

# THE MURINE H-2.7 SPECIFICITY IS AN ANTIGENIC DETERMINANT OF C4d, A FRAGMENT OF THE FOURTH COMPONENT OF THE COMPLEMENT SYSTEM\*

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The murine H-2.7 specificity was first defined by Hoecker et al. (1) on the basis of hemagglutination tests. It is found on haplotypes f, j, p, and s. David et al. (2) and Klein et al. (3) showed that, unlike other H-2 specificities, H-2.7 is expressed predominantly on erythrocytes, and mapped it to a new region designated *H-2G*, between *S* and *D*, based on reactivity of a recombinant strain A.TFR1. Recent studies have shown that the original mapping was wrong, and that H-2.7 actually maps in the *S* region (4) which controls two markers C4 (Ss) and the sex-limited protein (5). The H-2.7 antigen is also found in serum and plasma.

Similarly to H-2.7, the human HLA-linked Chido and Rodgers specificities are found on erythrocytes and serum. Recent observations have shown that these blood groups are antigenic determinants of a fragment of C4 (C4d) and that they can be passively incorporated onto erythrocytes after complement activation (6-8). In our paper, we examine the nature of the serum molecule that expresses H-2.7.

## Materials and Methods

**Reagents.** The chemicals used for isoelectric focusing (IEF)<sup>1</sup> and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from the following sources: N,N'-methylene-bisacrylamide, acrylamide, N,N,N',N'-tetramethylethylenediamine, and ammonium persulfate were obtained from Bio-Rad Laboratories, Richmond, Calif.; Ultrapur urea from Schwarz/Mann, Div. Becton, Dickinson & Co., Orangeburg, N. Y.; Ampholines, pH 3.5-10 and 5-7, from LKB Instruments, Inc., Rockville, Md.; Nonidet P-40, Shell Chemical Co., London. A low-molecular-weight calibration kit for SDS-PAGE (range: 94,000-14,400) and Sephadex G-200 were obtained from Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J. SDS and  $\epsilon$ -aminocaproic acid (EACA) were obtained from Sigma Chemical Co., St. Louis, Mo.; Diisopropyl fluorophosphate (DFP) and *Staphylococcus aureus*, Cowan I, (Staph A) from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.

**Buffers.** Dulbecco's phosphate-buffered saline (PBS) was obtained from Grand Island Biological Co., Grand Island, N. Y. PBS containing 2 mM Na<sub>3</sub>H-EDTA and 5 mM EACA, pH 7.4 (PBS-EDTA-EACA) was also used as a buffer.

**Animals.** Inbred mice were purchased from The Jackson Laboratories, Bar Harbor, Maine. Other inbred, congenic, and congenic recombinant inbred mice or blood samples were kindly

\* Supported by National Institutes of Health grants 13224 and 24473. Presented in part at The VIII International Complement Workshop, Key Biscayne, Florida, October 1979.

<sup>1</sup> *Abbreviations used in this paper:* DFP, diisopropyl fluorophosphate; EACA,  $\epsilon$ -aminocaproic acid; IEF, isoelectric focusing; PBS, phosphate-buffered saline; PBS-EDTA-EACA, PBS containing EDTA (2 mM) and EACA (5 mM); pI, isoelectric points; PVP, polyvinylpyrrolidone; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Slp, sex-limited protein; Staph A, *Staphylococcus aureus*, Cowan I strain.

donated by several colleagues: Doctors D. C. Shreffler, Washington University, St. Louis, Mo., (C3H.OH, C3H.OL, C3H.W7); J. Stimpfling, McLaughlin Research Institute, Great Falls, Mont., (B10.RIII, B10.RIII(20R), B10.BSVS); Martin Dorf, Harvard University Medical School, Boston, Mass., (A.TFR4, B10.TFR5); Howard Passmore, Rutgers University, New Brunswick, N. J., (A.BY, B10.LIB55, B10.STA12, B10.CAS2, B10.KPB128); Ross Basch, (B10.M, B10.Q), and G. Jeanette Thorbecke (A.TL, A.TH), New York University School of Medicine, New York; and Guido Biozzi, Institut Curie, Paris, France, (MP).

*Antisera.* Antisera to mouse C4 (C4c + C4d), C4c, C5, and SIp were prepared as described elsewhere (9-11). The anti-H-2.7 alloantiserum was generated in the congenic combination [B10.F(13R) × A/J]F<sub>1</sub> anti-B10.P(K<sup>P</sup>I<sup>S</sup>b<sup>D</sup>/K<sup>b</sup>I<sup>S</sup>d<sup>D</sup>) anti-H-2<sup>P</sup> such that the incompatibility was only at the *S* and *D* regions. By cytotoxicity, the antiserum reacts only with H-2D<sup>P</sup> (H-2.22). By hemagglutination, the reactivity with H-2<sup>f</sup>, H-2<sup>g</sup>, and H-2<sup>j</sup> is specific for H-2.7. In vivo absorption in H-2<sup>k</sup> can remove H-2.7 activity but not H-2.22. Hemagglutination with H-2<sup>P</sup> could be a result of both H-2.7 and H-2.22.

