MOLECULAR BIOLOGY

The Turkey Major Histocompatibility Complex: Identification of Class II Genotypes by Restriction Fragment Length Polymorphism Analysis of Deoxyribonucleic Acid

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ABSTRACT Using a chicken Class II MHC clone in Northern blot analysis, tissue-specific expression of turkey Class II MHC genes was observed in the embryonic bursa of Fabricius as well as in the adult spleen. In contrast, there was no detectable expression in the embryonic liver, brain, or spleen. Southern blot analysis of *Bam*HI-digested turkey DNA revealed two restriction fragment length polymorphism (RFLP) patterns that did not deviate significantly from single-gene Mendelian inheritance. Further analysis of *Pvu*II-digested DNA from 325 turkeys showed four distinct RFLP patterns that segregated within the turkey lines studied. Because the chicken Class II MHC clone hybridized specifically to mRNA in immune-associated tissues, and because it identified polymorphisms among turkeys, the chicken clone is suggested to identify four turkey Class II MHC genotypes. The current study provides good evidence that RFLP analysis of DNA can be used as a means for molecular genotyping at the MHC in turkeys.

(*Key words*: turkey, major histocompatibility complex, restriction fragment length polymorphism, molecular genotyping, Class II genes)

1992 Poultry Science 71:2083–2089

INTRODUCTION

The Class II MHC genes and their gene products have been studied intensively in several vertebrate species, and found to be highly conserved (Kroemer *et al.*, 1990). For example, there is 62% nucleotide sequence homology between the human and chicken Class II β genes (Bourlet *et al.*, 1988). In the chicken, the Class II proteins function as restriction elements for helper T cells during an immune response (Vainio *et al.*, 1984), i.e., during antigen presentation, helper T cells will recognize only foreign antigen in association with a Class II protein. The chicken Class II proteins are cell-surface heterodimers with polypeptide chains 30 to 34 kDa in size (Crone *et al.*, 1981; Guillemot *et al.*, 1986). Class II expression in chickens has been

Received for publication April 24, 1992.

Accepted for publication July 31, 1992.

[1]Salaries and research support provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University. Manuscript Number 124-92.

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observed in tissues of the immune system, such as the spleen and bursa of Fabricius, as well as in those tissues containing fixed macrophages (Ewert and Cooper, 1978).

There are variable numbers of Class II genes among the chicken MHC haplotypes, as multiple restriction fragments are observed by Southern blot analysis (Chausse *et al.*, 1989; Warner *et al.*, 1989). Several chicken Class II genomic clones have been isolated from the B^6 and B^{12} haplotypes, and restriction enzyme mapping and DNA sequence analysis suggest that these clones represent unique Class II genes. For example, there are three independent Class II β genes in the B^6 haplotype (Xu *et al.*, 1989) and five Class II β genes in the B^{12} haplotype (Guillemot *et al.*, 1988; Kroemer *et al.*, 1990). Therefore, direct isolation of Class II genes also suggests that there are variable numbers of genes among the chicken haplotypes. Similarly, variability at the protein level has also been observed among the chicken MHC haplotypes (Crone *et al.*, 1981; Guillemot *et al.*, 1986). These polymorphisms at the gene and protein levels among chicken MHC haplotypes probably contribute to the differences that are observed in immune response and disease resistance in chickens (Lamont, 1989).

One method that is commonly used to identify genetic variability among individuals is restriction fragment length polymorphism (RFLP) analysis of the DNA. The application of this procedure to poultry breeding has been described previously (Soller and Beckman, 1986). Hybridization of chicken Class II or Class IV MHC probes to chicken DNA indicated that there is a good correlation between the RFLP markers and serological analysis (Miller *et al.*, 1988; Lamont *et al.*, 1990). However, there is also evidence that demonstrates that DNA polymorphisms exist among birds within the same serologically defined haplotype (Chausse *et al.*, 1989; Hala *et al.*, 1989). Therefore, RFLP analysis of DNA may be the more accurate method for identifying differences among individuals at the MHC. The objective of the present research was to identify turkey MHC genotypes by RFLP analysis by taking advantage of the high degree of sequence conservation between the turkey MHC and a chicken Class II MHC genomic clone.

