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Immunology

Murine binding protein of the fourth component of complement: Structural polymorphism and its linkage to the major histocompatibility complex

(*H-2* complex/genetic polymorphism)

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ABSTRACT The binding protein of the fourth component of complement (C4-BP) is a regulatory protein of the complement system with specific affinity for the fourth component. This paper describes a structural polymorphism of murine C4-BP and its linkage to the major histocompatibility complex of the mouse (*H-2*). After isoelectric focusing of whole mouse plasma in low-endosmosis agarose, C4-BP was demonstrated as a single precipitin band by overlaying monospecific antiserum on the agarose gel. Two C4-BP patterns were distinguished among many strains of mice on the basis of isoelectric point—C4-BP a type, which has a pH range of 6.5–7.0 (exemplified by B10.BR and B10.AKM), and C4-BP b type, which has a pH range of 6.3–6.6 (exemplified by B10 and B10.M). Genetic crosses between two strains bearing distinct C4-BP types demonstrate a C4-BP pattern representative of both types. A linkage study was carried out in which progeny of two backcross combinations—[(B10 × B10.BR)F_1 × B10.BR] and [(B10.AKM × B10)F_1 × B10.AKM]—were phenotyped for C4-BP type and serum fourth-component level. Results were obtained suggesting that C4-BP patterns are inherited by a single codominant locus (*C4-Bp*) linked to the *H-2* complex. The recombination frequency between the *C4-Bp* locus and the *S* region was 0.017. By phenotyping appropriate intra-*H-2* recombinants of three different backgrounds (B10, A, and HT), this locus was assigned to the right of the *H-2D* region.

The major histocompatibility complex of higher vertebrates contains a remarkable cluster of genes that collectively control transplantation antigens, immune response, and biosynthesis of complement proteins. The complement system, one of the major defense mechanisms of vertebrates against microbial organisms, consists of perhaps 20 component proteins (e.g., C2, C3, C4) (1). Complement proteins can be activated by two pathways, the classical and the alternative. By either pathway, a unique enzyme complex (C3 convertase) is assembled. C3 convertase consists of either C2 and C4 (the classical pathway C3 convertase) or factor B and C3 (the alternative pathway C3 convertase) and plays a key role in the amplification process of complement activation (1). Studies from many laboratories, including ours, have demonstrated the linkages between the major histocompatibility complex and the loci controlling biosynthesis of these constituents of C3 convertase. Thus, the linkages to major histocompatibility complex of the genes controlling the following traits has been established: (*i*) serum C4 (Ss protein) level in mouse (2, 3); (*ii*) allotypic difference of C4 in mouse (4–6), guinea pig (7), and man (8–10); (*iii*) deficiency of C4 in man (11) and guinea pig (12); (*iv*) expression of a subclass of mouse C4 (sex-limited protein) (13); (*v*) allotype (14, 15) and deficiency (16) of human C2; (*vi*) serum C2 level in mouse (17); (*vii*) allotype of factor B in man (18–21), rhesus monkey (22), and guinea pig (12); and (*viii*) allotype of mouse C3 (23–26). It should be emphasized that, of the many complement proteins, none but these constituent proteins of the amplification C3 convertase are under the genetic control of major histocompatibility complex-linked loci (27–29).

We report here that murine C4-binding protein (C4-BP), a recently isolated complement protein that has regulatory functions for the C3 convertase of the classical pathway, is also controlled by a major histocompatibility complex-linked locus.

C4-BP is a macromolecular serum protein that has an electrophoretic mobility of β-globulin. It was first isolated from mouse plasma on the basis of its unique molecular structure and strong affinity for the activated form of C4 (Ss) protein (30). Then, a homologous protein was purified to homogeneity from human plasma and its structure and function were extensively studied (31–35). C4-BP has a M_r of ≈1 million as judged by elution profile in gel filtration and is composed of several polypeptide chains of 75,000 linked by disulfide bonds (human C4-BP) (31) or by noncovalent forces (mouse C4-BP) (36). C4-BP is an essential cofactor for C3b inactivator in the proteolytic cleavage of C4b (33, 35) and, to a lesser extent, of C3b (33) and functions as the regulator of C3 convertase of the classical pathway (34). Therefore C4-BP functions in a fashion similar to β 1H in the alternative pathway (37, 38). Our present study therefore implies that the locus controlling one of the regulatory proteins of C3 convertase is proximate to the loci that control the constituents of C3 convertase.

