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Assignment of *Rfp-Y* to the chicken major histocompatibility complex/*NOR* microchromosome and evidence for high-frequency recombination associated with the nucleolar organizer region

(trisomy mapping/ribosomal RNA genes)

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ABSTRACT ***Rfp-Y* is a second region in the genome of the chicken containing major histocompatibility complex (MHC) class I and II genes. Haplotypes of *Rfp-Y* assort independently from haplotypes of the *B* system, a region known to function as a MHC and to be located on chromosome 16 (a microchromosome) with the single nucleolar organizer region (*NOR*) in the chicken genome. Linkage mapping with reference populations failed to reveal the location of *Rfp-Y*, leaving *Rfp-Y* unlinked in a map containing >400 markers. A possible location of *Rfp-Y* became apparent in studies of chickens trisomic for chromosome 16 when it was noted that the intensity of restriction fragments associated with *Rfp-Y* increased with increasing copy number of chromosome 16. Further evidence that *Rfp-Y* might be located on chromosome 16 was obtained when individuals trisomic for chromosome 16 were found to transmit three *Rfp-Y* haplotypes. Finally, mapping of cosmid cluster III of the molecular map of chicken MHC genes (containing a MHC class II gene and two rRNA genes) to *Rfp-Y* validated the assignment of *Rfp-Y* to the MHC/*NOR* microchromosome. A genetic map can now be drawn for a portion of chicken chromosome 16 with *Rfp-Y*, encompassing two MHC class I and three MHC class II genes, separated from the *B* system by a region containing the *NOR* and exhibiting highly frequent recombination.**

Recently, Briles *et al.* (1) demonstrated by classical genetic testing within fully pedigreed families that a portion of the restriction fragments revealed in Southern blot hybridizations by chicken major histocompatibility complex (MHC) class I and class II probes is contributed by alleles within a second system of MHC-like genes that are genetically independent of the chicken MHC, the *B* system. The second system, designated *Rfp-Y*, was subsequently shown to correspond to cosmid cluster II/IV in the molecular map of chicken MHC genes (2, 3, 19), and hence *Rfp-Y* contains at least two MHC class I and two MHC class II loci along with a c-type lectin gene (*17.5*) and a gene (*17.8*) of unknown function (4).

Rfp-Y haplotypes are commonly found segregating in a variety of breeding stocks including experimental lines in which *B* system haplotypes have been fixed by selection. Although not direct evidence for function, this residual and commonly occurring polymorphism suggests that genetic variability in *Rfp-Y* system genes may be related to fitness.

Attempts to link *Rfp-Y* with other genetic markers in two reference mapping populations (5, 6) failed to demonstrate an association between *Rfp-Y* and any of >400 markers (4). A possible chromosomal assignment of *Rfp-Y* became apparent in a study of the Cornell Trisomic strain of chickens; a strain trisomic for chromosome 16, the microchromosome bearing the *B* system of histocompatibility and the single nucleolar organizer region (*NOR*) in the chicken genome (7). Enhancement of the intensity of the restriction fragments associated with *Rfp-Y* in aneuploid members of the Cornell Trisomic strain suggested that *Rfp-Y* might be located on chromosome 16 even though no assignment was evident in conventional linkage tests. Experiments described in this report were carried out to test this hypothesis.

MATERIALS AND METHODS

Animals. The chickens used in this study included the Trisomic (7, 38), PNU (8), and mono-PNU (9) strains from Cornell, UCD line 331 (10), UNH 6.6-2 (11), and a portion of family A186 as described (4). Various studies have shown conclusively that the MHC or *B* complex of chickens maps to a microchromosome that contains the 18S and 28S rRNA gene cluster, which is the nucleolar organizer region (*NOR*) (2, 7, 12, 13). The single *NOR* contains about 145 copies of the rRNA gene, occupying some 50–70% of this microchromosome (12). MHC class I, II, and IV (*B–G*) genes have been detected on this microchromosome (13–15). A genetic strain of chickens was developed at Cornell University with individual chickens having a trisomic condition for the MHC/*NOR* microchromosome (7). Trisomic individuals are viable and fertile. Crosses between trisomy 16 individuals generate a 1:2:1 ratio of disomic/trisomic/tetrasomic offspring, providing a chromosome 16 dosage series for mapping studies. Thus, stepwise enhanced hybridization intensities are produced in Southern blot analysis of MHC, rRNA, or other linked genes. This constitutes a rapid and accurate mapping method (trisomy mapping). Further selections from chickens trisomic for the *B*/*NOR* microchromosome have been made on the basis of nucleolar size to obtain new genetic lines, PNU (8) and mono-PNU (9), containing about 65 and 40 rRNA genes, respectively. These highly deleted *NOR* areas are carried in heterozygotes with *NOR* areas of normal size. The Cornell PNU strain was used to test for recombination frequencies between known *B* haplotypes (B^{15} and B^{6}) and a deleted rDNA cluster. Possible linkage between B^{6} and the reduced *NOR* was suspected since this condition was present in B^{6} containing stock and not in Trisomic strain B^{15} homozygotes. *B* haplotypes were determined by either standard *B* system hemagglutination methods (16) or molecular genotyping (17).