MATERIALS AND METHODS

Turkey Lines

Two randombred control lines (RBC1 and RBC2) maintained at the Ohio Agricultural Research and Development Center (Wooster, OH 44691) served as the base population for the present research. Both lines were derived from commercial strains, and have been maintained as closed populations since 1957 (RBC1) or 1966 (RBC2) (Nestor, 1977a). A paired mating system has been used to minimize inbreeding and genetic drift (Nestor, 1977b). In 1988, turkeys from these lines (25 pairs per line) were mated to produce the first generation of progeny for the preliminary studies of the turkey MHC. Full-sib matings were used to minimize differences due to minor histocompatibility loci, as well as to produce progeny that would be homozygous at the MHC. Four generations of selection for MHC type were completed in this subline of turkeys, and the subline is segregating for the MHC genotypes that are described in the present study. For the current study, DNA was isolated from pedigreed turkeys of Generations 2 to 4.

Deoxyribonucleic Acid Isolation

High molecular weight DNA was isolated from turkey erythrocytes. Blood samples were collected into PBS, pH 7.2, containing 34 m*M* sodium citrate, and then washed one time in PBS. Packed red blood cells (100 μL) were washed two to three times with a low-salt buffer (10 m*M* NaCl, 10 m*M* EDTA) to remove all hemoglobin. The cellular material and intact nuclei were then resuspended in 5 mL of lysis buffer (50 m*M* Tris-HCl, pH 8.0; .5% SDS, 100 m*M* EDTA) and homogenized using a tissue tearor.[4] Ribonuclease A[5] was added at a final concentration of 100 μg/mL, and samples were incubated at 37 C for 1 h.

[4]Biospec Products, Bartlesville, OK 74005.

[5]Sigma Chemical Co., St. Louis, MO 63178-9916.

Proteinase K[6] was added to a final concentration of 100 μg/mL and samples were allowed to digest overnight at 37 C. The samples were then extracted one time with phenol-saturated Tris-EDTA buffer (10 m*M* Tris-HCl, pH 8.0; 10 m*M* EDTA), and one time with chloroform-isoamyl alcohol (49: 1). The DNA was ethanol-precipitated by adding .1 vol of 4 *M* NaCl and 2 vol of 95% ethanol. The DNA pellet was washed sequentially, first with 70% ethanol and then with 95% ethanol. The DNA was freeze-dried and then allowed to dissolve in sterile deionized water (1 to 2 mL) at 4 C for at least 3 days.

Chicken Major Histocompatibility Complex Probe

A genomic clone (CCII-7-1) that contains the chicken Class II MHC β gene was used as a hybridization probe in all experiments described. The restriction map and nucleotide sequence of this clone have been reported previously (Xu *et al.*, 1989). The *Hin*dIII 2.3-kb insert that contains the *B-L* β gene was purified from the Bluescript vector by gel electrophoresis (Wieslander, 1979), and was used as the probe for Southern blot analysis. The probe was labeled with α ^{32}P-deoxycytidine triphosphate[7] to high specific activity (10^8 to 10^9 dpm/μg DNA) using DNA polymerase (Klenow fragment) and the random oligonucleotide labeling method (Feinberg and Vogelstein, 1983).

Deoxyribonucleic Acid Digestion and Southern Blot Analysis

Turkey DNA (10 μg) was digested overnight at 37 C with restriction endonucleases, *Bam*HI or *Pvu*II[6] at a final concentration of 4 U enzyme/μg DNA (Maniatis *et al.*, 1982). The DNA samples were electrophoresed for 30 h at 30 V in .8% agarose gels, which were then depurinated, denatured, and neutralized as previously described (Maniatis *et al.*, 1982). The DNA was then transferred in 10× SSC (1× SSC = .15 *M* NaCl, .015 *M* sodium citrate) to a nitrocellulose membrane by the Southern blot method for at least 18 h (Maniatis *et al.*, 1982). After transfer, nitrocellulose membranes were dried under vacuum at 80 C for 2 h.

