al., 1991; Plachy et al., 1992; Yamamoto et al., 1995; Pharr et al., 1997). In these studies, three cDNA clones, *gene 8.5* (Kaufman et al., 1989), *bg 28* (Goto et al., 1988), and *bg 32.1* (Miller et al., 1988), were used as probes. In most of these studies, the investigators examined RFLP patterns from only one *B-G* clone of *B* genotype identified by hemagglutination (Goto et al., 1988; Chaussé et al., 1989; Kuragaki et al., 1991; Plachy et al., 1992; Briles et al., 1993; Landesman et al., 1993).

Nakaki et al. (1997) defined the DNA sequences of these three probes. The *bg 28* cDNA clone coded for the extracellular domain of the *B-G* gene, whereas the *gene 8.5* and *bg 32.1* clones were primarily from the intracellular domain. Because antisera and monoclonal antibodies usually examine the extracellular regions of *B-G* gene products (Briles et al., 1982), the *bg 28* gene would be the most efficient of these three probes for *B* genotyping. A question to consider is: What is the efficiency of *B-G* cDNA clones for the determination of *B* genotypes in the same chicken population? The present study evaluates the utility of these three probes for determining *B* genotypes using RFLP analysis. In randomly mating populations of chickens in which the *B* genotypes are still segregating, RFLP analysis was performed on the parents and their offspring.

MATERIALS AND METHODS

Chickens

The lines used in this study and their *B* genotypes are listed in Table 1. Serological *B* haplotypes were determined by the hemagglutination test with antisera prepared at the Laboratory of Animal Breeding and Genetics, Hiroshima University.

The four lines, GVHR-HA, LA, HG, and LG were established from the N line of Hokkaido University, Sapporo,

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Abbreviation Key: RFLP = restriction fragment length polymorphism; GVHR = graft-versus-host reaction.

TABLE 1. Line and *B* genotypes of chickens

Line	*B* Genotype	Breed	Sampling location
Selected line			
GVHR-HA	$B^{15}B^{15}$	White Leghorn	Hiroshima University
GVHR-LA	$B^{15}B^{15}$	White Leghorn	Hiroshima University
GVHR-HG	$B^{9}B^{9}$	White Leghorn	Hiroshima University
GVHR-LG	$B^{9}B^{9}$	White Leghorn	Hiroshima University
IgG-H	$B^{31}B^{31}$	White Rock	Hiroshima University
IgG-L	$B^{32}B^{32}$	White Rock	Hiroshima University
Inbred line			
CB	$B^{12}B^{12}$	White Leghorn	Hiroshima University
RPRL-151_5	$B^{15}B^{15}$	White Leghorn	Saitama Medical University
GSP	$B^{21}B^{21}$	Fayoumi	Nippon Institute for Biological Science
B-Homozygous line			
HB-2	$B^{2}B^{2}$	White Leghorn	Hiroshima University
HB-15	$B^{15}B^{15}$	White Leghorn	Hiroshima University
Cornell-P	$B^{19}B^{19}$	White Leghorn	Saitama Medical University
Cornell-N	$B^{21}B^{21}$	White Leghorn	Saitama Medical University
Randomly mated population			
N	Segregating	White Leghorn	Hiroshima University
C	Segregating	Cross bred	Hiroshima University

Japan, by selection for high and low competencies of splenomegaly in graft-versus-host reaction (GVHR) (Okada and Mikami, 1974). The IgG-H and -L lines were developed by selection for high (H) and low (L) levels, respectively, of IgG at 10 wk of age (Tamaki, 1980). The IgG-H and IgG-L lines contained serological B^{31} and B^{32} haplotypes, respectively. The CB line established by Hések et al. (1966) was introduced to Hiroshima University in 1985 by K. Hála, University of Innsbruck, Austria, and has been described by Hála (1987). The HB-2 (formerly called line P) and HB-15 (formerly line V) lines were developed by Toivanen et al. (1981). They were imported from Hy-line, adapted in Turku. The HB-2 and HB-15 were the *B* homozygous lines, B^2 and B^{15}, respectively (Hála, 1987). These were kindly provided by the Immunobiology Laboratory, Hiroshima University, Japan.