MATERIALS AND METHODS

Reagents. Sources of most reagents used for isoelectric focusing (IEF) have been described (5). Nonidet P-40 was purchased from Shell Chemicals (London). Ultrapure urea was purchased from Schwarz/Mann. Neuraminidase (*Costridium perfinges*, type V) was obtained from Sigma.

Mice. Sources of mice used are listed in Table 3.

Plasma. Blood samples were collected from the hearts of individual mice into syringes moistened with 0.4 M EDTA, immediately made 2.5 mM in phenylmethylsulfonyl fluoride, and centrifuged. Mouse plasma was treated with neuraminidase and then tested immediately for C4-BP type.

Antisera. Potent antiserum monospecific for mouse C4-BP was prepared by immunizing rabbits with highly purified mouse C4-BP. Preparation and characterization of purified C4-BP and monospecific antiserum have been described (36). Monospecificity of the antiserum was ensured by the following criteria.

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Abbreviations: C2, C3, C4, second, third, and fourth components of complement, respectively; C4-BP, C4-binding protein; IEF, isoelectric focusing; Ss, serum serologic substance (e.g., mouse C4).

First, the antiserum formed only a single precipitin line against whole mouse serum or plasma, as well as against highly purified and functionally active C4-BP, in immunodiffusion and immunoelectrophoresis. Second, the antiserum precipitated from mouse plasma only a single protein that migrated in $NaDodSO_4$/polyacrylamide gel electrophoresis (either in the reduced or in the unreduced form) as a protein of M_r 80,000. Third, the antiserum demonstrated complete identity with reference antiserum against mouse C4-BP (kindly supplied by Victor Nussenzweig, New York University). Rabbit antiserum monospecific for mouse C4 (Ss) protein was prepared and characterized as described (5).

Demonstration of Structural Polymorphism of Murine C4-BP. IEF in low-endosmosis agarose gel followed by immunofixation (39) was performed as described (5) with slight modifications. To prepare the agarose gel, 20 ml of agarose (0.5% IsoGel, Marine Colloid Division, Rockland, ME)/3 M urea/0.5% Nonidet P-40/3% Ampholines (pH 3.5–10) was poured onto a glass plate (100 × 130 mm). EDTA/mouse plasma was incubated at 4°C for 18 hr with neuraminidase at a final concentration of 5 units/ml and then mixed with an equal volume of precooled 6 M urea/1% Nonidet P-40. IEF was carried out at 4°C for 5 hr with a constant voltage gradient of 17 V/cm. After IEF, one portion of the gel was used for determining the pH gradient by cutting out 0.5-cm segments and eluting the Ampholines in distilled water. The rest of the gel was overlayed with anti-mouse C4-BP, washed, and stained with Coomassie blue as described (5).

Assay for C4 (Ss) Level. C4 (Ss) level in mouse plasma was determined by Laurell rocket immunoelectrophoresis in Svendsen buffer (40) using rabbit anti-mouse C4 (Ss) protein.

Linkage Tests. Progeny of two different backcross combinations—[(B10 × B10.BR)F_1 × B10.BR] and [(B10.AKM × B10)F_1 × B10.AKM]—were tested for C4-BP phenotype and the expression of Ss trait (serum Ss level), by immunofixation IEF and rocket immunoelectrophoresis, respectively. To ensure the full expression of Ss trait (2, 3), only male mice were tested and only after they reached an age of 3 months. The significance of the linkage of the two traits and the recombination frequency were calculated as described (41).

RESULTS

Demonstration of Structural Polymorphism of Murine C4-BP. By immunofixation IEF, mouse C4-BP was demonstrated as a broad precipitin band in neuraminidase-treated EDTA/plasma of all the mouse strains tested. Fig. 1 shows the C4-BP patterns of several congeneic strains of B10 background. Two C4-BP patterns were distinguished among B10 congeneic lines on the basis of isoelectric point. A C4-BP type having a pH range of 6.5–7.0 (termed C4-BP a) was observed in plasma of B10.BR and B10.AKM strains. A C4-BP type having a more acidic pH range (6.3–6.6) (termed C4-BP b) was observed in plasma of B10 and B10.M strains. The genetic cross (B10 × B10.AKM)F_1 demonstrated a C4-BP pattern representative of both C4-BP a type and C4-BP b type. Essentially, no difference was observed between male and female mice of the same strain with respect to C4-BP patterns (data not shown), although a significant difference was noted between C4-BP level of male and female mouse (30).