*Hemagglutination Test.* The polyvinylpyrrolidone (PVP) test was performed according to Stimpfling (12) with some modifications. Briefly, serial dilution of antiserum was made in 0.9% PVP solution (0.9% PVP in PBS, pH 7.5, that contained 0.2% bovine serum albumin) starting with a 1:10 dilution. The 2% suspension of washed erythrocytes (twice in PBS, once in 0.85% NaCl) was prepared in 0.85% NaCl. 0.05 ml of the respective erythrocyte suspensions was added to the diluted antisera (0.1 ml) in each tube (75 × 10 mm). The serum-erythrocyte mixtures were incubated for 2 h at room temperature. After the incubation, the tubes were centrifuged for 30 sec at 1,000 *g*, and read by gently flushing the sediment with 0.85% NaCl.

*Hemagglutination-inhibition Test.* Mice were bled from the tail vein and artery into the tubes in ice that contained Na<sub>3</sub>H-EDTA, pH 7.5, to a final concentration of 0.01 M. Immediately after the centrifugation, plasma was used for the hemagglutination-inhibition test. The alloantiserum was serially diluted in PVP solution as in the hemagglutination test. An equal volume of EDTA plasma or serum fraction was mixed with the antiserum, and then the 2% erythrocyte suspension was added. The mixtures were incubated, centrifuged, and the results read as described above.

*Partial Purification of C4.* Mouse blood was collected in 0.010 M Na<sub>3</sub>HEDTA, pH 7.5, and 0.005 M EACA. After the plasma was separated, DFP was added to a final concentration of 0.002 M. The plasma (2 ml) was filtered through a Sephadex G-200 column (2.5 × 100 cm) equilibrated in the PBS-EDTA-EACA buffer described above. The fractions of the ascending limb of the second OD peak, which contained the C4 antigen, were pooled, and DFP was added to a final concentration of 0.002 M.

*Purification of C4c.* This was done as described elsewhere (11), using serum from MP mice.

*Partial Purification of C4d (α<sub>2</sub>) Fragment.* Serum from DBA/2J or SJL mice was collected in glass tubes and allowed to clot at room temperature for 20 min, in ice for 1 h, and at 37° C for 1 h. The serum was stored in the refrigerator overnight, and then passed through a Sephadex G-200 column. The third protein peak was concentrated (PM 30; Amicon Corp., Scientific Sys. Div., Lexington, Mass.) to the original volume of serum.

*Radiolabeling.* The partially purified C4, C4c, and C4d preparations were radiolabeled by the method of Hunter and Greenwood (13). Before labeling, preparations were incubated for 30 min at 0° C with excess Staph A for removing contaminating IgG. After dialysis to remove free iodine, a second absorption with Staph A was performed.

*Immunoprecipitation.* This was performed as described by Kessler (14). When necessary, the contaminating sex-limited protein (Slp) was removed by incubating the radiolabeled fraction for 10 min in ice with an excess of antiserum to Slp, followed, after 10 min, by addition of Staph A to remove the immune complexes. The depletion of Slp from these samples was confirmed by the absence of the characteristic Slp-α- and γ-chain spots in the radioautograms of the second dimension (15).

The C4-, C4c-, or C4d-positive radiolabeled material was incubated for 10 min at 0° C with the antisera, with occasional stirring. Then Staph A was added and incubated for an additional 10 min and centrifuged. The pellet containing the immune complexes was washed several times by centrifugation. The radiolabeled antigen was eluted by incubating for 20 min, room temperature, in IEF sample buffer containing 1% (wt:vol) Ampholine (pH 5-7), 5% (vol:vol)

$\beta$ -2 mercaptoethanol, 2.5% (vol/vol) Nonidet P-40, and 9 M urea, or by boiling for 3 min in SDS-PAGE sample buffer (16).

Controls included immunoprecipitation with: normal mouse serum, normal rabbit serum, alloantiserum to C5, and anti-Slp, in samples from genetically or phenotypically Slp-negative mice.

*Analytical Procedure.* SDS-PAGE was performed as in (16). 3% stacking gels and 7–15% linear-gradient running gels were used under reducing conditions in a slab system. IEF was performed in a vertical slab gel made of 4.5% acrylamide with a pH gradient between 3.5 and 7.9. Samples were applied into wells formed by polymerization of the acrylamide around plexiglass combs placed at the cathodic extreme of the gels. IEF was performed at 500 V for 16 h.