Blood samples from RPRL-$15I_5$, Cornell-P, and Cornell-N were kindly provided by K. Kanki at Saitama Medical School, Japan. Cornell-P and Cornell-N are the international standard lines for the B^{19} and B^{21} haplotypes, respectively (Briles et al., 1982). The RPRL-$15I_5$ was an inbred line with B^{15} haplotype (Hála, 1987). These lines had been determined by monoclonal antibody detection of B-G and B-F molecules as B^{15}, B^{19}, and B^{21}-homozygous. Although the Cornell-N line was typed as B^{21}, it reacted to both antibodies of anti-B-G^{21} and anti-B-F^{21}, whereas the GSP line with B^{21} haplotypes had been typed as *B*-F^{21} but not as *B*-G^{21} (Kanki and Mizutani, 1987). The GSP line was provided by M. Mizutani at the Nippon Institute for Biological Science, Japan.

The N line was a White Leghorn line that was maintained by random mating by the Laboratory of Animal Breeding and Genetics, Hiroshima University, Japan. The C line was maintained by random mating from a cross-bred population of White Leghorns and White Rocks in the same laboratory. In these two randomly mated lines, several *B* alleles are still segregating.

Genomic DNA Extraction

Genomic DNA from three chickens in each line was prepared from peripheral red blood cells according to the methods described by Nishibori *et al.* (1997). Concentration and purity were measured by a spectrophotometer.

B-G Probes

The chicken MHC class IV (*B-G*) cDNA clones of *gene 8.5* (Kaufman et al., 1989), *bg 28* (Goto et al., 1988), and *bg 32.1* (Miller et al., 1988) were subcloned into pBluescript II[2]. The clone of *gene 8.5*, kindly provided by C. Auffray, CNRS, France, had 600 bp. The clones of *bg 28* and *bg 32.1*, kindly provided by M. M. Miller, Beckman Research Institute of the City of Hope, Duarte, CA, 91010-0269, had 500 bp and 650 bp, respectively. The sequences and location of these three probes in the structure of the *B-G* gene were reported previously (Nakaki et al., 1997). The probes were labeled with [α-^{32}P]dCTP by the random primer method using the Multiprime DNA labeling system[3].

Southern Blotting and RFLP

Genomic DNA (20 ng) was digested with the restriction endonucleases *Eco*RI, *Bgl*II, *Hae*III, and *Pvu*II. Digested DNA fragments were electrophoresed in 1% agarose gels (15 cm) in TAE buffer (40 m*M* Tris-acetate 1 m*M* Na_2EDTA) at 20 V for approximately 24 h. The DNA in the gel was denatured using an alkaline buffer (0.2 *M* sodium hydroxide, 0.6 *M* sodium chloride) and transferred to Hybond-N^+ positively charged nylon membranes[3] with 20 × SSC (3 *M* sodium chloride, 0.3 *M* sodium citrate). Hybridization was carried out using chicken *B-G*

[2]Strategene, La Jolla, CA 92307.
[3]Amersham, Arlington Heights, IL 60005.

cDNA-labeled probes. Membranes were washed at room temperature in 2 × SSC with 0.1% SDS for 10 min, at 65°C in 1 × SSC with 0.1% SDS for 15 min, and at 65°C in 0.7 × SSC with 0.1% SDS for 15 min and then were subjected to autoradiography exposure for 1 or 2 d at –80°C.

