Serologic analyses of antisera raised in mouse strain combinations differing at the major histocompatibility complex (H-2) indicate that each H-2 haplotype is characterized by numerous antigenic specificities. It has been proposed that the data are most easily explained in terms of two tightly linked loci, H-2K and H-2D (1-3). This "two-gene" model implies that the product of each H-2K or H-2D region bears several alloantigenic specificities. Antigenic specificities unique for a single H-2K or H-2D region, or regions presumed to be of a common origin, have been designated "private" specificities, while antigenic specificities shared between different regions or different alleles of a region have been designated "public" specificities. The molecules determined by the H-2K and H-2D region are glycoproteins of an apparent mol wt of 45,000 daltons. Cullen et al. (4) analyzed the soluble H-2 molecules by coprecipitation with various antisera and demonstrated that, in the combinations tested, each H-2K or H-2D product bore private and public specificities. This was also demonstrated by lymphocyte redistribution studies in several strains (5, 6). After reviewing the data for and against the two-gene model, Klein noted that the great preponderance of data supported the model (7). Recently, however, co-capping (8) and immunoprecipitation techniques (9) have suggested that antisera to private and antisera to public specificities detect two separable molecules associated with the D region of the H-2d haplotype.

An additional approach to the question of the number of genes associated with the Dd region recently became possible through the discovery of the BALB/c, H-2 loss mutant, designated BALB/c-H-2db (10). Skin from BALB/c was quickly rejected by the mutant (11-12 days), but grafts in the opposite direction survived indefinitely. No serological differences were detectable between wild type and mutant with the notable exception that the mutant did not react with one antiserum detecting public H-2 specificities (11). However another antiserum presumably of similar specificity reacted equally well with both strains. Skin graft complementation studies localized the antigenic loss of the BALB/c-H-2db mutant to the D end of its H-2 haplotype (11). Since the mutation occurred in an H-2d strain and since recent precipitation studies from this laboratory (9)
indicated that the $D^d$ region had two antigenically separable gene products, we have isolated the H-2 products of BALB/c-$H-2^{db}$ and compared them to those of BALB/c.

We report here that the $H-2$-associated mutant BALB/c-$H-2^{db}$ is missing an antigenic specificity(ies) on an additional molecule chemically separable from the molecules bearing the private H-2 specificities. The gene determining this additional molecule maps within or close to the $D$ region and is therefore genetically separable from the $K$, $I$, $S$, $G$, $Qa-2$, and $Tla$ regions. The implications for the chromosomal fine structure and two-gene model of the major histocompatibility complex are discussed.

Materials and Methods

Mice. The BALB/c and BALB/c-$H-2^{db}$ mice were of the subline Kh from the colony of H. I. Kohn, and the B6.K1 and B6.K2 mice were from the colony of L. Flaherty. Mice of strains A, A.AL, B6-$H-2^s$, C3H, and C3H.OH were from the colony of D. H. Sachs.

Alloantisera. The immune sera and the strain combinations they were produced in are listed in Table I. The anti-H-2.4(D4) was produced by The Jackson Laboratory, Bar Harbor, Maine, under contract from the National Institute of Allergy and Infectious Diseases, and the other two reagents listed in Table I were produced in this laboratory according to previously published methods (12).

Absorptions of Alloantisera. In vivo absorptions were performed by injecting mice intraperitoneally with 0.5 ml of immune sera, waiting 2 h, and then bleeding from the orbital sinus. In vitro absorptions were carried out as outlined in the legend of Fig. 4.

Cytotoxicity Assay. Cytotoxic testing was done by the two-stage trypan blue assay as previously described (13). Selected newborn rabbit serum, diluted 1:5, was used as a source of complement.

Chemical Analyses. Radiolabeling, solubilization, precipitation, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were all performed by previously described methods (14). Briefly, spleen cells ($5 \times 10^7$/ml) were cultured for 5 h in leucine-free Eagle's minimum essential medium supplemented with glutamine and 200 $\mu$Ci/ml $^3$H-leucine (New England Nuclear, Boston, Mass.). The radiolabeled cells were solubilized with 0.5% Nonidet P-40 (Shell Chemical Company, New York) in 0.01 M Tris-0.15 M NaCl, pH 7.4. The lysate was centrifuged at 100,000 $g$ for 1 h, after which the supernate was applied to a lentil lectin-Sepharose 4B affinity column. The fraction eluted with 0.1 M alpha methyl mannoside was concentrated and stored at $-70^\circ$C until use.

