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Role of nonclassical class I genes of the chicken major histocompatibility complex *Rfp-Y* locus in transplantation immunity

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Abstract The chicken major histocompatibility complex (*MHC*) genes are organized into two genetically independent clusters which both possess class I and class II β genes: the classical *B* complex and the Restriction fragment pattern-*Y* (*Rfp-Y*) complex. In this study, we have examined the role of *Rfp-Y* genes in transplantation immunity. For this we used three sublines, *B19H1*, *B19H2* and *B19H3*, derived from a line fixed for *B19*. Southern blots, PCR-SSCP assays using primers specific for *Rfp-Y*

genes, and *Rfp-Y* class I allele-specific sequencing show that the polymorphisms observed in *B19H1*, *B19H2* and *B19H3* are due to the presence of three different *Rfp-Y* haplotypes. The *Rfp-Y* class I (*YF*) alleles in these three haplotypes are highly polymorphic, and RT-PCR shows that at least two *YF* loci are expressed in each subline. The three sublines show *Rfp-Y*-directed alloreactivity in that *Rfp-Y*-incompatible skin grafts are rejected within 15 days, a rate intermediate between that seen in *B*-incompatible rejection (7 days) and that observed for grafts within the sublines (20 days). We conclude that *Rfp-Y* has an intermediate role in allograft rejection, likely to be attributable to polymorphism at the class I loci within this region.

The sequence data reported are available in the GenBank database under the accession numbers AY257165 (*YFVw*15*), AY257166 (*YFVw*16*), AY257167 (*YFVw*15*), AY257168 (*YFVw*17*), AY257169 (*YFw*16*), and AY257170 (*YFw*17*)

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Chickens possess two major histocompatibility complex (*MHC*) gene clusters: the classical *B* complex and the Restriction fragment pattern-*Y* (*Rfp-Y*) complex, detected initially by polymorphic class I and class II β gene restriction fragment patterns presented by members of fully pedigreed families (Briles et al. 1993). Each cluster possesses at least two class II β (*BL* or *YL*) and two class I (*BF* or *YF*) genes (Guillemot et al. 1988; Miller et al. 1994a, 1994b, 1996). While sharing overall sequence homology, the class I and class II β genes of the *B* and *Rfp-Y* systems separate into subfamilies, reflecting the gene cluster to which they belong (Zoorob et al. 1993; Miller et al. 1994a). At least one *Rfp-Y* class I locus (*YFV*) is transcribed in nearly all organs, exhibits allelic sequence diversity, and can be translated as a mature *YFV* protein associated with β_2 -microglobulin, which is recognized as an alloantigen in vivo (Afanassieff et al. 2001; Miller et al. 2001). Because of the sequence particularities of its antigen binding region, *YFV* appears to be a nonclassical class I locus and *Rfp-Y* a region

containing *MHC* genes of specialized function (Afanassieff et al. 2001).