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Abbreviation: MHC, major histocompatibility complex.

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Southern Blot Analysis. Probes included a cDNA clone *B-LBII* (18), *18.1* (4, 19), a rDNA probe (20), and an 800-bp *Eco*RV subclone from cosmid cβ13 (2). Southern blots were prepared as described (4).

RESULTS

***Rfp-Y* Haplotype Segregation in the Cornell Trisomic, PNU, and Mono-PNU Strains.** Although failing to show linkage with the *B* complex (1), the possibility that the *Rfp-Y* class I and class II genes might be located on the *B/NOR* microchromosome had to be considered when it was observed in Southern blot hybridizations that the intensity of MHC class II gene restriction fragments associated with the *Rfp-Y* system increased with increases in copy number of the *B/NOR* microchromosome in trisomic and tetrasomic chickens. The observation that first suggested the *Rfp-Y* genes might be associated with chromosome 16 was made when comparing the pattern of MHC gene restriction fragments from an individual tetrasomic for chromosome 16 with those of disomic members of the PNU and mono-PNU strains (Fig. 1*A.1*). While the chickens in this test with a MHC class II probe, *B-LBII*, were uniformly homozygous for the B^{15} haplotype, as determined by blood typing and supported by the uniform presence of a 4.3-kb *Bgl* I restriction fragment (Fig. 1*A.1*, the 1.9-kb *Bgl* I fragment is monomorphic), the *Bgl* I restriction fragments of 9.5, 9.0, 6.5, 6.0, and 5.5 kb associated with *Rfp-Y* haplotypes were found to be segregating. Among the samples from the disomic individuals of the mono-PNU and PNU strains, the intensities of *Rfp-Y*-associated bands are relatively uniform. This is particularly apparent when identical restriction fragment patterns are compared, such as the seven patterns labeled 1/5 and three labeled 3/5 in Fig. 1*A.1*. In contrast, the pattern from a tetrasomic individual exhibited enhanced intensity not only for the 4.3-kb restriction fragment typical of class II *B* system genes and the monomorphic 1.9-kb fragment but also for three out of four restriction fragments associated with *Rfp-Y* (Fig. 1*A.1*). The enhanced intensity of bands associated with *Rfp-Y* suggested that there might be an increase in copy number of the *Rfp-Y* system in this tetrasomic individual.

To interpret the various patterns of restriction fragments revealed by the *B-LBII* probe in the *Bgl* I-digested DNA from the Cornell strains in terms of individual *Rfp-Y* haplotypes, additional hybridizations were carried out with DNA from a number of families within the Trisomic strain. Segregation of the fragments within these families (data not shown) defined four *Rfp-Y* haplotypes, Y^1, Y^3, Y^5, and Y^6. Y^1 and Y^3 have been described (1) and are defined by cosegregation of 9.5- and 6.0-kb and 9.5- and 5.5-kb bands, respectively (1). The Y^5 and Y^6 haplotypes, to our knowledge, have not been described and are defined by the cosegregation of 9.0- and 6.5-kb and 9.0- and 6.0-kb bands, respectively. With the number of haplotypes and their patterns determined, the five patterns of restriction fragments present in Fig. 1*A.1* can be interpreted in terms of