Linkage of the Locus Controlling C4-BP Polymorphism to the *H-2* Complex. The results shown in Fig. 1 suggested that C4-BP patterns are associated with the *H-2* complex, because these strains carry an identical genetic background but distinctive *H-2*. To determine the mode of inheritance of mouse C4-BP and also to confirm a genetic linkage of C4-BP polymorphism to the *H-2* complex, we carried out a linkage study. Backcross progeny of two different combinations of B10 congeneic strains were tested for C4-BP pattern and serum level of C4 (Ss) protein. In backcross progeny, C4-BP type segregated into F_1 type and a parental type (Fig. 2). The segregation ratios of a parental (homozygous) type versus F_1 (heterozygous) type were 1.1 and 1.3 for the first and the second backcross combinations, respectively. Table 1 shows a close association of C4-BP type with *H-2*. C4-BP a was inherited in association with Ss-low trait, and C4-BP b type was inherited with Ss-high trait. The results shown in Table 1 are compatible with the idea that murine C4-BP is controlled by a single codominant locus linked to *H-2*. We

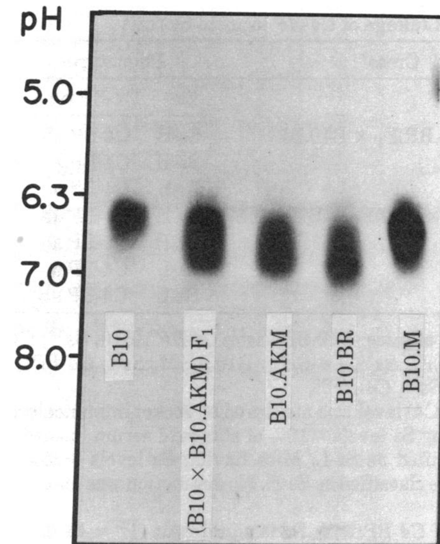

FIG. 1. C4-BP patterns of B10 congeneic mice as demonstrated by immunofixation IEF. EDTA/plasma (20 μl) of B10 congeneic lines was treated with neuraminidase and electrofocused in 0.5% low-endosmosis agarose gels. After IEF, the gel plate was overlayed with rabbit antiserum monospecific for mouse C4-BP. The plate was then washed, dried, and stained with Coomassie blue. B10.AKM and B10.BR demonstrate C4-BP pattern with a pH range of 6.5–7.0 (C4-BP a type). B10 and B10.M demonstrate more acidic C4-BP pattern with a pH range of 6.3–6.6 (C4-BP b type). (B10 × B10.AKM)F_1 demonstrate a C4-BP pattern representative of these two patterns with a pH range of 6.3–7.

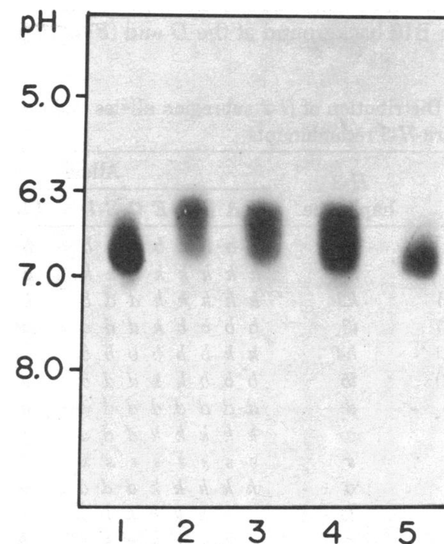

FIG. 2. Segregation of C4-BP patterns in backcross progeny. C4-BP patterns of selected backcross progeny of [(B10 × B10.BR)F_1 × B10.BR] are shown. Mice 1 and 5 demonstrate C4-BP a type, and mice 2–4 demonstrate C4-BP ab type.