Studies of *Rfp-Y* immune functions conducted with chickens carefully defined for their *B* and *Rfp-Y* haplotypes show the influence of *Rfp-Y* incompatibility on transplantation immunity (Pharr et al. 1996). To gain further insight into the immune functions of *Rfp-Y* genes, we have analyzed a serologically defined *B19* chicken line (French 1975), which shows diversity in the restriction fragment patterns typical of *Rfp-Y* genetic polymorphism. Southern blot analysis using *BL β* and *BF* probes has provided evidence for the presence of three different patterns, called *H1*, *H2* and *H3* (Chaussé et al. 1990). By breeding males and females homozygous for the same restriction fragment patterns, we derived three different sublines: *B19H1*, *B19H2* and *B19H3*. To demonstrate that the variation between these sublines was due to *Rfp-Y*, genomic DNA from *B19H1*, *B19H2* and *B19H3* homozygous birds was analyzed by Southern hybridization and polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) assays (Fig. 1). All birds shared identical *Bgl*I restriction patterns with the *BG*-specific probe, confirming the initial serological typing (Fig. 1a). The PCR-SSCP patterns obtained with primers specific for *BF* or *BL* exon 2 (Fig. 1b) showed that *B19H1*, *B19H2* and *B19H3* have identical *B* class I and class II β genes. In contrast, Southern blotting with *163/164f*, a *YF*-specific probe (Fig. 1a), and PCR-SSCP assays using a primer pair amplifying exon 2 of the *YF* genes (Fig. 1b) showed that these regions are polymorphic in the *B19H1*, *B19H2* and *B19H3* birds. The PCR-SSCP assays demonstrated that *YF* genes are polymorphic in exon 2, which encodes a portion of the class I antigen binding region. Southern blotting with a *BL β II* probe that hybridizes to class II restriction fragments in both the *B* and *Rfp-Y* genes (Fig. 1a) showed polymorphisms in the *Bgl*I fragments (9.5, 6.5 and 6.0 kb) associated with *Rfp-Y* (Miller et al. 1996) that distinguish *B19H1* and *B19H3* from *B19H2* (Fig. 1a). Whether these restriction fragment polymorphisms result in any functional differences among the *Rfp-Y* class II β alleles remains to be determined. However, since there is little evidence of polymorphism in the PCR-SSCP patterns derived from the exon 2 fragments corresponding to the antigen binding region of *YL* genes (Fig. 1b), it is unlikely that the *Rfp-Y* class II β loci in *B19H1*, *B19H2* and *B19H3* display allelic polymorphisms similar to those shown by *B* class II β and other classical class II β loci. These results are consistent with the limited sequence diversity of the *YL* genes described earlier by Zoorob and co-workers (1993). The finding of three *Rfp-Y* haplotypes is compatible with earlier Southern patterns reported by Chaussé et al. 1990. We will henceforth refer to these haplotypes as *Yw*15*, *Yw*16* and *Yw*17* respectively, adding three more haplotypes to those already in the literature.

Gene activity and function in the *Rfp-Y* region is still poorly understood. Only one *YF* gene (*YFV*) was found to be transcribed in the *B12/Yw*7.1* CB chicken line. The second locus (*YFVI*), in the *Yw*7.1* haplotype, is disrupt-