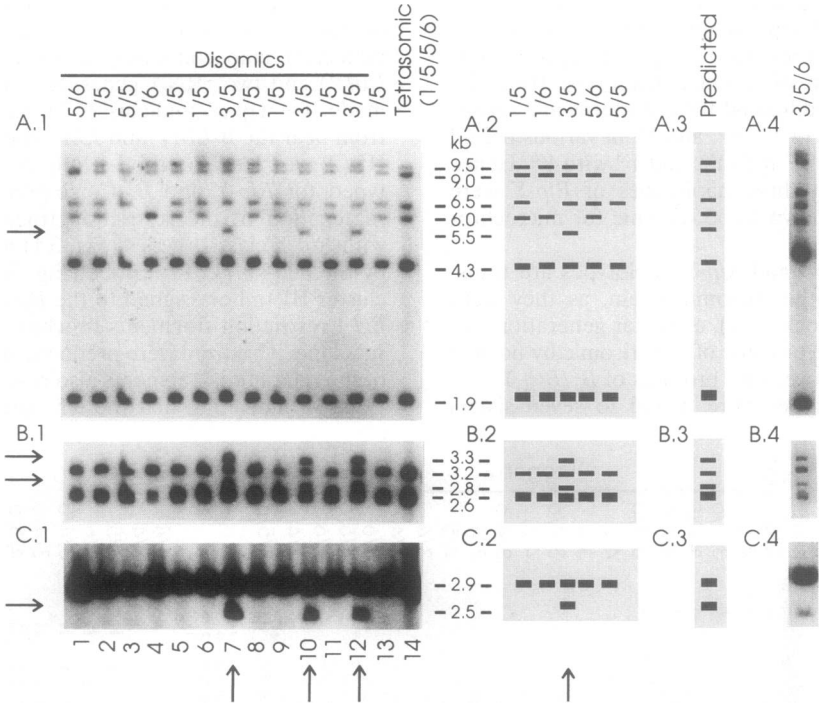

FIG. 1. Patterns of restriction fragments displayed by the Cornell mono-PNU (lanes 1–6), PNU (lanes 7–12), and Trisomic (lanes 13 and 14) strains. (*A.1* and *A.2*) Five MHC class II gene restriction fragment patterns corresponding to segregation of four *Rfp-Y* haplotypes, Y^1, Y^3, Y^5, and Y^6, are revealed in *Bgl* I-digested DNA of disomic members of the mono-PNU (lanes 1–6) and PNU lines (lanes 7–12), as well as in DNA two members of the Trisomic strain, disomic (lane 13) and tetrasomic (lane 14), for the *B/NOR* microchromosome. The restriction fragments of 9.5, 9.0, 6.5, 6.0, and 5.5 kb originate from the *Rfp-Y* system. Haplotypes Y^1, Y^3, Y^5, and Y^6 are defined by cosegregation of 9.5- and 6.0-kb, 9.5- and 5.5-kb, 9.0- and 6.5-kb, and 9.0- and 6.0-kb restriction fragments, respectively. Arrow at left denotes the 5.5-kb *Bgl* I restriction fragments associated with Y^3. The 4.3-kb fragment is from the *B* system. The 1.9-kb fragment is monomorphic. (*A.3*) If *Rfp-Y* is located on the MHC/*NOR* microchromosome, a pattern of MHC class II gene restriction fragment patterns containing five fragments is predicted to occur occasionally among trisomic (and tetrasomic) individuals in families in which Y^1, Y^3, Y^5, and Y^6 are segregating. (*A.4*) As illustrated here, patterns containing five restriction fragments were found among the trisomic (and tetrasomic) individuals in these families. (*B.1* and *B.2*) Restriction fragment pattern polymorphisms were also revealed by *18.1*, a probe associated with the *Rfp-Y* system c-type lectin gene. Restriction fragments of 3.2 and 2.6 kb are present in all samples. Fragments of 3.3 and 2.8 kb (as noted by arrows at left) are present only in samples from birds bearing the Y^3 haplotype. (*B.3* and *B.4*) The predicted and observed pattern of *18.1* restriction fragments for a trisomic individual carrying a Y^3 haplotype. (*C.1* and *C.2*) Restriction fragment patterns revealed *Pvu* II-digested DNA by an rRNA probe. All samples contain a 2.9-kb fragment. An additional restriction fragment of 2.5 kb (noted by arrows at left and below) is present only in the samples from Y^3 individuals. (*C.3* and *C.4*) The predicted and observed pattern of rRNA gene restriction fragments for a trisomic individual carrying Y^3.