After the run, one portion of the slab gel was fixed in a mixture of methanol (25%), acetic acid (12%), and water (63%); dried; and subjected to radioautography using intensifying screens (Cronex; DuPont Instruments, Wilmington, Del.). A second portion of the gel was used for determining the pH gradient by cutting 0.5-cm segments and eluting the Ampoline in degassed distilled water. When necessary, a third portion of the IEF slab was cut and subjected to a second electrophoretic separation by molecular weight in SDS-PAGE after the procedure of O'Farrell (17).

## Results

*Correlation between the H-2.7 Specificity and Allotypes of Murine C4.* We have described a polymorphism of mouse C4 on the basis of differences of isoelectric points of their  $\gamma$ -chains as detected by the two-dimensional O'Farrell technique (17). The genetic control of this structural variation was mapped to the S region of H-2 (18).

Here we show a striking correlation between the C4 allotypes and the presence of the H-2.7 specificity (Table I). Mice which bear the p, f, s, and j alleles in the S region of H-2 have C4 of the  $\gamma_1$  type and are H-2.7 positive. Conversely, the presence of

TABLE I  
*Relationship between the S-Region-controlled Variants of the  $\gamma$ -Chain of C4 and the Expression of the H-2.7 Alloantigen*

Allele at the S region	Type of C4- $\gamma$ -chain*	H-2.7 antigen
p	1	Positive
f	1	Positive
s	1	Positive
j	1	Positive
Unknown (MP)	1	Positive
k	2	Positive‡
d	2	Negative
r	2	Negative
u	2	Negative
q	2	Negative
b	2	Negative
w7	2	Negative
Wild, nondesignated (B10.LIB55 and B10.STA12)	3	Negative

\* Types 1, 2, and 3 with isoelectric points at pH 7.4, 6.9, and 6.5, respectively (18).

‡ Negative on erythrocytes, negative or weakly positive in serum by inhibition of hemagglutination; positive by specific in vivo absorption of the alloantiserum (2, 3).

other alleles in the *S* region is associated with C4- $\gamma_2$  or C4- $\gamma_3$  and H-2.7 negativity, with the exception of *S<sup>k</sup>* mice, which bear C4- $\gamma_2$  and nevertheless have been typed as H-2.7 positive by in vivo absorption of anti-H-2.7 (2, 3).

*The Alloantiserum to H-2.7 Immunoprecipitates C4 Molecules from Mouse Plasma.* EDTA-plasma from H-2.7-positive (SJL) and -negative (DBA/2J) mice was subjected to molecular-sieve chromatography on Sephadex G-200, and the fractions assayed for the presence of H-2.7 specificity by inhibition of hemagglutination, as described in Materials and Methods. Strong activity was found only in the ascending limb of the second protein peak originating from SJL mice, which also contains most of the C4 molecules. The positive tubes were pooled, radiolabeled and immunoprecipitated with rabbit antisera to C4 as well as with alloantiserum to H-2.7. Another portion was incubated with C $\bar{1}$ s before immunoprecipitation. The immune complexes, eluted from Staph A, were subjected to SDS-PAGE, or to IEF under denaturing conditions, followed by radioautography. Fig. 1 demonstrates that the antiserum to H-2.7 immunoprecipitates from SJL plasma fraction three polypeptide chains with molec-