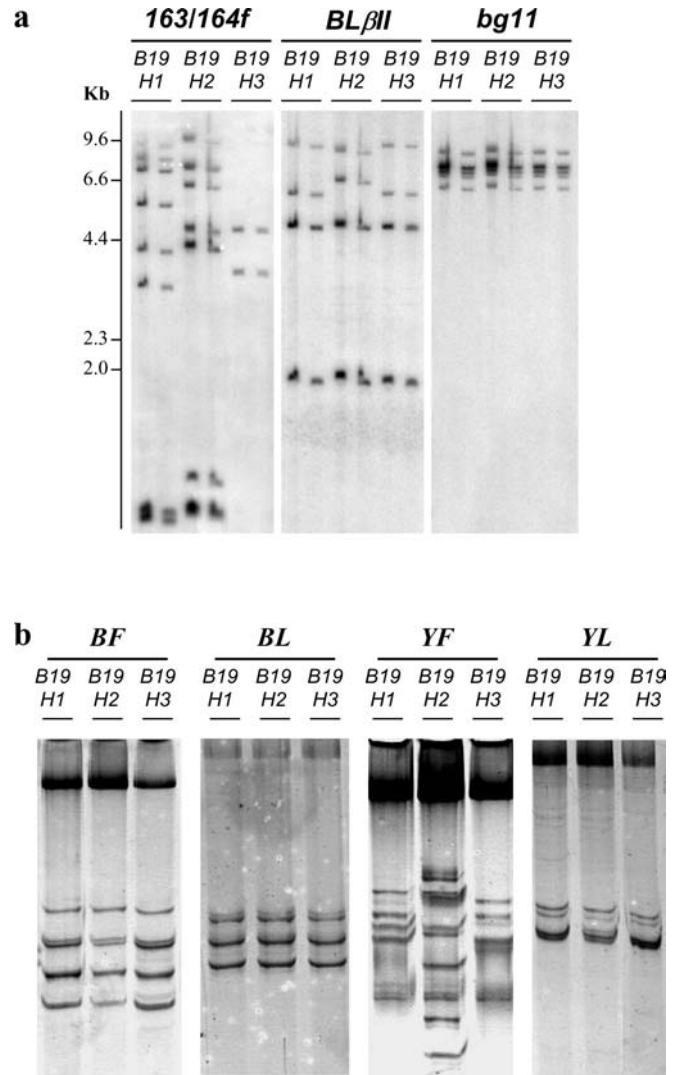


Fig. 1a, b Analysis of genomic DNA of *B19H1*, *B19H2* and *B19H3* homozygous birds. **a** Southern blot analysis. The three *Rfp-Y* haplotypes found segregating in the *B19* line are defined by *Bgl*I restriction fragments hybridizing to a *Rfp-Y* class I gene-specific probe, *163/164f* (GenBank AF493428). *Bgl*I restriction fragment patterns obtained with probes specific for class II β genes, *BL β II* (Briles et al. 1993) and *BG* genes, *bg11* (GenBank AF493427) are also provided. **b** PCR-SSCP assays. DNA from chickens of the three *B19* sublines was amplified with primer pairs specific to exon 2 of *B* or *Rfp-Y* class I and class II β genes, respectively. Amplification products were then denatured and electrophoresed on polyacrylamide gels under non-denaturing conditions. *BF* and *BL*: PCR-SSCP patterns obtained with primers specific for *B* complex class I and class II β genes, respectively. *YF* and *YL*: PCR-SSCP patterns obtained with primers specific for *Rfp-Y* system class I and class II β genes, respectively

ed and is apparently unexpressed (Afanassieff et al. 2001). Here, RNA extracted from the spleens of *B19H1*, *B19H2* and *B19H3* birds was reverse transcribed, PCR-amplified using exon 2–5 spanning primer pairs specific for *YFV* and *YFVI*, cloned, and sequenced. The deduced amino acid sequences are presented in Fig. 2 together with those of *YFVw*7.1* and *YFVIw*7.1* from the CB

Fig. 2a–c Comparison of predicted amino acid sequences of spleen *YF* transcripts from *B19H1*, *B19H2* and *B19H3* sublines with the sequences of *YFVw*7.1* (GenBank AF218783) and *YFVIw*7.1* (GenBank AF218784). **a** *YFV*-specific sequences obtained after RT-PCR using primers specific for the *YFV* gene (*YFa1-5'/YFVTM-3'*). **b** *YFVI*-specific sequences obtained after RT-PCR using primers specific for *YFVI* gene (*YFa1-5'/YFVITM-3'*). **c** *YF*-specific sequences obtained after RT-PCR using broadly hybridizing *YFa1*-specific primers (*YFa1-5'/YFa1-3'*). - designates an amino acid identical to that in the consensus sequence shown in the top row. Primers used to amplified *YF*-specific transcripts are indicated in *bold italic*

a. *YFV*-specific sequences

YFa1-5' primer >

α1 10. 20. 30. 40. 50. 60. 70. 80.
 Consensus GSHSLSRYFLTGMTDPGPGMPRFVIVGYVDDK**IFGI**YDYSKSR**TAQPI**VEMLPQEDQEHWAQTQKARGGERDFDWLGR**LP**ERYNKSK
 H1-*YFVw*15* -----A-----R-----
 H2-*YFVw*16* -----A-----F-----C-I-G
*YFVw*7.1* T-N-----D-----Q-----N-N-----

α2 90. 100. 110. 120. 130. 140. 150. 160. 170.
 Consensus GSHTLQMMFGCDILEEDGSIIRGYDQYAFDGRD**YIAFDMDTMTFTAADPVAEIT**KRRWETEGTYAERWKHELGTVCVQNLRRY**LE**HGKAALKRR
 H1-*YFVw*15* -----K-----
 H2-*YFVw*16* -----RII-----
*YFVw*7.1* -----M-----FL-----