RESULTS

Typical RFLP patterns for each serological *B* haplotype digested with *Pvu*II and hybridized with *gene 8.5*, *bg 28*, and *bg 32.1* are shown in Figure 1. The RFLP patterns were clearly different among several serological *B* haplotypes for the three probes and the four restriction enzymes (the results of digestion with *Bgl*II, *Eco*RI, and *Hae*III are not shown). Unique patterns were found for B^2, B^9, B^{12}, B^{15}, B^{19}, B^{31}, and B^{32}, whereas B^{21} had two patterns. The GVHR-HA, -LA, HB-15, and RPRL-15I_5, which were determined serologically to be B_{15}, had the same RFLP patterns on different genetic backgrounds. The GSP line showed different RFLP patterns from the Cornell-N line in the experimental conditions, although both have been typed serologically as B^{21}. The RFLP patterns of the IgG-H and -L lines were clearly different, despite that the lines were derived by two-way selection from the same base population.

Mendelian inheritance of RFLP hybridized with *gene 8.5* digested by *Bgl*II and with *bg 28* digested by *Pvu*II was confirmed in the mating among N and among C lines consisting of six and eight individuals from two generations, respectively (Figures 2A and 3A). For two combinations of probe and restriction endonuclease, that is *bg 28* and *Hae*III and *bg 32.1* and *Eco*RI, two new unexplained bands appeared in the C line family (Figures 4 and 5). The RFLP patterns of the N line parents were the same as that of their progeny (Figures 2A and 3A). Because of the intricate nature of the RFLP patterns in the C line family, schematic representations of the patterns are presented in Figures 2B and 3B. Polymorphic bands of the sire and dam are indicated by arrows on the right (sire present, dam absent) or left (sire absent, dam present) sides. Some bands present in both parents were present in some offspring [#3, #4, and #8 (Figure 2B) and #1 and #4 (Figure 3B)] and missing in other offspring. These results suggest that the parents were heterozygous for these *B-G* fragments.

DISCUSSION

The *B* blood typing of chicken by hemagglutination with allo-antisera presents some difficulties in the identification of the *B* haplotypes of individuals from populations with different genetic backgrounds. For example, it was difficult to judge the *B* haplotype of some red jungle fowl and green jungle fowl because they reacted to all of the allo-antisera prepared from closed populations of domestic fowl (Nishibori, unpublished data). Establishment of a method for identifying the *B-G* haplotype by RFLP typing would solve the problem of typing cross-reactivity encountered when using *B* allo-antisera hemag-