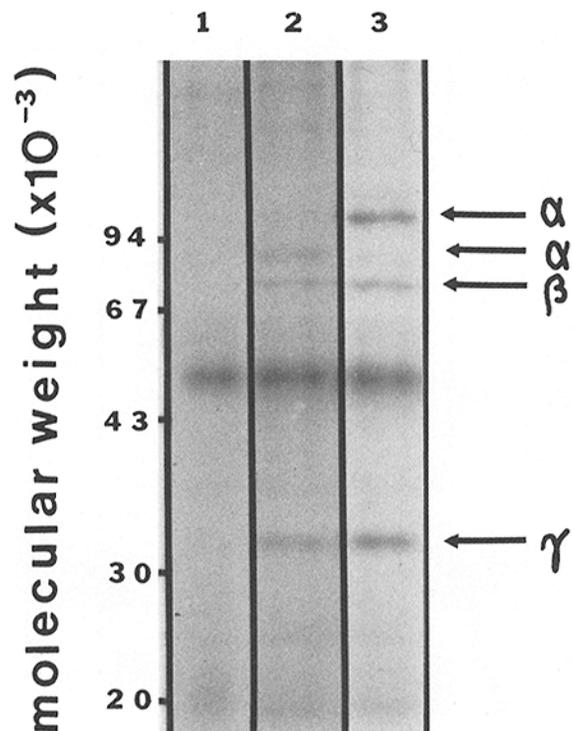


FIG. 1. Radioautogram of a 7-15% SDS-PAGE linear gradient of H-2.7-positive fractions obtained after passage of SJL EDTA-plasma through a Sephadex G-200 column, and immunoprecipitated with anti-H-2.7 alloantiserum. This experiment is representative of results obtained with EDTA-plasma from strains carrying the s, p, j, and f alleles at the *S* region of the H-2 complex. Track 1, immunoprecipitation with normal mouse serum. Tracks 2 and 3, immunoprecipitation with anti-H-2.7. The material loaded in track 2 was treated with C $\bar{1}$ s before immunoprecipitation. C $\bar{1}$ s cleaved a small fragment from the  $\alpha$ -chain. The comparison between tracks 1 and 3 shows that the alloantiserum immunoprecipitated C4 from SJL plasma ( $\alpha$ -,  $\beta$ -, and  $\gamma$ -chains with 98,000, 77,000 and 34,000 mol wt, respectively). Track 2 shows that the alloantiserum also immunoprecipitated the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -chains of C4b.

ular weights identical to those of the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -chains of C4 (Fig. 1 track 3) (19). The  $\alpha$ -chain is cleaved by C $\bar{1}$ s, and a fragment with a molecular weight identical to the  $\alpha'$ -chain of C4 is generated (Fig. 1 track 2). Under nonreducing conditions, anti-H-2.7 immunoprecipitates a single molecule with an apparent 205,000 mol wt (data not shown).

As expected from the results shown in Table I, the C4 molecules immunoprecipitated by anti-H-2.7 contain  $\gamma$ -chains of the  $\gamma_1$ -type as determined by IEF (Fig. 2).

*Identification of the C4 Fragment Which Bears the H-2.7 Specificity.* The experiments described indicate that, with one exception, the H-2.7 specificity is found only on C4 (or C4b) molecules bearing  $\gamma$ -chains with isoelectric points (pI) = 7.4 ( $\gamma_1$ ). To determine whether the  $\gamma_1$ -chains themselves bear the alloantigenic determinant, we analyzed the C4c and C4d fragments of C4b that were generated from its interaction with C4b-inactivator and C4-binding protein. The purified C4c fragments were obtained from serum of MP mice (H-2.7 positive) by the procedure described