α3 180. 190. 200. 210. 220. 230. 240. 250. 260. 270.
 Consensus VQPEVRVWGKEADGILTL**S**CHAHG**FYPR**PIA**IS**WMKDG**MVR**DOETHWGGI**VP**NSDGT**YH**ASA**AI**DV**LP**EDG**DKY**CR**VE**HAS**LP**Q**GL**FSW
 H1-*YFVw*15* -----Y-----V-----R-----T-----L-----
 H2-*YFVw*16* -----N-----R-----
*YFVw*7.1* -----E-----T-----R-----W-----

<*YFVTM-3' primer*

TM & Cyto 1-3 280. 290. 300. 310. 320. 330.
 Consensus EPQPNL**IP**IEVAGAVVA**IA**V**IA**AV**GL**VVWKS**SG**KEK**GYE**AAAGHDGESSGSATGSEPSI
 H1-*YFVw*15* -----A-----G-----
 H2-*YFVw*16* -----
*YFVw*7.1* -----V-----

b. *YFVI*-specific sequences

YFa1-5' primer >

α1 10. 20. 30. 40. 50. 60. 70. 80.
 Consensus GSHSLSRYFLTGMTDPGPGMPRFVIVGYVDDK**IFGI**YDYSKSR**TAQPI**VEMLPQEDQEHWAQTQK**AG**GERDFDW**FL**SRL**LP**ERYNK**SG**
 H1-*YFVIw*15* -----
 H3-*YFVIw*17* -----
*YFVw*7.1* -----D-----

α2 90. 100. 110. 120. 130. 140. 150. 160. 170.
 Consensus GSHTMQMMIGCDILEEDGSIIRGYDQYAFDGRD**FLAFDMDTMTFTAADPVAEIT**KRRWETEGTYAERWKHELGTVCVQNLRRY**LE**HGKA**AA**VKRR
 H1-*YFVIw*15* -----
 H3-*YFVIw*17* -----
*YFVw*7.1* -----

α3 180. 190. 200. 210. 220. 230. 240. 250. 260. 270.
 Consensus VQPEVRVWGKEADGILTL**S**CHAHG**FYPR**PIA**IS**WMKDS**MV**QD**Q**ETRWGGI**VP**NRDGT**YH**TSA**AI**DV**LP**EDR**DKY**CR**VE**HAS**LP**Q**GL**FSW
 H1-*YFVIw*15* -----
 H3-*YFVIw*17* -----
*YFVw*7.1* -----

<*YFVITM-3' primer*

TM & Cyto 1-3 280. 290. 300. 310. 320. 330.
 Consensus EPQPNL**IP**IEAWLVVPLV**V**LF**V**AL**IA**L**LV**WK**FL**SGKEK**GYE**AAAGHDG**V**SSGSATGSEPSI
 H1-*YFVIw*15* -----
 H3-*YFVIw*17* -----
*YFVw*7.1* -----

c. *YF*-specific sequences

YFa1-5' primer > <*YFa1-3' primer*

α1 10. 20. 30. 40. 50. 60. 70. 80.
 Consensus GSHSLSRYFLTGMTDPGPGMPRFVIVGYVDDK**IFGI**YDYSKSR**TAQPI**VEMLPQEDQEHWAQTQK**AG**GERDFDW**FL**X**LR**LP**ERY**NK**SG**
 H1-*YFVw*15* -----R-----RG-G-
 H1-*YFVIw*15* -----F-----F-S-
 H3-*YFVIw*17* -----F-----F-S-
 H2-*YFw*16* -----R-----RD-LE-CGL-G-
 H3-*YFw*17* -----R-----RD-LE-CGL-G-
*YFVw*7.1* T-N-----DT-----N-N-
*YFVIw*7.1* -----D-----F-S-