the four haplotypes (Fig. 1*A.2*). Twelve of the 13 disomic individuals are heterozygotes. Seven are Y^1/Y^5, three are Y^3/Y^5, one is Y^1/Y^6, and one is Y^5/Y^6. Only a single individual presents a pattern (9.0 and 6.5 kb) consistent with homozygosity, in this instance for the Y^5 haplotype. Taking into account the four bands in the *Rfp-Y* pattern of the tetrasomic individual and the enhanced intensity of three out of four of these bands, the tetrasomic individual might be carrying one copy of Y^1, two of Y^5, and one of Y^6.

Given that restriction fragments of five sizes define the four *Rfp-Y* haplotypes in the Trisomic strain and its derivatives, occasionally restriction fragment patterns containing all five restriction fragments, as diagrammed in Fig. 1*A.3*, should be displayed by trisomic and tetrasomic individuals if there are indeed three and four copies of the *Rfp-Y* system present in these aneuploid animals. These were observed as illustrated in Fig. 1*A.4*.

Trisomic × Normal Diploid Cross. To determine whether trisomic individuals presenting the five band patterns transmit a multiplicity of restriction fragment patterns consistent with the presence of three *Rfp-Y* haplotypes, trisomic males from the Trisomic strain displaying five band patterns were mated to B^2/B^2 (line UNH6.6-2) hens homozygous for Y^4. The results of the Southern blot analysis of one such family, a cross between a Y^4/Y^4 dam and a $Y^3/Y^5/Y^6$ sire, are presented in Fig. 2. Y^4 contributed by the dam is represented in *Bgl* I/*B-LBII* hybridizations by 9.5- and 5.3-kb restriction fragments and her B^2 haplotype is represented by a 4.6-kb band. Six patterns were observed among the 18 progeny. The 5.3- and 9.5-kb bands of the Y^4 haplotype was present in all six patterns. Three of the six patterns of restriction fragments appear to be the result of transmission of a single haplotype from the sire—Y^3, Y^5, or Y^6. The additional three patterns are consistent with the transmission of Y^3, Y^5, and Y^6 in various paired combinations, Y^3 with Y^5, Y^3 with Y^6, and Y^5 with Y^6. Hence it can be concluded that three haplotypes of *Rfp-Y* were transmitted by the sire known to be trisomic for microchromosome 16.

To determine whether *B* and *Rfp-Y* haplotypes are transmitted independently in the Trisomic strain, as they were observed to be in other stock (1, 4), a further generation was produced from among the progeny of the trisomic by normal diploid cross described above. Two brothers of B^2/B^{15}, Y^4/Y^5 and B^2/B^{15}, Y^3/Y^4 genotypes were mated to seven sisters bearing B^2/B^{15} and Y^3/Y^4, Y^4/Y^5, or Y^4/Y^6. If *B* and *Y* are genetically linked, the linkage phases of the original (grand) parents should predominate among the gametes transmitted to the F_2 progeny. Only 56% of 106 informative gametes were found to carry the original combination of *B* and *Y* haplotypes, demonstrating once more the highly frequent recombination between *B* and *Y*.

Cosegregation of *Rfp-Y* and *NOR* Polymorphic Markers. While the most parsimonious interpretation of the patterns of segregation observed for *Rfp-Y* in the Trisomic strain is the presence of *Rfp-Y* on the MHC/*NOR* microchromosome, more direct evidence was sought for the association of *Rfp-Y* system with the *B*/*NOR* microchromosome. A polymorphism in the rRNA genes (Fig. 1*C.1–4*) was found to be associated with *Rfp-Y* haplotype Y^3 (Fig. 1*A.1–4*) and with a corresponding polymorphism associated with the c-type lectin gene located in *Rfp-Y* (Fig. 1*B.1–4*). The linkage of these three polymorphisms was verified in Southern blot hybridizations analyzing their segregation in fully pedigreed families (not illustrated). Thus, it can be concluded that the *Rfp-Y* system is on the MHC/*NOR* microchromosome closely linked to the *NOR* and separated from the *B* system by a region of high recombination. This conclusion is further supported by additional observations of polymorphisms within the rRNA genes segregating in concert with *Rfp-Y* haplotypes in the East Lansing reference population (ref. 6 and S. Lamont and N. Bumstead, personal communications).