Specific antigens were isolated by indirect precipitation. Radiolabeled antigen was incubated with specific alloantisera, after which an excess amount of protein A-bearing Staphylococcus aureus Cowan I (SaCI) was added to precipitate antigen-antibody complexes. The SaCI-precipitates were washed and the radiolabeled antigens were eluted with 2% SDS and 2% 2-mercaptoethanol. The eluates were analyzed on 10% polyacrylamide SDS gels.

Results

Sequential precipitations were performed to characterize the H-2 molecules associated with the wild-type BALB/c, and the mutant BALB/c-$H-2^{db}$ in terms of their reactivity with antisera to public and private H-2 specificities. Antisera detecting $K^d$ and $D^d$ private specificities (H-2.31 and H-2.4, respectively) and an antiserum known to detect both $K^d$ and $D^d$ public specificities (H-2.6, 27, 28, 29) were used (Table I). These antisera were made in strain combinations such that

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1 Abbreviations used in this paper: NMS, normal mouse serum; SaCI, Staphylococcus aureus Cowan I; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
TABLE I

<table>
<thead>
<tr>
<th>Strain combination</th>
<th>Haplotypes involved</th>
<th>Specificity on BALB/c*</th>
</tr>
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<tbody>
<tr>
<td>(B10.AKM × 129) anti-B10.A§</td>
<td>(H-2° × H-2°) anti-H-2*</td>
<td>Private of D region (H-2.4)</td>
</tr>
<tr>
<td>(B10 × A) anti-B10.D2</td>
<td>(H-2° × H-2°) anti-H-2*</td>
<td>Private of K region (H-2.31) and Ia.11 (weak)</td>
</tr>
<tr>
<td>C3H anti-C3H.SW</td>
<td>H-2° anti-H-2*</td>
<td>Public of K and D regions (H-2.6, 27, 28, 29) and Ia.8</td>
</tr>
</tbody>
</table>

* The H-2 alloantigenic specificities present on BALB/c cells which can possibly be detected by each immune serum, as predicted by the H-2 chart.

† Produced by The Jackson Laboratory, Bar Harbor, Maine, under contract from the National Institute of Allergy and Infectious Diseases.

when tested on H-2d strains the antisera to either of the private specificities would not contain antibodies to public specificities and vice versa, as predicted by the H-2 chart.

For the initial precipitation step antigen from BALB/c or the mutant was divided into two aliquots. One aliquot was pretreated with normal mouse serum (NMS) as a control and the other aliquot was pretreated with a mixture of an excess amount of both the antiserum to the private K region specificity and antiserum to the private D region specificity. After incubation, precipitation was carried out with an excess amount of protein A-bearing SaCl. Both supernates were then subdivided into three aliquots and tested for residual antigenic activity in a final precipitation step. The first aliquot was tested with the antiserum to the D region private specificity, the second with the antiserum to the K region private specificity, and the third with the antiserum detecting public specificities of both K and D regions. The products of this final precipitation step were analyzed by SDS-PAGE.

The electrophoretic migration patterns for the sequential precipitation of wild-type antigen, BALB/c, are shown in Fig. 1. In the controls, the anti-H-2.4 and anti-H-2.31 immune sera precipitated the expected H-2 molecules (panels no. 1 and 2, respectively), as did the antiserum to public specificities H-2.6, 27, 28, 29 (panel no. 3). The depletion of molecules bearing the private D and K region specificities (panels no. 4 and no. 5, respectively) did not remove all molecules reactive with the antiserum to public specificities (panel no. 6). These results indicate that there is a population of molecules which reacts with the antiserum detecting public H-2 specificities, but neither of the antisera to private H-2 specificities. Therefore, these antisera define three antigenically separable molecules associated with BALB/c, all of which have the same apparent mol wt (45,000 daltons) as determined by SDS migration patterns.