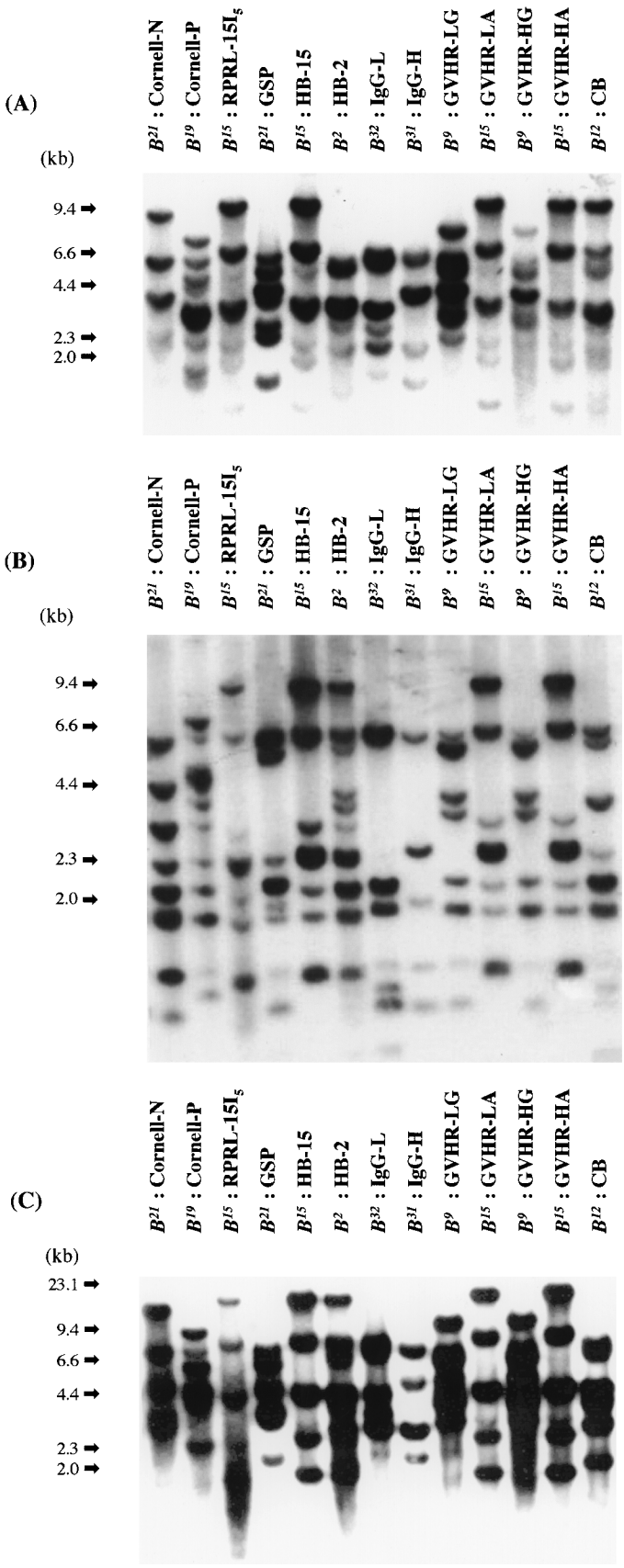

FIGURE 1. Restriction fragment length polymorphisms of *B-G* genes. Genomic DNA was digested with *Pvu*II and hybridized to (A) *gene 8.5*, (B) *bg 28*, and (C) *bg 32.1*. Lambda phage DNA digested with *Hind*III was used as a molecular size marker.

glutination. Recently, RFLP has been widely used in laying chickens to analyze MHC genes by using cDNA probes such as the *gene 8.5* (Kuragaki et al., 1991; Yamamoto et al., 1995), bg *11* (Miller et al., 1988; Briles et al., 1993), *bg 28* (Goto et al., 1988; Miller et al., 1988), and *bg 32.1* (Chaussé et al., 1989; Plachy et al., 1992; Pharr et al., 1997). All four probes have been successfully analyzed