Mapping Cosmid Cluster III to the *Rfp-Y* Region. With the location of *Rfp-Y* on microchromosome 16 established, the assignment of cosmid cluster III, a cluster in the chicken MHC molecular map containing a single class II MHC gene (*B-LBIV*) and two rRNA genes, to *B* or *Rfp-Y* remained to be made. To map cosmid cluster III, an 800-kb *Eco*RV fragment from near the *B-LBIV* and *13.1* genes of cβ13 clone in cluster III (2) was used to probe a fully pedigreed family previously typed for *Rfp-Y* and *B*. Correspondence was found in the segregation of the restriction fragment patterns associated with *Rfp-Y* class II genes (Fig. 3*A*) and the patterns revealed by the 800-kb *Eco*RV probe (Fig. 3*B*), thus allowing cosmid cluster III to be assigned to the *Rfp-Y* system. Moreover, the *Bgl* I restriction fragments to which the 800-kb *Eco*RV cβ13 subclone hybridized were predominantly of 9.5 and 9.0 kb, two of the restriction fragments also revealed by the *B-LBII* probe (Fig. 3), indicating that the 9.5- and 9.0-kb fragments likely

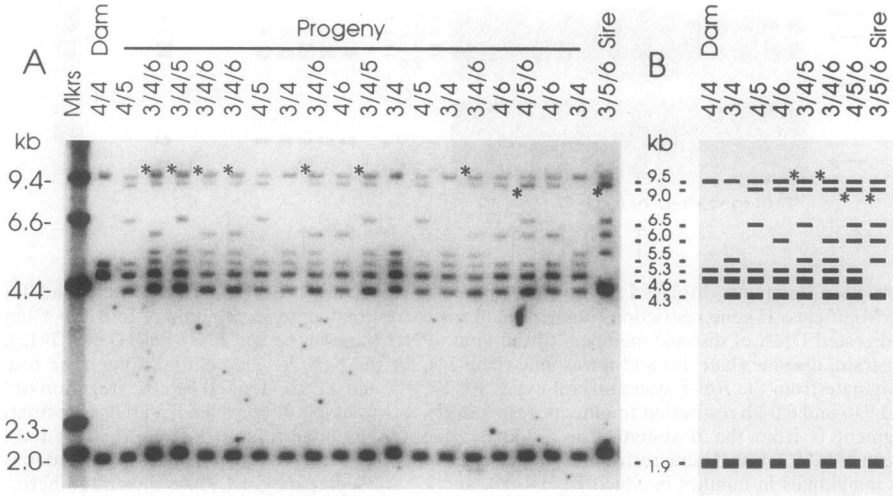

FIG. 2. (*A*) Patterns of restriction fragments revealed by the *B-LBII* probe in *Bgl* I-digested DNA in a family with 18 progeny produced in a cross between a trisomic ($B^{15}/B^{15}/B^{15}$) sire tentatively typed as carrying $Y^3/Y^5/Y^6$ on the basis of the presence of five polymorphic *Bgl* I/*B-LBII* restriction fragments and a B^2/B^2, Y^4/Y^4 dam. (*B*) Patterns of restriction fragments in the progeny, dam, and sire are consistent with transmission of Y^4 to all progeny from the dam and with one or two copies of Y^3, Y^5, and Y^6 variously transmitted by the sire. Restriction fragments in the patterns of trisomic animals representing two different *Rfp-Y* haplotypes are noted by asterisks. Though not marked with an asterisk, the 9.5-kb fragment present in the Y^3/Y^4 patterns represents both haplotypes.