Since BALB/c-H-2db carries a loss mutation which apparently occurred in the H-2 complex, it was of interest to determine if the three above described molecules associated with the H-2 haplotype of BALB/c were also detectable in the mutant. The results of the co-precipitation analysis with antigen from BALB/c-H-2db are shown in Fig. 2. In the controls, in which antigen from the mutant was pretreated with NMS, antibodies to the D and K region private specificities also precipitated the expected H-2 molecules (panels no. 1 and no. 2, respectively). However, with BALB/c-H-2db antigen, complete removal of mole-
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FIG. 1. Electrophoretic migration patterns of BALB/c antigen sequentially precipitated with anti-H-2 immune sera (Table I). 3H-leucine-labeled antigen was pretreated by precipitation with the reagent or reagents indicated on the left and the supernates from this first precipitation step were then tested for residual reactivity with the reagent indicated above the panels. The test precipitates were solubilized with SDS and subjected to disk electrophoresis on 10% polyacrylamide gels. Using this system the H-2 molecules migrate to about 4 cm, the Ia molecules migrate between 5 and 7 cm, and a nonspecific running front (RF) appears at about 8 cm of gel. The top panels show that BALB/c antigens were precipitated by antisera to private (panels no. 1 and 2) and public (panel no. 3) specificities. The lower three panels show that while pretreatment with antiserum to the private specificities H-2.4 and 31 removed all molecules bearing the private specificities (panels no. 4 and 5, respectively), the antiserum to public specificities still precipitated some molecules of the same molecular weight (panel no. 6). Therefore, there are three distinct classes of 45,000 mol wt molecules associated with BALB/c, those that react with either of the private specificities (panels no. 1 and 2) and those that react only with the antiserum to public specificities (panel no. 6). As indicated in Table I the antiserum to the private specificity H-2.31 also contains a low titer of anti-Ia.11 antibodies, resulting in the small poorly resolved Ia peak in panels no. 2 and 5. The antiserum to public specificities used in these gels contains anti-Ia.8 antibodies, resulting in the Ia peaks in panels no. 3 and 6.

Cules bearing the private specificities caused concomitant removal of molecules detected by the antiserum to public specificities (Fig. 2, panel no. 6). This is a strikingly different result than was seen with BALB/c (compare panel no. 6 of Fig. 1 and Fig. 2). Therefore, in BALB/c a population of molecules (of 45,000 mol wt) was detected which did not bear either of the private H-2 specificities, whereas in the mutant BALB/c-H-2\textsuperscript{db} there were no detectable molecules of this type.

Genetic Mapping of the Antigenic Loss in the Mutant. When the C3H (H-2\textsuperscript{k}) anti-C3H.SW (H-2\textsuperscript{k}) serum was absorbed in vivo in mutant mice, cytotoxic activity on cells from the mutant was removed, however some of the activity on the wild type, BALB/c, was retained. The retained antibodies were cytotoxic for greater than 80% of BALB/c spleen cells, a reaction like that seen with antibodies to H-2K and H-2D specificities. Therefore, this absorbed antiserum appeared to be specific for an antigen(s) that was lost as a result of the mutation and by inference is specific for a molecule that does not bear private H-2 specificities.

To map the genetic locus coding for the antigen which is absent in the BALB/c-H-2\textsuperscript{db} mutant, recombinant mouse strains were tested. While BALB/c (H-2\textsuperscript{k}) mice were positive with the C3H anti-C3H.SW serum absorbed in the mutant,
Fig. 2. Electrophoretic migration patterns of BALB/c-H-2\textsuperscript{db} antigen sequentially precipitated with anti-H-2 immune sera (Table I). This experiment was carried out identically to the one shown in Fig. 1 except that antigen from the mutant strain rather than the strain of origin was used. The top panels show that BALB/c-H-2\textsuperscript{db} antigens were precipitated by antiserum to private (panels no. 1 and 2) and antiserum to public (panel no. 3) specificities. The lower three panels show that pretreatment with antisera to the private specificities (panels no. 4 and 5, respectively), concomitantly removed H-2 molecules precipitated by the antiserum to public specificities. Therefore only two classes of 45,000 mol wt molecules were detectable with antigen from the mutant BALB/c-H-2\textsuperscript{db}, those that bear either of the private specificities (panels no. 1 and 2). The third class of 45,000 mol wt molecules found in BALB/c that reacted only with the antiserum to public specificities (Fig. 1, panel no. 6) was undetectable in the mutant (Fig. 2, panel no. 6).

C3H (H-2\textsuperscript{k}) mice were negative (as expected). Therefore the $H-2^d/H-2^k$ recombinants C3H.OH and A.AL were informative. As shown in Fig. 3, C3H.OH which is identical with the H-2\textsuperscript{d} haplotype except for its D region which came from H-2\textsuperscript{k}, did not react with absorbed antiserum. The strain A.AL which is identical with the H-2\textsuperscript{k} haplotype except for its D region, which came from H-2\textsuperscript{d}, did react with this absorbed antiserum. Therefore both of these recombinants map the antigenic loss of the mutant in or to the right of the D region.