line (Afanassieff et al. 2001). Evidence for two different transcripts was found with each primer pair: *YFVw*15* (*B19H1*, GenBank AY257165) and *YFVw*16* (*B19H2*, GenBank AY257166) with the *YFV*-specific primers (Fig. 2a), and *YFVIw*15* (*B19H1*, GenBank AY257167) and *YFVIw*17* (*B19H3*, GenBank AY257168) with the *YFVI*-specific primers (Fig. 2b). All of these transcripts share high homology with the CB line sequences: 91.5%, 89.6%, and 99.6%, respectively. No evidence of *YFV* transcripts was obtained from *Yw*17*. Similarly no *YFVI* transcript was obtained from *Yw*16*. We were also unable to amplify these regions from genomic DNA (data not shown), suggesting that these loci may be missing in these haplotypes.

To look more generally for evidence of *Yw*15*, *Yw*16*, and *Yw*17* class I gene expression, a third primer pair designed to hybridize to the *YF* exon 2 ($\alpha 1$ domain), a region generally conserved in *YF* sequences, was used to analyze cDNAs from the *B19H1*, *B19H2*, and *B19H3* chickens. Three sequences were obtained with this primer pair (Fig. 2c). The first, obtained from *B19H1* birds,

corresponds to *YFVw*15*. The second, obtained from *B19H1* and *B19H3* birds, corresponds to the *YFVI* sequence found in *Yw*15* and *Yw*17*. The last, a more divergent sequence, was obtained from both the *B19H2* and the *B19H3* cDNAs. This third sequence (GenBank AY257169 and AY257170) is 70.5% and 77.2% homologous to the corresponding regions of *YFVw*7.1* and *YFVIw*7.1*, respectively (Fig. 2c), and may represent a third *YF* locus. These results indicate that the *B19H1*, *B19H2* and *B19H3* sublines each possess distinguishing polymorphic *YF* genes. At least two *YF* genes are transcribed in each haplotype. While some are more similar to the *YFV* and *YFVI* loci described earlier by Afanassieff and co-workers (2001), the additional *YF* sequences obtained for *Yw*16* and *Yw*17* using the broadly hybridizing *YFa1*-specific primers provides evidence that there is at least one additional expressed locus in these haplotypes.

To test for a role of *Rfp-Y* in alloreactivity, skin graft survival assays were conducted following a previously established wattle-to-shank technique (Bacon and Craig

Table 1 Influence of *Rfp-Y* and *B* haplotypes on skin graft survival in *B19* chicken sublines. Viable grafts were assigned a macroscopic score according to Polley et al. 1960, ranging from 0 (complete rejection and scar formation) to 6 (normal skin). The day of rejection was taken to be the first day when the bulk of the graft was assigned a score of 3. The median survival time (MST) for which the macroscopic score was below 3, and the confidence limits of the MST are given in days

Type of graft	<i>Rfp-Y</i> haplotypes of skin-grafted animals		Median survival time (days)	
	Donor	Host	Median	Confidence limit
<i>Rfp-Y</i> -compatible ^a	<i>H1</i>	<i>H1</i>	27	13–59
	<i>H2</i>	<i>H2</i>	19	13–27
	<i>H3</i>	<i>H3</i>	16	13–20
	Median <i>Rfp-Y</i> -compatible		20*	–
	<i>Rfp-Y</i> -incompatible ^b	<i>H1</i>	<i>H2</i>	15
<i>H1</i>		<i>H3</i>	14	13–17
<i>H2</i>		<i>H1</i>	15	13–17
<i>H2</i>		<i>H3</i>	15	13–17
<i>H3</i>		<i>H1</i>	14	10–17
<i>H3</i>		<i>H2</i>	15	13–17
Median <i>Rfp-Y</i> -incompatible		15*	–	
Controls, by compatibility type				
Autograft ^c	<i>H1</i>	<i>H1</i>	59 ^d	–
	<i>H2</i>	<i>H2</i>	59	–
	<i>H3</i>	<i>H3</i>	59	–
	Median syngenic		59*	–
<i>B</i> -incompatible ^e	<i>B19H1</i>	<i>B14</i>	6	–
	<i>B19H2</i>	<i>B14</i>	8	6–10
	<i>B19H3</i>	<i>B14</i>	8	6–10
	Median <i>B</i> -incompatible		7*	–