Table 1. Linkage of *C4-BP* locus to Ss trait

Cross*	Phenotype		Incidence
	Ss†	C4-BP type	
[(B10 × B10.BR)F_1 × B10.BR]	Ss-H	C4-BP ab	31
	Ss-H	C4-BP a	1
	Ss-L	C4-BP a	32‡
[(B10.AKM × B10)F_1 × B10.AKM]	Ss-H	C4-BP ab	50
	Ss-H	C4-BP a	1
	Ss-L	C4-BP a	64
	Ss-L	C4-BP ab	1§

* Genotypes of mice: B10.BR, Ss^{ll}, $C4\text{-}BP^a$; B10, Ss^{hh}, $C4\text{-}BP^b$; (B10 × B10.BR)F_1, Ss^{hl}, $C4\text{-}BP^{ab}$; B10.AKM, Ss^{ll}, $C4\text{-}BP^a$; (B10.AKM × B10)F_1 Ss^{hl}, $C4\text{-}BP^{ab}$.

† Serum Ss (C4) level was measured by rocket immunoelectrophoresis. Mice having Ss levels <10% of standard serum (pooled B10 serum) were classified as Ss-L. Mice having Ss levels >25% of standard serum were classified as Ss-H. No distinction was made between Ss^{hh} and SS^{hl}.

‡ Linkage of *C4-BP* with Ss is significant ($\chi^2 = 62.02$; df = 1; $P < 0.001$). Recombination frequency is 0.016.

§ Linkage of *C4-BP* with Ss is significant ($\chi^2 = 112.03$; df = 1; $P < 0.001$). Recombination frequency is 0.017.

tentatively termed this locus *C4-BP* and have given designations $C4\text{-}BP^a$ and $C4\text{-}BP^b$ to the alleles that determine allotypic variation of C4-BP a and C4-BP b types, respectively. However, a few mice were detected among backcross progeny that bear phenotypes suggesting a recombination of *C4-BP* and *Ss* traits. The recombination frequencies of these two traits are 0.016 and 0.017 for the first and the second backcross combinations, respectively. Based on these recombination values, we assigned *C4-BP* to the proximity of *H-2* complex but outside the *H-2D* to *H-2K* boundaries, because the genetic distance between the *K* and *D* regions is known to be 0.3 centimorgans (41, 42).

Mapping of *C4-BP* Locus to the Right Side of *H-2D* Region. To determine whether *C4-BP* is located in the centromeric or telomeric side of *H-2*, we analyzed the distribution of *C4-BP* alleles among appropriate intra-*H-2* recombinants of three different genetic backgrounds. Table 2 shows that B10.A (3R) and B10.A (5R), carrying alleles from B10 background at the *K* end (*K–J* regions) of the *H-2* complex, bear C4-BP a type (non-B10 type). On the other hand, B10.A (2R) and B10.A (4R), carrying alleles from B10 background at the *D* end (*B–D* regions), bear C4-BP b type (B10 type). The result suggested that *C4-BP* is located not near the *K* end but near the *D* end of *H-2* complex. The C4-BP patterns of other recombinant strains of HT and A background shown in the lower part of Table 2 are compatible with this hypothesis. In further support of this hypothesis, we observed a striking correlation between *C4-BP a* and *Tla.a* or *Tla.c* on the one hand and between *C4-BP b* and *Tla.b* on the other. The *Tla* region is known to be located on the right side of *H-2* complex with a genetic distance of 1.5 centimorgans (41, 42).

Table 2. Distribution of *H-2* subregion alleles and *C4-BP* alleles among intra-*H-2* recombinants

Strain	*H-2* haplotype	Allele									
		K	*A*	*B*	*J*	*E*	*C*	*S*	*D*	*Tla*	*C4-BP*
B10	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*
B10.BR	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*a*	*a*
B10.A (2R)	*h2*	*k*	*k*	*k*	*k*	*k*	*d*	*d*	*b*	*b*	*b*
B10.A (3R)	*i3*	*b*	*b*	*b*	*b*	*k*	*d*	*d*	*d*	(*a*)	*a*
B10.A (4R)	*h4*	*k*	*k*	*b*	*b*	*b*	*b*	*b*	*b*	(*b*)	*b*
B10.A (5R)	*i5*	*b*	*b*	*b*	*k*	*k*	*d*	*d*	*d*	*a*	*a*
B10.D2	*d*	*d*	*d*	*d*	*d*	*d*	*d*	*d*	*d*	*c*	*a*
B10.A	*a*	*k*	*k*	*k*	*k*	*k*	*d*	*d*	*d*	*a*	*a*
B10.S	*s*	*s*	*s*	*s*	*s*	*s*	*s*	*s*	*s*	*b*	*b*
A	*a*	*k*	*k*	*k*	*k*	*k*	*d*	*d*	*d*	*a*	*a*
A.TL	*t1*	*s*	*k*	*k*	*k*	*k*	*k*	*k*	*d*	*c*	*a*
A.SW	*s*	*s*	*s*	*s*	*s*	*s*	*s*	*s*	*s*	*b*	*b*
A.TH	*t2*	*s*	*s*	*s*	*s*	*s*	*s*	*s*	*d*	*a*	*a*
HTG	*g*	*d*	*d*	*d*	*d*	*d*	*d*	*d*	*b*	*b*	*b*
HTH	*h*	*k*	*k*	*k*	*k*	*k*	*d*	*d*	*b*	*b*	*b*
HTI	*i*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*d*	*a*	*a*