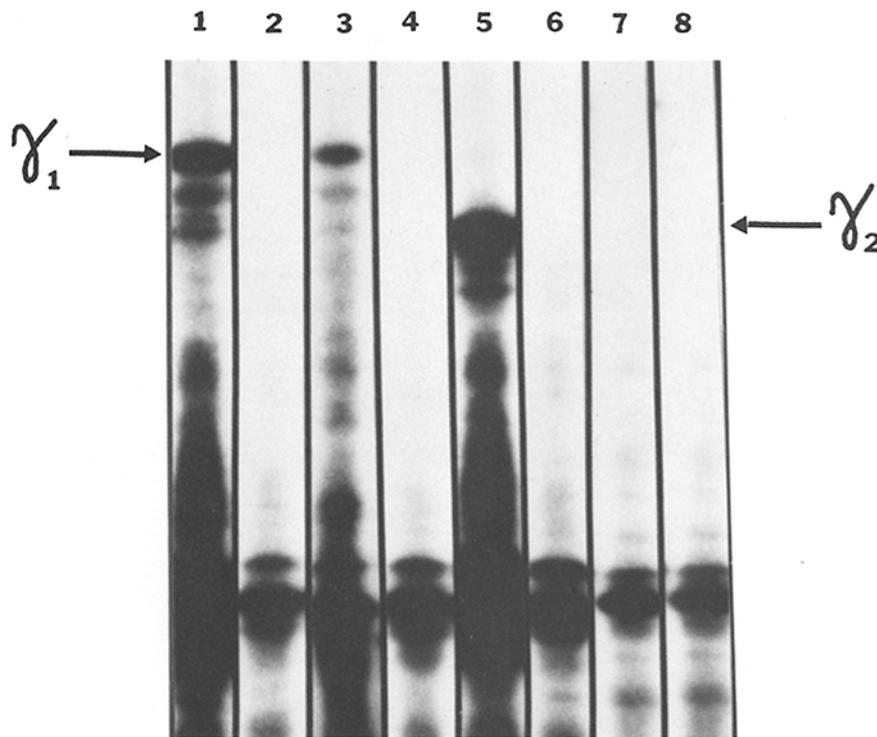


FIG. 2. IEF radioautogram of mouse C4. C4-positive plasma fractions obtained after passage of EDTA-plasma through a Sephadex G-200 column were immunoprecipitated, denatured, and focused at 500 V for 16 h in a vertical slab IEF acrylamide gel containing 9.5 M urea and 2% Ampholine, with an effective pH gradient between 4.9 and 7.9. Tracks 1-4 show the results obtained with SJL strain (representative of C4- $\gamma_1$ , H-2.7-positive strains) and tracks 5-8 show results obtained with DBA/2J strain (representative of C4- $\gamma_2$ , H-2.7-negative strains). Immunoprecipitation was performed with rabbit anti-mouse C4 (tracks 1 and 5), normal rabbit serum (tracks 2 and 6), mouse anti-H-2.7 (tracks 3 and 7), and normal mouse serum (tracks 4 and 8). The heteroantiserum immunoprecipitates both C4- $\gamma_1$  and C4- $\gamma_2$  (tracks 1 and 5). Anti-H-2.7 immunoprecipitates C4 of the  $\gamma_1$  type (track 3) but not C4 of the  $\gamma_2$  type (track 6). The additional specific bands in tracks 1, 3, and 5 are  $\alpha$ -chain fragments (18).

elsewhere (11). Although they contained intact  $\gamma$ - and  $\beta$ -chains and fragments of the  $\alpha$ -chain ( $\alpha_3$  and  $\alpha_4$  peptides) they were H-2.7 negative by two criteria: they were inactive in the inhibition of hemagglutination assay at concentrations close to those found in normal serum, and could not be immunoprecipitated by anti-H-2.7.

These results suggested that the H-2.7 antigenic determinant is not associated with C4c, but instead is associated with C4d. To clarify this question we subjected mouse serum from SJL (H-2.7 positive) and DBA/2J (H-2.7 negative) mice to chromatography on Sephadex G-200 and assayed for the presence of H-2.7 by inhibition of hemagglutination. (In mouse serum, C4 is readily activated and fragmented into C4c and C4d unless special precautions are taken [19].) In contrast to the results obtained after fractionation of EDTA-plasma in Sephadex G-200, strong H-2.7 activity was found only in the third OD peak. The positive tubes were pooled, radiolabeled, and immunoprecipitated separately with anti-H-2.7 and anti-C4.

The results of the radioautographs after SDS-PAGE are shown in Fig. 3. A single specific band of apparent 45,000 mol wt was revealed by immunoprecipitation with both anti-H-2.7 and anti-C4 antisera (Fig. 3, tracks 2 and 3). That it was the same polypeptide that was recognized by anti-C4 and anti-H-2.7 was demonstrated by sequential immunoprecipitation. First, the radiolabeled material was incubated with antisera to C4 and C4c in antibody excess, or with normal rabbit serum, and the

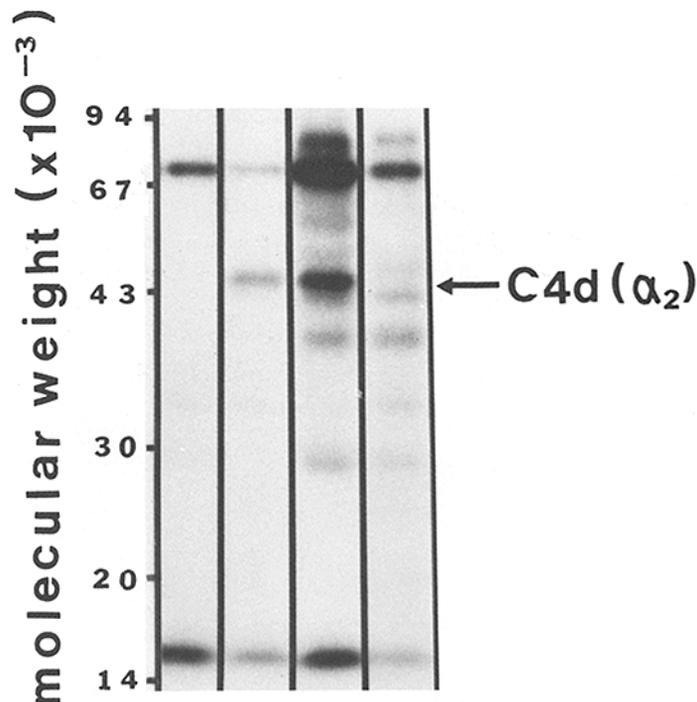


FIG. 3. Radioautogram of a 7-15% SDS-PAGE linear gradient of partially purified mouse C4d ( $\alpha_2$ ) fragment. Mouse serum from SJL mice (H-2.7 positive) was passed through a Sephadex G-200 column in the presence of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  ions. The third protein peak was radiolabeled and immunoprecipitated with normal mouse serum (track 1), mouse anti-mouse H-2.7 (track 2), rabbit anti-mouse C4 (track 3), and normal rabbit serum (track 4). A 45,000-dalton fragment was specifically immunoprecipitated by both anti-C4 and anti-H-2.7 (C4d or  $\alpha_2$ ).

immune complexes were removed with Staph A by centrifugation. Next, the supernates were incubated with alloantibodies to H-2.7, and again treated as above. The immune complexes eluted from the bacteria were subjected to SDS-PAGE and radioautography. We found that pretreatment of the sample with anti-C4, but not with anti-C4c or normal rabbit serum, removed the specific band immunoprecipitated by anti-H-2.7 (data not shown).