Prehybridization and hybridization solutions and conditions were similar to those described previously (Maniatis *et al.*, 1982). The ^{32}P-labeled chicken MHC probe was used at a final concentration of 20 ng/cm^2 in the hybridization solution. Following hybridization, nitrocellulose membranes were washed sequentially (2× SSC, .1% SDS at 25 C, then at 42 C; and .2× SSC, .1% SDS at 25 C, then at 42 C) to remove unbound radioactivity, prepared for autoradiography, and exposed to Kodak X-OMAT AR5 films for 24 to 72 h at –80 C with intensifying screens.[8]

Northern Blot Analysis

Various tissues, including liver, brain, spleen, and bursa of Fabricius from 25-day-old turkey embryos, as well as spleens from adult turkeys were collected and were immediately placed in liquid nitrogen. Tissues were stored at –80 C until required. Total cellular RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). The poly-A+ RNA was purified from cellular RNA using oligo-dT-cellulose (Chirgwin *et al.*, 1979). A total of 3 μg of poly-A+ RNA per tissue was subjected to electrophoresis at 20 V for 18 h in formaldehyde-agarose (1%) gels (Rosen *et al.*, 1990). Following electrophoresis, the RNA was transferred in 20× SSC to Zetaprobe membranes.[9] Prehybridization and hybridization solutions contained 1% SDS, 50% formamide, and Blotto (12× SSC, .04 *M* sodium phosphate, pH 6.5, and .5% nonfat dry milk powder; Johnson *et al.*, 1984). The probe, hybridization, and stringency of wash conditions were similar to those used for Southern blot analysis.

Statistical Analysis

Chi-square analysis was used to evaluate the goodness of fit for RFLP segregation

[6]Boehringer-Mannheim Biochemicals, Indianapolis, IN 46250.
[7]ICN Biomedicals, Inc., Costa Mesa, CA 92626.
[8]Dupont, Wilmington, DE 19898.
[9]Biorad, Richmond, CA 94804.

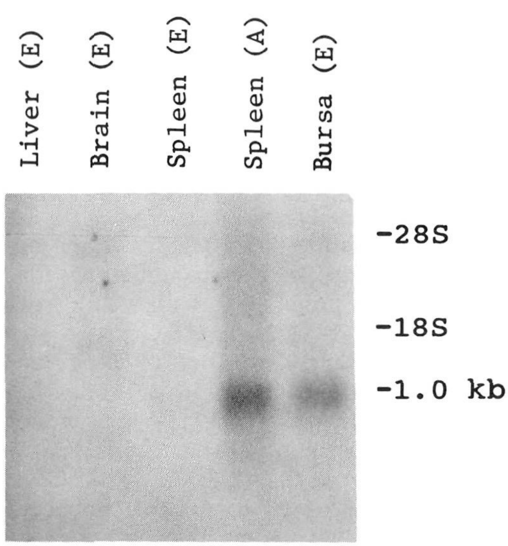

FIGURE 1. Northern blot analysis of turkey messenger RNA that was isolated from adult (A) or embryonic (E) tissues. The hybridization probe was a chicken Class II MHC genomic clone. The 18S and 28S are the internal ribosomal RNA molecular weight markers.

patterns. The expected ratios were based on classical Mendelian genetics.

RESULTS

Tissue-specific expression of Class II MHC genes in turkeys was observed as an approximate 1.0 kb mRNA species in the adult spleen and the embryonic bursa of Fabricius (Figure 1). There was no detectable Class II mRNA in embryonic liver, brain, or spleen.