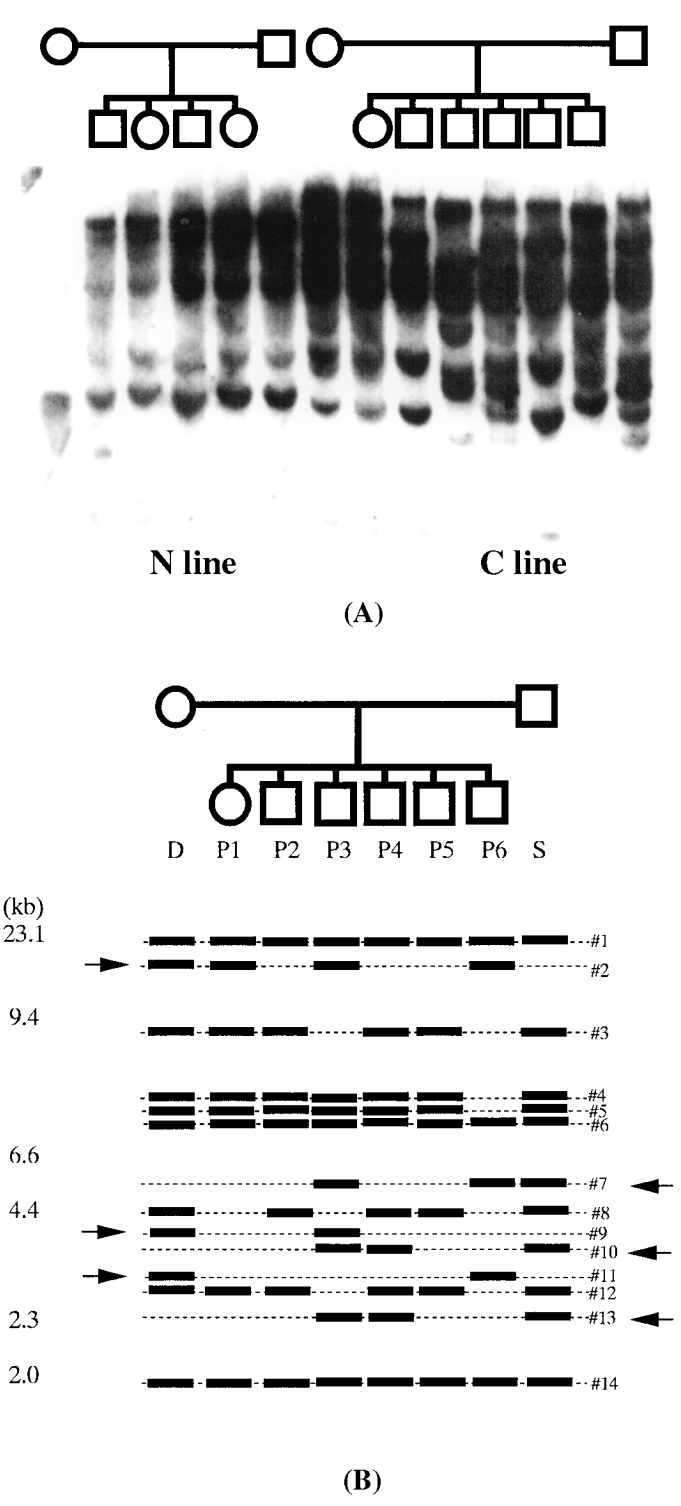

FIGURE 2. (A) Restriction fragment length polymorphisms (RFLP) of two families of N and C lines following hybridization with the *B-G* cDNA probe *gene8.5*. Genomic DNA was digested with *Bgl*II. (B) Diagrammatic representations of the RFLP patterns found among individuals of the family of C lines. D = dam (○), S = sire (□), P1 to 6-progeny, #1 to #14 indicate the number of bands. Molecular size markers (kb) are based on a *Hind*III digestion of lambda phage DNA.