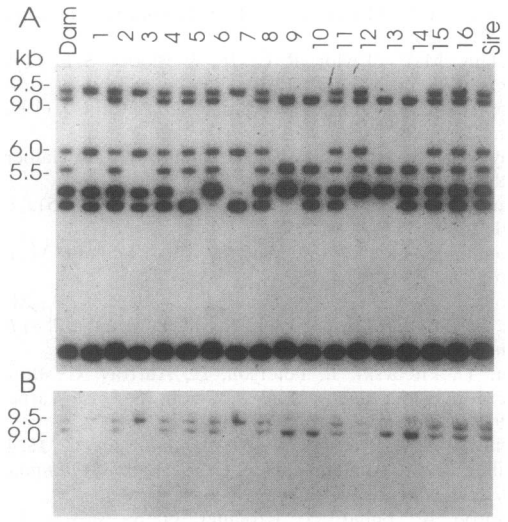

FIG. 3. (*A*) Class II MHC restriction fragment patterns revealed in *Bgl* I-digested DNA of members of a family (A186) previously typed for *Rfp-Y* and *B* (4). (*B*) Restriction fragment patterns revealed in the same family by an *Eco*RV fragment subcloned from cβ13 of chicken MHC cosmid cluster III (2).

contain the *B-LBIV* gene and adjacent DNA. Assignment of cosmid cluster III to the *Rfp-Y* system places all three members of the *B-LBIII* gene family (*B-LBIII, B-LBIV*, and *B-LBV*, ref. 21, which should now more appropriately be termed *Y-LBIII, Y-LBIV*, and *Y-LBV*) within the *Rfp-Y* system.

Recombination Between *B* and the *NOR/Rfp-Y* Regions. With the location of *Rfp-Y* on the MHC/*NOR* microchromosome established, a point for highly frequent recombination must necessarily lie between *Rfp-Y* and *B* to account for the lack of observable linkage. To test for possible high rate of recombination between the *B* system and the *NOR*, genetic analysis was performed with chromosomes marked for both regions (22). The specific cross involved B^{15} homozygotes having normal *NOR* genes (B^{15}/B^{15}, +/+) × $B^{15}B^{6}$ heterozygotes having a deleted *NOR* ($B^{15}B^{6}$, $+/p^{1}$). Initially, it was not known if, in the heterozygous parental type, the deleted *NOR* (p^{1}) region was linked to B^{15} or B^{6}. If B^{6} and p^{1} were linked, as was initially suspected since the p^{1} mutation was identified in stock carrying the B^{6} haplotype, then all B^{6} progeny ($B^{15}B^{6}$ birds) would have the rDNA deletion p^{1}. Alternatively, if B^{15} and p^{1} were linked in the heterozygous parent, then the $B^{15}B^{15}$ progeny would have the deletion and the $B^{15}B^{6}$ progeny would not.

The genetic analysis of this cross revealed the expected 1:1 ratio of the initial parental genotypes B^{15}/B^{15}, +/+ and $B^{15}B^{6}$, $+/p^{1}$ in an F_1 generation (Table 1). However, the alternative recombinant genotypes—$B^{15}B^{6}$, +/+ and B^{15}/B^{15}, $+/p^{1}$—were also recovered, and their frequency approached 50% (Table 1). This indicates a high rate of recombination between the *B* system and the deleted *NOR*. Since *Rfp-Y* also shows a high rate of recombination with *B* and maps in close proximity to the *NOR*, it is most likely associated with a region on the opposite or distal end of the *NOR* relative to *B*.

Table 1. Test for recombination between the *B* system and the *NOR* containing the 18S and 28S rRNA gene cluster

Progeny type	Normal nucleoli (+/+), no.	Reduced nucleoli ($+/p^{1}$), no.
B^{15}/B^{15}	26*	29
B^{15}/B^{6}	27	19*
Total†	53	48

B haplotypes were defined by serological typing. The normal *NOR* or rDNA gene cluster is designated as + and the normal genotype as +/+. The reduced *NOR* containing a deletion in rDNA genes is designated as p^{1} and the heterozygote with one normal and one reduced *NOR* is $+/p^{1}$. The parental genotypes were B^{6}/B^{15}, $+/p^{1}$, and B^{15}/B^{15}, +/+.

*Progeny having the parental genotypes. The remaining two classes are recombinant types.

†The 1:1 ratios of parental genotypes and also recombinant genotypes were obtained as determined in a χ^2 test.