TABLE 1. Molecular weight of DNA restriction fragments

*Bam*HI RFLP pattern[1]		*Pvu*II RFLP pattern[1]			
1	2	A	B	C	D
(kb)					
10.0	9.4	5.0	6.0	5.0	5.1
5.6	7.0	4.1	4.2	4.5	4.3
		3.3	3.4	3.4	3.4
			2.2	2.6	3.1

[1]RFLP = restriction fragment length polymorphism.

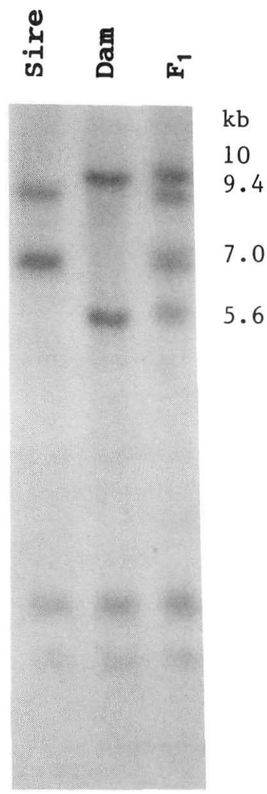

FIGURE 2. Southern blot analysis of *Bam*HI-digested turkey DNA from sire, dam, and the F_1 progeny. The hybridization probe was a chicken Class II MHC genomic clone.

Southern blot analysis identified two distinct patterns of turkey DNA digested with *Bam*HI, and probed with the chicken Class II MHC β clone (Figure 2). The DNA patterns represented are from two homozygous turkeys, and the heterozygous F_1 offspring. Molecular weights of the restriction fragments of the *Bam*HI RFLP patterns (designated 1 and 2, respectively) are shown in Table 1. Five mating types were examined for segregation of the *Bam*HI DNA patterns, and chi-square analysis for goodness of fit indicates that the patterns do not deviate from Mendelian inheritance (Table 2). Southern blot analysis of DNA from turkeys (n = 325) that represented three generations of pedigreed families made it possible to identify homozygous turkeys, and subsequently, distinct *Pvu*II RFLP patterns. Digestion of

TABLE 2. Segregation of *Bam*HI restriction fragment length polymorphism (RFLP) patterns in select families of turkeys

Mating type[1]	Number of families	Progeny			Chi-square[3]
		1/1	1/2	2/2	
2/2 × 2/2	3	0 (0)[2]	0 (0)	20 (20)	.0125
1/1 × 2/2	1	0 (0)	7 (7)	0 (0)	.0357
1/2 × 1/2	8	10 (12)	26 (24)	12 (12)	.3021
1/2 × 2/2	6	0 (0)	11 (12.5)	14 (12.5)	.1600

[1]Numeric designations for genotypes are the *Bam*HI RFLP patterns, as described in Table 1.

[2]Numbers in parentheses represent expected number of progeny for each genotype.

[3]Significance was determined at the chi-square critical value (P = .05) of 5.99. None of these were significant.

turkey DNA with the restriction enzyme, *Pvu*II, and hybridization with the chicken Class II MHC probe identified four different RFLP patterns (designated A to D) in this population of turkeys (Figure 3). As expected, the *Pvu*II RFLP patterns also followed Mendelian inheritance. The molecular weights of the *Pvu*II restriction fragments are presented in Table 1.