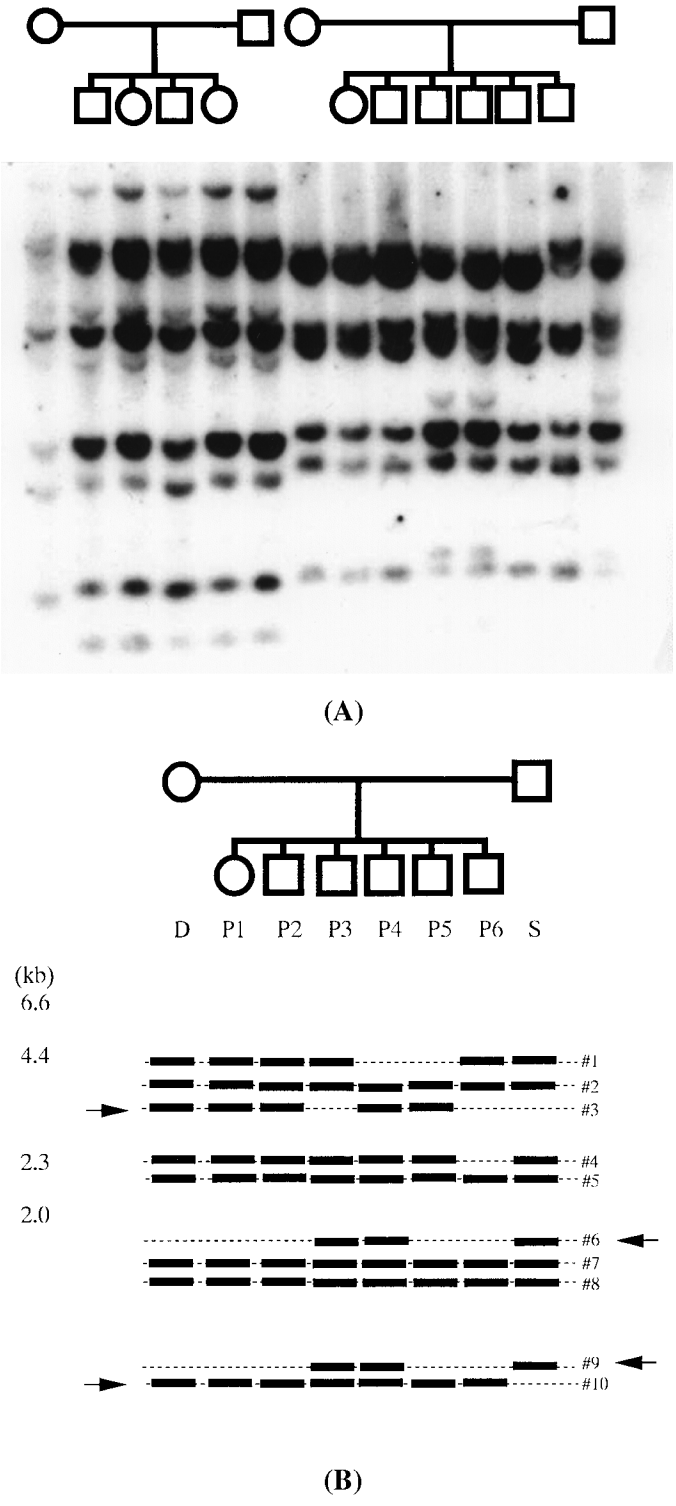

FIGURE 3. (A) Restriction fragment length polymorphisms (RFLP) of two families of N and C lines following hybridization with the *B-G* cDNA probe *bg 28*. Genomic DNA was digested with *Pvu*II. (B) Diagrammatic representations of the RFLP patterns found among individuals of the family of C lines. D = dam (○), S = sire (□), P1 to 6-progeny, #1 to #10 indicate the number of bands. Molecular size markers (kb) are based on a *Hind*III digestion of lambda phage DNA.