It should be pointed out that, as expected, the C4d fragment from H-2.7 negative mice was immunoprecipitated by anti-C4, but not by anti-H-2.7. Table II summarizes the results of the immunoprecipitation with various antisera of C4 and C4 fragments from H-2.7 positive and negative mice.

*Absence of Trans Effects in the Expression of H-2.7 Determinants.* Some of the variations observed in the structure of C4 could be post-translational in nature. For example, as suggested by Huang et al. (20), the H-2.7 antigenic determinants might be generated perhaps after cleavage of C4 chains by enzymes under S-region control. Therefore it was of interest to search for trans effects in F<sub>1</sub> hybrids between SJL (H-2<sup>s</sup>, H-2.7 positive, C4- $\gamma_1$ ) and DBA/2J (H-2<sup>d</sup>, H-2.7 negative, C4- $\gamma_2$ ). No such effects were observed. As shown in Fig. 4, track 2, although both types of C4 ( $\gamma_1$  and  $\gamma_2$ ) can be immunoprecipitated from the serum of these mice by the heteroantiserum, only the C4- $\gamma_1$  molecules were H-2.7 positive (Fig. 4, track 3).

*Presence of the H-2.7 Antigenic Determinants and of  $\gamma_2$ -Type Chains in C4 from Mice Bearing H-2<sup>k</sup>.* Mice bearing the k allele in the S region have unusually low levels of circulating C4, as shown by hemolytic titrations (19) or by rocket immunoelectrophoresis (11) using heteroantisera. The C4-low mice can be shown to bear H-2.7 by specific absorption of the alloantiserum in vivo (2, 3). Direct hemagglutination reactions are usually negative, and very low H-2.7 activity is found in their serum by the hemagglutination-inhibition tests. We show here that C4 molecules from the serum of these mice are exceptional in that they bear H-2.7 alloantigen as well as the  $\gamma_2$ -type chains. C4 from B10.HTT (S<sup>k</sup>) mice was specifically immunoprecipitated by anti-H-2.7. As shown in IEF, the  $\gamma$ -chains were of the  $\gamma_2$ -type (Fig. 4, track 5). The

TABLE II  
Summary of Results of Immunoprecipitation of C4 and C4 Fragments from H-2.7-positive and -negative Mice Using Various Specific Antisera

Mouse strains*	Nature of C4 fragment	Immunoprecipitation with antiserum to:		
		C4 (C4c + C4d)‡	C4c‡	H-2.7§
H-2.7 positive	C4 or C4b	Positive	Positive	Positive
	C4c	Positive	Positive	Negative
	C4d	Positive	Negative	Positive
H-2.7 negative	C4 or C4b	Positive	Positive	Negative
	C4c	Positive	Positive	Negative
	C4d	Positive	Negative	Negative

\* Immunoprecipitation of native C4 with the three antisera was carried out with preparations from many strains of mice bearing the different alleles in the S region as shown in Table I. Immunoprecipitation of C4b, C4c, and C4d was carried out in preparations originating from SJL, MP (H-2.7 positive), and DBA/2J (H-2.7 negative) mice.

‡ Heteroantisera (9, 11).

§ Alloantiserum (Materials and Methods).