Strain Distribution of *C4-BP a* and *C4-BP b* alleles. Table 3 shows the distribution of C4-BP alleles among 27 mouse strains. Here we show a striking correlation between the *C4-BP* allele and *H-2D* alleles but little correlation between the *C4-BP* allele and *H-2K* alleles. Mouse strains carrying D^b, D^f, or D^s bear C4-BPb type. Conversely, mouse strains carrying D^d bear C4-BPa type. On the other hand, $H\text{-}2^k$ strains form two groups with regard to C4-BP type—B10.BR, C3H/HeJ, CBA/J, and C57BR bear C4-BP a type, while AKR/J and C58/J bear C4-

Table 3. Strain distribution of *C4-BP a* and *C4-BP b* alleles

Strain	Haplotype	*H-2K*	*H-2D*	*Tla*	*C4-BP**	Source of mice†
B10.A	*a*	*k*	*d*	*a*	*a*	S, M, J
B10.A (3R)	*i3*	*b*	*d*	*a*	*a*	S, M
B10.A (5R)	*i5*	*b*	*d*	*a*	*a*	M
HTI	*i*	*b*	*d*	*a*	*a*	S, O
A.TH	*t2*	*s*	*d*	*a*	*a*	S, M
B10.D2	*d*	*d*	*d*	*c*	*a*	S, M, J
BALB/cJ	*d*	*d*	*d*	*c*	*a*	M
DBA/2J	*d*	*d*	*d*	*c*	*a*	S, M
A.TL	*t1*	*s*	*d*	*c*	*a*	S, M
A	*a*	*k*	*d*	*c*	*a*	S, M
B10.AKM	*m*	*k*	*q*	*a*	*a*	S
B10.BR	*k*	*k*	*k*	*a*	*a*	S, J
CBA	*k*	*k*	*k*	*b*	*a*	S, M
C3H/HeJ	*k*	*k*	*k*	*b*	*a*	S
C57BR	*k*	*k*	*k*	*a*	*a*	S, M
AKR/J	*k*	*k*	*k*	*b*	*b*	S
C58/J	*k*	*k*	*k*	*a*	*b*	S
B10	*b*	*b*	*b*	*b*	*b*	S, M, J
B10.A(2R)	*h2*	*k*	*b*	*b*	*b*	S, M
B10.A(4R)	*h4*	*k*	*b*	*b*	*b*	H
HTG	*g*	*d*	*b*	*b*	*b*	S, O
HTH	*h*	*k*	*b*	*b*	*b*	S, O
C3H.Sw	*b*	*b*	*b*	*b*	*b*	S
C57BL/6J	*b*	*b*	*b*	*b*	*b*	S, K
A.CA	*f*	*f*	*f*	*d*	*b*	S, M
B10.M	*f*	*f*	*f*	*d*	*b*	S, M
B10.S	*s*	*s*	*s*	*b*	*b*	O, Sh
A.SW	*s*	*s*	*s*	*b*	*b*	S, M

* For determining C4-BP phenotypes, at least 10 individual mice were tested by more than five IEF runs. When available, strains from more than one source were tested. C4-BP patterns thus obtained were consistent for each strain.

† S, K. Suzuki of the Institute for Medical Sciences, Tokyo University; M, K. Moriwaki of the National Institute of Genetics; O, K. Okuda of the Yokohama Medical College; K, K. Kondo of the Faculty of Agriculture, Nagoya University; H, T. Hamaoka of the Faculty of Medicine, Osaka University; Sh, D. C. Shreffler of Washington University, St. Louis, MO; J, Jackson Laboratory.