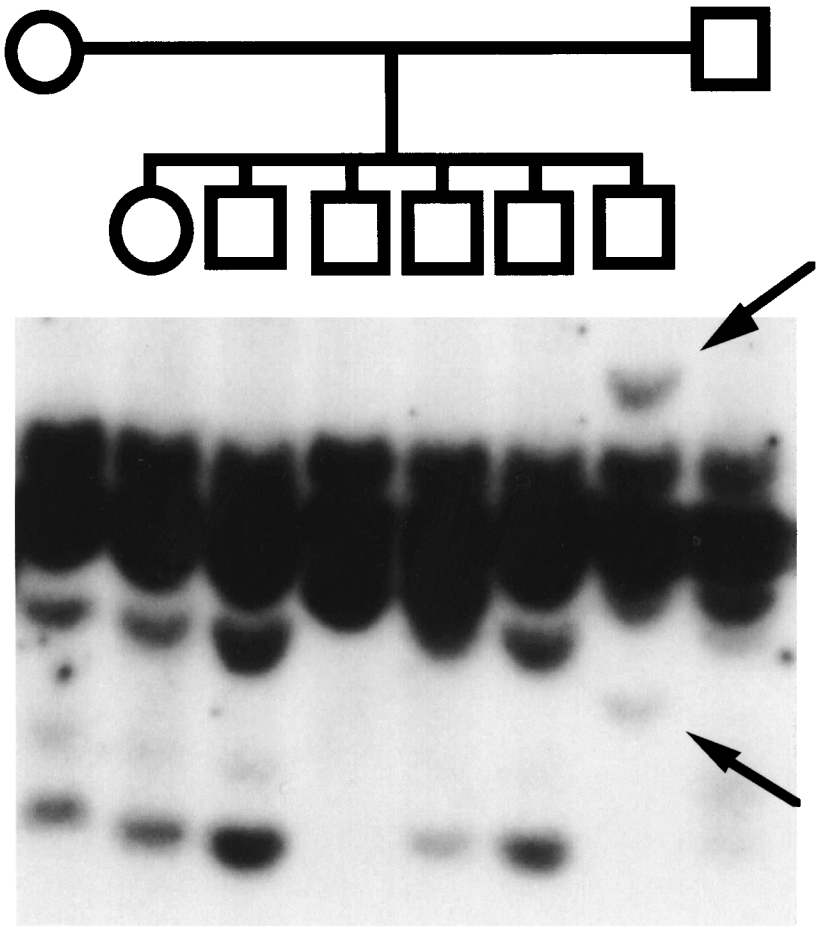

FIGURE 4. Restriction fragment length polymorphisms of the family of C lines following hybridization with the *B-G* cDNA probe *bg 28*. Genomic DNA was digested with *Hae*III. The arrow indicates the hereditarily unexplainable band in this family.

in RFLP typing. This study showed that each of three *B-G* cDNA, *gene 8.5*, *bg 28*, and *bg 32.1*, was a useful tool with which to identify the *B* genotypes in inbred, highly selected, and *B*-homogyzous lines (Figure 1). As shown in Figure 1, four lines with the serological B^{15} haplotype had the same RFLP patterns on different genetic backgrounds.

In spite of GSP having been previously characterized as *B-F*[21] (M. Mizutani, 1999, Nippon Institute for Biological Science, Kobuchizawa, Yamanashi, Japan, 408-0041, personal communication), its *B-G* RFLP pattern was different from that of Cornell-N having the same *B* genotype (Figure 1). However, Kanki and Mizutani (1987) mentioned that the GSP line has been determined as *B-F*[21] not *B-G*[21] by using monoclonal antibodies of B-F and B-G, respectively. In the present study, the *B-G* haplotype of the GSP line was not *B-G*[21].

In the B^{31} and B^{32} haplotypes, the IgG-H and IgG-L lines differed in their RFLP pattern (Figure 1) in spite of being derived by two-way selection from the same base population. Although both lines reacted similarly with all of our antisera in the hemagglutination test, the RFLP technique was able to identify IgG-H as the B^{31} haplotype and IgG-L as the B^{32} haplotype. Thus, RFLP analysis with a *B-G* cDNA is a more powerful means of identifying of *B-G* genotype than hemagglutination test with alloantisera.

Identification of the *B* genotype by RFLP typing with *B-G* cDNA was difficult in the randomly bred population (Yamamoto et al., 1995). In this experiment, the RFLP patterns of N and C randomly bred lines differed from those of inbred and selected lines (Figures 2A and 3A). Uni et al. (1992) and Landesman et al. (1993) reported that MHC class IV haplotypes were identified in a population of meat-type chickens by RFLP analysis with the *bg 32.1* probe and suggested that the RFLP technique could be applied to the determination of *B-G* genotypes in the chicken; however, they did not identify *B* genotypes by hemagglutination test. Because the standard RFLP pat-

terns for each *B* haplotype are not known yet, it was impossible to compare the RFLP pattern of the randomly bred population with that of inbred or highly selected chicken lines. Thus, additional studies with many chicken populations will be needed to establish the standard RFLP patterns for each of the *B* haplotypes.

With some combinations of probes and restriction endonucleases, rare bands that were difficult to explain hereditarily as shown by RFLP in the family of C lines appeared (Figures 4 and 5). In short, some bands observed in some of the progeny were not present in both of the parents. The main reason for this is that the B-G antigen shows a polymorphism at the level of the polypeptide structure or gene organization. It is possible that the chicken population with segregating *B* genotypes is highly polymorphic. Kaufman et al. (1991) reported that 20 *B-G* genes were located in the B-G region, some of which were certainly active. It might be that several B-G molecules with different sizes exist in lines that are not inbred (Kaufman et al., 1990; Miller et al., 1991; Kaufman and Salomonsen 1992). Kaufman and Lamont (1996) suggested that the polymorphisms of the *B-G* gene were maintained either by a high mutation rate or by "genetic hitchhiking." In this regard, RFLP analysis with the *B-G* cDNA probe could be said to have given a hereditarily unexplainable band in populations that are not inbred.