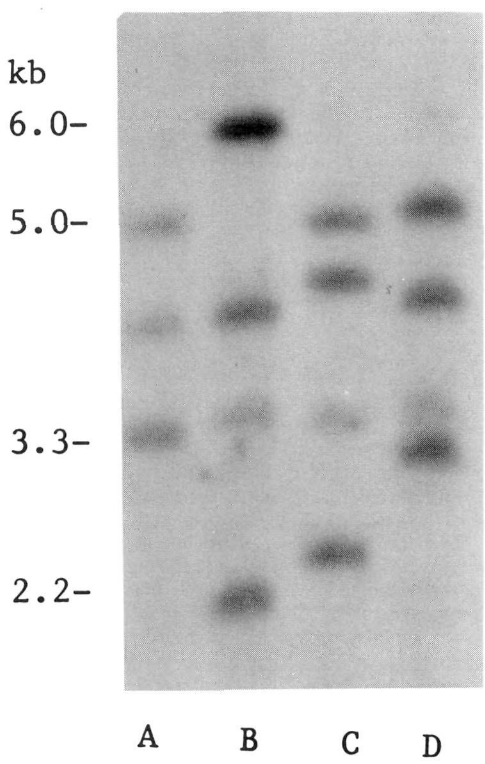

FIGURE 3. Southern blot analysis of *Pvu*II-digested turkey DNA. The hybridization probe was a chicken Class II MHC genomic clone. Lane A = restriction fragment length polymorphism (RFLP) Pattern A; Lane B = RFLP Pattern B; Lane C = RFLP Pattern C; Lane D = RFLP Pattern D.

DISCUSSION

Very little research has been undertaken to investigate the turkey MHC. Two reports have described the graft versus host reaction in turkeys, suggesting that there are Class II MHC differences in the turkey (Shoffner, 1964; Cherms, 1977). Similarly, the transplantation antigen (Class I) was identified in turkeys using a skin graft procedure (Palmer and Nordskog, 1980). Evidence was also presented that suggests that turkey erythrocytes do not express Class I proteins, or alternatively, there are low levels of Class I proteins on the erythrocyte surface (Palmer and Nordskog, 1980).

The present research examined the turkey MHC at the genetic level. Because the MHC is highly conserved between species (Kroemer *et al.*, 1990), it was anticipated that there would be high homology between the chicken and turkey MHC genes. This hypothesis was confirmed by the intense hybridization of a chicken Class II genomic clone to turkey messenger RNA (by Northern blot analysis) and turkey DNA (by Southern blot analysis). In addition, it was found that tissue-specific Class II gene expression in turkeys was similar to that of the chicken. A 1.0-kb message was observed in the adult turkey spleen and late embryonic bursa of Fabricius. However, negligible levels of Class II mRNA were observed in the turkey embryonic spleen. This finding was to be expected, because only 20 to 25% of chicken late embryonic spleen cells are positive for Class II, whereas 50 to 60% of adult chicken spleen cells are positive (Ewert and Cooper, 1978). Thus, the chicken Class II genomic clone appears to be identifying turkey Class II MHC genes and their transcripts.

The chicken Class II genomic clone also identified polymorphic restriction fragments in *Bam*HI- and *Pvu*II-digested turkey DNA. In the current study, there were four turkey Class II genotypes (RFLP Patterns A to D, respectively) that were identified by RFLP analysis of *Pvu*II-digested DNA. In chickens, the number of restriction fragments that were identified by Class I or Class II probes varied with the MHC haplotype (Chausse *et al.*, 1989; Hala *et al.*, 1989). In the present study, the number of restriction fragments per turkey genotype remained constant at three or four bands. The level of polymorphism in the turkey Class II region is low, and is limited to six or seven polymorphic restriction fragments among the turkey Class II genotypes studied. However, analysis of other commercial or experimental turkey lines may reveal additional Class II MHC genotypes, and shed light on the diversity of the MHC in turkeys.

In the present research, the four Class II genotypes were identified by molecular analysis of DNA. It is a well-known concept that alterations in the DNA may not necessarily reflect protein (or phenotypic) changes. For example, within the serologically defined B^{21} chicken MHC haplotype, there were five different subtypes based on DNA analysis (Chausse *et al.*, 1989). However, for the turkey MHC genotypes, histocompatibility data (Emara *et al.*, 1992) provides evidence that the Class II genotypes are phenotypically distinct.

ACKNOWLEDGMENTS

The authors wish to thank Linda Foster for her technical advice, and assistance in the preparation of the chicken Class II clone. Partial financial support for this research was provided by United States Department of Agriculture Competitive Grant 8801815. The work was performed by the senior author in partial fulfillment for the Ph.D. degree in Poultry Science, at The Ohio State University, Columbus, OH 43210.

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