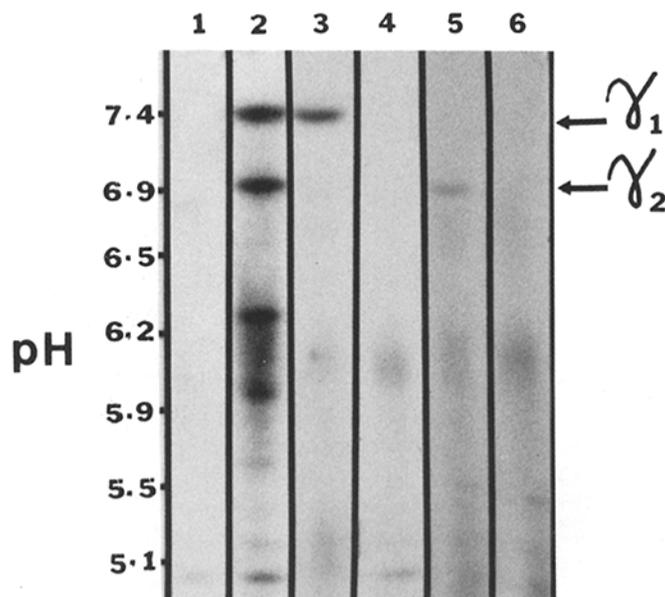


FIG. 4. IEF radioautogram of mouse C4. Experimental conditions as in Fig. 2. Tracks 1-4 show the results obtained with C4 from an F<sub>1</sub> hybrid between SJL (*S<sup>a</sup>*, H-2.7 positive, C4- $\gamma_1$ ) and DBA/2J (*S<sup>d</sup>*, H-2.7 positive, C4- $\gamma_2$ ). Tracks 5 and 6 show results obtained with B10.HTT (*S<sup>a</sup>*, C4- $\gamma_2$ , H-2.7 positive). Track 1, immunoprecipitation with normal rabbit serum, track 2 with rabbit anti-C4, tracks 3 and 5 with mouse anti-H-2.7, and tracks 4 and 6 with normal mouse serum. Both  $\gamma_1$  and  $\gamma_2$  types of C4 were immunoprecipitated from the serum of the F<sub>1</sub> hybrid by the heteroantiserum, but only C4- $\gamma_1$  bears the H-2.7 alloantigen. (This is shown by the comparison between tracks 2 and 3.) The specific bands focusing between pH 6.2 and 5.9 represent degradation products of the  $\alpha$ -chain. This figure also shows that C4 from B10.HTT mice bear  $\alpha_2$  chains (track 5). The difference in intensity between the  $\gamma$ -chain bands in track 5 and those present in tracks 2 and 3 is expected, because *S<sup>a</sup>* mice have very low levels of C4.

apparent molecular weights of the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -chains were identical to those of C4 from other strains of mice (Fig. 5, tracks 2 and 3), but as expected, the intensity of the bands was much weaker.

### Discussion

We show here that the H-2.7 alloantigenic determinant is present on a fragment of C4. This conclusion is based on several lines of evidence. In the absence of complement activation, the H-2.7 alloantiserum identifies a plasma molecule with the structural and functional characteristics of C4, as indicated by the molecular weight of its three disulfide-linked polypeptide chains ( $\alpha$ ,  $\beta$ , and  $\gamma$ ), by the pI of its  $\gamma$ -chain, and by the C $\bar{1}$ s sensitivity of its  $\alpha$ -chain.

In serum, the H-2.7 specificity is mainly associated with a smaller peptide (4). We have identified this peptide (45,000 mol wt) as a fragment of the  $\alpha$ -chain of C4 (C4d or  $\alpha_2$ ): it could be immunoprecipitated by a heteroantiserum to mouse C4, but not to mouse C4c. Conversely, purified C4c from H-2.7-positive mice could not be immunoprecipitated by the alloantiserum. Also, a peptide with an identical molecular weight on SDS-PAGE was generated from partially purified mouse C4 from treatment with purified human C $\bar{1}$ s and the mouse control proteins C4b-inactivator and C4-

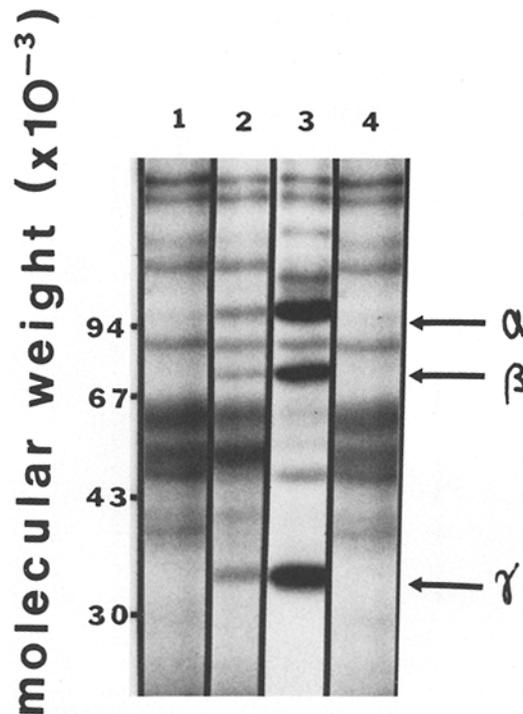


FIG. 5. Radioautograms of 7-15% SDS-PAGE slab gels. Experimental conditions were identical to those described in the legend of Fig. 1. Tracks 1 and 2 show results from immunoprecipitation of C4-positive fractions from B10.HTT ( $S^k$ , C4-low, H-2.7 positive). Tracks 3 and 4, SJL ( $S^s$ , C4-high, H-2.7 positive). Tracks 2 and 3 originate from immunoprecipitation with anti-H-2.7 and tracks 1 and 4 are controls with normal mouse serum. The apparent molecular weights of the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -chains of C4 from  $S^k$  mice and  $S^s$  mice are identical and in agreement with previous findings (19).