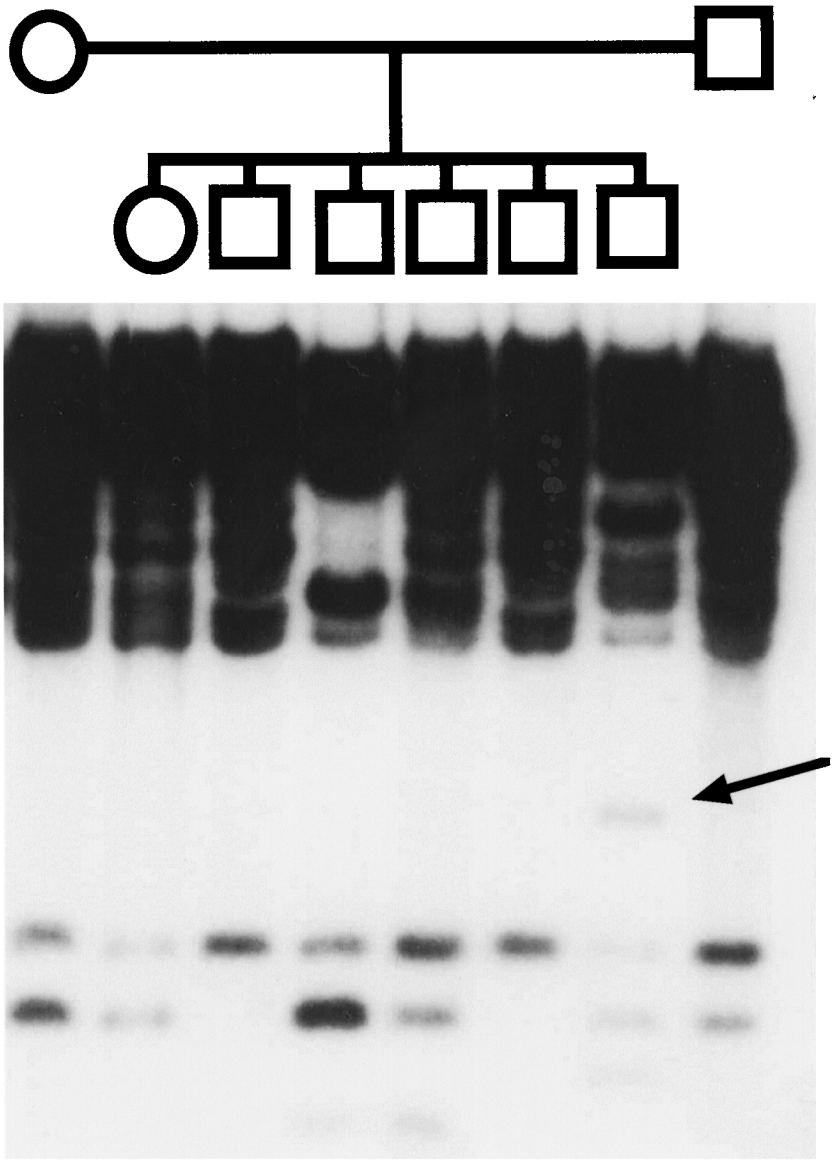

FIGURE 5. Restriction fragment length polymorphisms of the family of C lines following hybridization with the *B-G* cDNA probe *bg 32.1.* Genomic DNA was digested with *Eco*RI. The arrow indicates the hereditarily unexplainable band in this family.

The results of this study are summarized as follows: 1) the *B-G* cDNA gene as a DNA probe is a powerful tool for identifying *B* genotypes in inbred or highly selected lines by RFLP analysis and 2) chickens from random mating populations have several polymorphic *B-G* genes and manifest several structurally different B-G antigens.

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