DISCUSSION

Trisomy mapping of *Rfp-Y* to the same microchromosome occupied by the *B* system and the *NOR* allows the map of known chicken MHC genes to be unified on a single chromosome, even though the three gene regions cannot yet be precisely oriented with respect to the centromere (Fig. 4). The frequent recombination (approximately 50%) between *B* and the *NOR* (and *Rfp-Y*) suggests that the *B* and the *Rfp-Y* system are located on opposite sides of the *NOR*. Cosmid cluster III containing two rRNA genes represents one margin of the *NOR*. Cosmid cluster II/IV is placed near cosmid cluster III since the *Y-LIV* gene maps to *Rfp-Y*. The *NOR*, occupying at least 5.8 megabases of DNA (7, 24), is located an unknown distance away from the *B* system here represented by cosmid cluster I. One crossover per meiosis in the region intervening between the *NOR* and cosmid cluster I would account for the observed frequency of recombination between *B* on the one hand and the *NOR* and *Rfp-Y* on the other. Regular meiotic recombination apparently occurs frequently in chicken microchromosomes. One recombination nodule per microchromosome (25) and one chiasma per microchromosome (26) are commonly observed. The order of the genes within the *B* region with respect to the *NOR* remains to be determined. It is not known whether the *B–G* genes, represented in Fig. 4 by the single member of this gene family found in cosmid cluster I, are located at the proximal or distal end of *B* with respect to the *NOR*. Low-frequency recombination occurs between the *B–F* and the *B–G* regional genes (27, 28, 39).

To our knowledge, the domestic chicken is the first species in which both MHC class I and class II genes have been found to be organized together into two genetically independent units. How frequently this arrangement will be found in other species is not known. A *Rfp-Y*-like gene system or yet another alternative arrangement of genes at multiple sites in addition to a *B* system may be possessed by one of the closest relatives of the chicken, the ring-necked pheasant (29, 30). Generally, the picture of major histocompatibility genes that is emerging as more species are examined is one in which the gene number and location is, in a sense, highly unstable. That is, the number, arrangement, exon make-up, and chromosomal location of MHC genes may be far more varied than has been previously

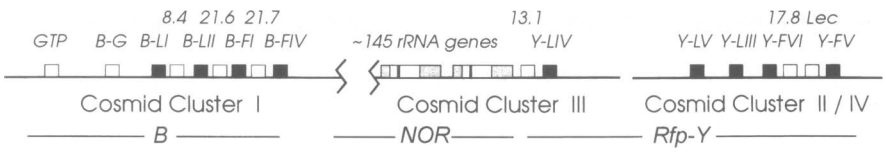

FIG. 4. Diagram of a portion of chicken chromosome 16 locating *B* and *Rfp-Y* genes with respect to the *NOR*, as well as to the positions of the chicken MHC cosmid clusters I, III, and II/IV (2). GTP is a GTP-binding protein gene (23) and *Lec* is the *17.5* gene encoding a c-type lectin (19).

suspected. For example, very recently an expressed human MHC class I gene that closely resembles the class I gene family found within the HLA has been located on human chromosome 1 (31). A large family of nonclassical MHC class I genes in *Xenopus* are located in a linkage group separate from the MHC (32). The apparent adaptation of the *B–G* gene family by means of exon shuffling (ref. 33 and R.M.G., Laura J. Hidas, Susan I. Jarvi, and M.M.M., unpublished data) is yet another means by which MHC genes may evolve. Of particular interest, of course, is the determination of the selective forces underlying the evolution of MHC genes.

Perhaps a selective advantage is provided by the arrangement of the MHC genes into two genetic units. If the MHC class I and class II genes in the *B* and *Rfp-Y* system function identically, then more genetic diversity would be provided to a population from a relatively small number of loci by arrangement into freely recombining units without sacrificing whatever advantage is provided by the clustering of MHC class I and class II genes in chromosomal regions.

It is possible that genes within *Rfp-Y* differ in function from their counterparts within *B*. The *Rfp-Y* genes, including the c-type lectin gene located in *Rfp-Y*, may represent an earlier form of the MHC, one that is based in innate immunity. Although the nature of the MHC class I and class II genes present within the *Rfp-Y* system is still under investigation, both classes of genes are at least transcribed and both show patterns of sequence specialization separating them from the *B* system genes (ref. 21 and M. Afanassieff, J. Ha, R.M.G., R.Z., C.A., and M.M.M., unpublished data). Given the strong influence of the chicken *B* system in genetic resistance to viral diseases (34–37), it will be interesting to see if *Rfp-Y* has a demonstrable influence as well.

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