* Groups that differ significantly ($P < 0.05$) in their rejection times

^a Twenty-five *Rfp-Y*-compatible grafts were done

^b Fifty-three *Rfp-Y*-incompatible grafts were done

^c Skin graft on the same chicken (autograft); 17 autografts were done

^d Skin grafts were all accepted beyond 59 days

^e Histocompatible lines *B19* and *B14* differ in their *B* haplotypes but the *B14* line has not been defined for *Rfp-Y* haplotypes; 12 allogenic grafts were done

1969). Five reciprocal grafts were exchanged between each group of chickens of the same sex, with each chicken receiving an auto-, syngeneic or allogeneic graft. The grafts were observed every 3 or 4 days until 59 days post-grafting. Reciprocal grafts exchanged between chickens from the three different sublines were rejected with a median survival time (MST) of 15 days (53 grafts in total within these three sublines as noted in Table 1). In contrast, reciprocal grafts within sublines were rejected with a MST of 20 days, and *B*-incompatible grafts at 7 days (Table 1). All autografts were still in place at 59 days (Table 1). These results show that differences at *Rfp-Y* among the three sublines have a moderate, but significant, influence on the survival of skin transplants. Similar results were obtained in the closed line N, where *B* is fixed and three *Rfp-Y* haplotypes segregate (Pharr et al. 1996). In this line, *Rfp-Y* incompatibilities resulted in graft rejections that were essentially about twice as fast as those attributable to the potentially numerous minor histocompatibility (mH) antigens.

Skin graft rejection is attributable mainly to classical class I antigens (Bjorkman and Parham 1990). However, other blood-group antigens have additional moderate effects in chickens (Schierman and Nordskog 1965; Bacon and Craig 1969). Our results are in agreement with previous observations (Bacon et al. 1987; Pharr et al. 1996) and, in addition, show that it is likely to be the class I genes located in the *Rfp-Y* system that account for the observed moderate influence of *Rfp-Y* in skin graft rejection. Indeed, the three sublines have a maximum

inbreeding level of 66.54% (Pinard-van der Laan, personal communication), and thus are likely to segregate for *mH* loci, including the *C* blood-group locus (Dambrine, unpublished data) that can account for the level of incompatibility seen in animals matched for both *B* and *Rfp-Y* haplotypes. However, analysis of the genomic DNA samples and the *YF* gene transcripts from the *B19* birds has shown that the three sublines possess polymorphic *YF* genes and also have variable patterns of *YF* locus expression. This result is in agreement with a role for *YF* genes in skin graft rejection. Similar variation in *YF* gene expression may be present in the lines used by Pharr and co-workers (1996) for their initial description of the role of *Rfp-Y* genes in transplantation immunity.

The *Rfp-Y* haplotypes in the current investigation are involved in skin graft rejections. This result, when taken together with the conclusions of Pharr and co-workers (1996), indicate that genes within the *Rfp-Y* system have an influence on immune responses. It seems most likely that *Rfp-Y* acts to complement the *B* complex-mediated mechanisms, rather than to duplicate the actions of the *B* complex. The description of *YF* genes as nonclassical class I genes (Afanassieff et al. 2001) strongly supports this hypothesis. Moreover, the influence of *Rfp-Y* genes on immune functions may be linked both to their degree of genetic polymorphism and the levels of expression of the *YF* and *YL* loci, respectively. Additional studies are necessary to define the influence of both individual *Rfp-Y* alleles on immune responses, and possible interactions between specific *Rfp-Y* and *B* genotypes.

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