binding protein (data not shown). Huang et al. (20) and Huang and Klein (21) found that the H-2.7 alloantigen can be passively incorporated in vitro and in vivo onto H-2.7-negative cells. This observation is substantiated by our findings, because the C4d fragment, which bears the H-2.7 alloantigen, is known to mediate the binding to cell membranes of the C4b molecules generated following complement activation (22). As indicated in Table I (with one exception, to be discussed below), C4 from different mouse strains can be either H-2.7 positive and  $\gamma_1$ -bearing, or H-2.7 negative and  $\gamma_2$ - or  $\gamma_3$ -bearing. These variations in the pI of the  $\gamma$ -chain and in the expression of H-2.7 antigenic determinant are independent of each other, because the C4d fragment that expresses H-2.7 originates from the  $\alpha$ -chain. It is difficult to explain these multiple structural variations on the basis of a single locus with multiple alleles. It could be argued that some of the  $\alpha$ - or  $\gamma$ -chain modifications result from post-translational events such as, for example, glycosylation. However, we found no evidence of trans genetic effects, which could be expected if an enzyme controlled by the  $S$  region was involved in the generation of the C4 variants (Fig. 4). Indeed,  $F_1$  hybrids between mice bearing H-2.7 positive, C4- $\gamma_1$ , and H-2.7-negative, C4- $\gamma_2$ , had in their serum the same two kinds of C4 ( $\gamma_1$  and  $\gamma_2$ ), only one expressing H-2.7 on its  $\alpha$ -chain ( $\gamma_1$ ) with no evidence of recombination of markers.

Carroll and Capra (23) recently reported structural differences in the  $\beta$ -chain of C4

from mice with the d, s, and p haplotypes, as compared to C4 originating from strains with the b and k haplotypes. Perhaps the C4 variants are complex allotypes, similarly to what has been reported for the products of other loci within the major histocompatibility complex (24).

Another problem concerns the multiple anomalies associated with the  $S^k$ . Mice bearing  $S^k$  have been identified as H-2.7 positive on the basis of the specific absorption of the alloantiserum in vivo. However, by hemagglutination or inhibition-of-hemagglutination assays, the alloantigen could not usually be detected on their erythrocytes or serum (2, 3). The most likely explanation for these contradictory findings resides in the very low levels of C4 found in the serum of these mice. As shown in Fig. 5, we could demonstrate the presence of the alloantigen on their C4 by means of a sensitive immunoprecipitation technique.

Unexpectedly, however, the C4 molecule of  $S^k$  mice is associated with  $\gamma_2$  chains, whereas all other H-2.7-positive strains bear  $\gamma_1$  (Table I). Possible reasons for this unusual combination of markers within the C4 molecule are under investigation in our laboratory. Further advances will depend on the clarification of the biochemical basis for the polymorphisms of C4. One interesting possibility is that the structural gene for C4 in  $S^k$  mice originated from an intracistronic recombination between genes for C4- $\gamma_1$  and C4- $\gamma_2$  and that this event is related to the C4-deficiency observed in these animals.

Finally, on the basis of the present results and the fact that the *S* and *G* regions cannot be separated by recombination (4), the existence of an independent *G* region is not substantiated.

### Summary

The *S* region of *H-2* controls a polymorphism of the  $\gamma$ -chain of C4 ( $\gamma_1$ ,  $\gamma_2$ , and  $\gamma_3$ ) as shown by differences in their isoelectric points. The *G* region of *H-2* was defined by the presence of an alloantigen (H-2.7) on erythrocytes and serum. We found that antisera to H-2.7 immunoprecipitated C4 and no other protein from mouse EDTA-plasma. Furthermore, all H-2.7-positive strains bear C4- $\gamma_1$ , and conversely, H-2.7-negative mice bear C4- $\gamma_2$  or  $\gamma_3$  (with one exception; see below). The H-2.7 specificity resides on C4d, a 45,000-mol wt fragment generated from the cleavage of the  $\alpha'$ -chain of C4b by serum control proteins. Because the C4d fragment bears the labile binding site of C4 for cell membranes, it is likely that the erythrocyte alloantigen is acquired from serum as a result of the activation of C4. On the basis of these findings, the existence of a separate *G* locus is unlikely.

Our results also show that C4- $\gamma_1$  and C4- $\gamma_2$  differ from each other at least in their  $\alpha$ - and  $\gamma$ -chains, and may represent complex allotypes. No trans effects were observed in  $F_1$  hybrids between H-2.7-positive and -negative mice.

Mice that bear the *k* allele in the *S* region are exceptional in two respects: they are C4-deficient and their C4 molecules bear  $\gamma_2$  chains and the H-2.7 alloantigen. Perhaps the low levels of C4 are a consequence of the genetic event leading to this unusual  $\alpha$ - $\gamma$ -chain combination.

We wish to thank Dr. Shumpei Yokota for hemagglutination tests, M. D. Eichinger for excellent technical work, and J. Joseph for manuscript preparation.

*Received for publication 14 February 1980.*

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