BP b type. These results are compatible with the idea that *C4-BP* is near the *D* end but outside the *K–D* boundaries.

DISCUSSION

The observed polymorphism of C4-BP can be explained by allotypic variation in the amino acid sequence or by posttranslational modification such as glycosylation and deamination. However, we definitely excluded the possibility that the C4-BP polymorphism described here is in reality artifactual heterogeneity caused by technical irregularities.

(*i*) The monospecificity of anti-mouse C4-BP serum used for detection of polymorphism has been firmly established (36); therefore, it is unlikely that we are detecting polymorphism of an unidentified serum protein reactive with contaminating antibody. We emphasize that our anti-C4-BP antiserum showed antibody specificity distinct from antiserum directed to the H-2-linked complement proteins (C4, C3, and factor B) (36).

(*ii*) We used neuraminidase-treated plasma as test sample for demonstrating C4-BP polymorphism because we found, in preliminary experiments, that removal of sialic acid resulted in better resolution of banding patterns. However, the C4-BP polymorphism described here is not artifactual heterogeneity caused by neuraminidase treatment; two comparable C4-BP patterns could be distinguished, even when untreated plasmas were subjected to IEF (data not shown).

(*iii*) The initial paper on mouse C4-BP stated that mouse C4-BP appeared polymorphic in serum because C4 tended to be activated in mouse serum, and the resultant complex of C4-BP and activated C4 showed a characteristic shift in electrophoretic mobility (30). We definitely excluded the possibility that we are detecting such pseudopolymorphism. First, we took great care to prevent activation of C4 during experimental procedures. We used EDTA/plasma supplemented with phenylmethylsulfonyl fluoride, instead of serum, as test samples, and we carried out IEF strictly at 4°C. With these precautions, we could effectively prevent activation of C4 and subsequent complex formation between C4-BP and C4. This was shown by the following control experiment. Plasmas of several B10 congeneic lines were electrofocused in duplicates. After IEF, one plate was immunofixed with anti-C4-BP and the second plate was immunofixed with anti-mouse C4 (Ss). In agreement with our previous results (5), each strain exhibited only a single precipitin band of C4; there were no additional patterns representing a C4–C4-BP complex or cleavage products of C4. Furthermore, the protein band of C4 showed a pH range characteristic of each haplotype (5) and distinct from that of C4-BP band. These results clearly indicated that C4 was not activated under our experimental conditions and that C4-BP was not coelectrofocused with C4 (Ss) protein. Furthermore, C4-BP patterns showed no correlation with plasma C4 (Ss) level. This was illustrated by the strain distribution of C4-BP type (see Table 3). For example, many Ss-high strains had the same C4-BP type as certain Ss-low H-2^k strains. In fact, H-2^k strains formed two groups with regard to C4-BP type. To further exclude the influence of C4 on C4-BP patterns, we performed the following control experiment. B10 plasma was incubated in the presence of 10 mM EDTA with various amounts of the IgG fraction of rabbit anti-mouse C4 (Ss) at 4°C overnight. After removing precipitates by centrifugation, supernates were assayed for residual C4 (Ss) level by rocket immunoelectrophoresis and for C4-BP type by immunofixation IEF. Addition of 0.1 ml of IgG (10 mg/ml) to 0.1 ml of B10 plasma resulted in complete depletion of C4 (Ss) protein from plasma. Notwithstanding, the C4-BP pattern remained unchanged (data not shown).

By phenotyping backcross progeny (see Table 1) and appropriate intra-*H-2* recombinant strains (see Table 2), we demonstrated that C4-BP is controlled by a single codominant locus (*C4-BP*) located in the right side of the *H-2D* region. Strain distribution of *C4-BP* alleles among many mouse strains was compatible with the assumed localization of *C4-BP* (see Table 3). It is interesting to note that H-2^k strains form two groups with regard to C4-BP allotype. This finding was not totally unexpected, because it is known that these strains are identical, with the loci between the *K–D* regions but they may differ with regard to loci localized between the *H-2D* and the *Tla* regions (41, 42). The locus controlling allotypes of murine C3 (*C3-1* locus) has been mapped to the position 10 centimorgam telomeric to the *H-2* complex (24, 26). We have recently established a B10 congeneic line that carries a nonparent type C3 allotype. Phenotyping of this congeneic strain confirmed the gene order of S, *H-2D*, *C4-BP*, *C3-1* (unpublished observation).

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