The C3H and C3H.SW, like most congenic strains differ not only by H-2 genes but also by genes closely linked to H-2. Recently the Qa-1 (15) and Qa-2 (16) regions have been defined by antigens which map to the right of H-2 between the D and Tla regions. To map the antigenic loss of the BALB/c-H-2\textsuperscript{db} mutant relative to these other loci the B6.K1 and B6.K2 recombinants were tested. To assure that only the relevant antigen was being assayed, the C3H anti-C3H.SW serum absorbed in the mutant was then absorbed in vitro with cells from B6, B6.H-2\textsuperscript{k}, B6.K1, and B6.K2 and tested for residual cytotoxicity against BALB/c cells. As shown in Fig. 4 the B6.K1- and B6.K2-recombinant strains have segments of chromosome from B6, A, and B6.H-2\textsuperscript{k}. Cells from strains B6 and A reacted with the antiserum absorbed in the mutant, while cells from B6.H-2\textsuperscript{k} did not. The B6.K1 and B6.K2 strains both derived their Tla region from B6.H-2\textsuperscript{k}, a strain that typed negative with the absorbed antiserum and yet both the B6.K1 and B6.K2 strains typed positive. This result maps the antigenic loss in the BALB/c-H-2\textsuperscript{db} mutant to the left of the Tla region. Differences in a serologically detected antigen, Qa-2, as well as a minor histocompatibility antigen, have been demonstrated between the B6.K1 and B6.K2 strains (16). The Qa-2 region of
Fig. 3. Genetic mapping of the antigenic loss of the BALB/c-H-2k mutant using intra-H-2 recombinant mouse strains. BALB/c mice have the H-2s haplotype and C3H mice have the H-2k haplotype. Both the C3H.OH and A.AL strains arose from recombination between the H-2k haplotype of C3H and the H-2s haplotype of DBA/2. The antiserum absorbed in the mutant mice was cytotoxic for greater than 80% of spleen cells with a titer of 1:16 on strains BALB/c and A.AL. No lysis above background (less than 10%) was seen at a 1:2 dilution on spleen cells from C3H, C3H.OH, and BALB/c-H-2k.

B6.K1 came from B6.H-2k, a strain that was negative with the antiserum absorbed in the mutant and yet B6.K1 was positive. Therefore the antigen present in BALB/c and not the mutant BALB/c-H-2db is not Qa-2 and maps to the left of the Qa-2 region. Because the A and B6 strains both reacted with the antiserum absorbed in the mutant, mapping studies using the B6.K1 and B6.K2 strains cannot genetically map the antigenic loss in the mutant relative to the Qa-1 region. Therefore, combining the results of Figs. 3 and 4, the antigenic loss in BALB/c-H-2db can be mapped into the D-Qa-1 interval.

Discussion

The data presented here compare the H-2 molecules of BALB/c and its coisogenic mutant, BALB/c-H-2db. Sequential precipitation experiments using antisera to private and public H-2 specificities, demonstrated three separable

<table>
<thead>
<tr>
<th>Strain</th>
<th>Reactivity with antiserum absorbed in the mutant</th>
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<tbody>
<tr>
<td>BALB/c</td>
<td>+</td>
</tr>
<tr>
<td>C3H</td>
<td>-</td>
</tr>
<tr>
<td>C3H.OH</td>
<td>-</td>
</tr>
<tr>
<td>A.AL</td>
<td>+</td>
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<table>
<thead>
<tr>
<th>Strain</th>
<th>Reactivity with antiserum absorbed in the mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6</td>
<td>+</td>
</tr>
<tr>
<td>B6.H-2k</td>
<td>-</td>
</tr>
<tr>
<td>A</td>
<td>+</td>
</tr>
<tr>
<td>B6.K1</td>
<td>+</td>
</tr>
<tr>
<td>B6.K2</td>
<td>+</td>
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</table>
MUTATION IN A NEW H-2-ASSOCIATED HISTOCOMPATIBILITY GENE

populations of H-2 molecules associated with BALB/c. Two of the classes of molecules are characterized by their reactivity with antisera to either K or D region private specificities and also antisera to public specificities. These by definition are the classical transplantation antigens, the products of the H-2K and H-2D regions. A third class of molecules does not bear either private specificity but reacts with an antiserum to public specificities. As determined by migration in SDS-PAGE all three populations of H-2 molecules have the same apparent mol wt of 45,000 daltons. These results are consistent with those reported previously which demonstrated two separable products associated with the D end of the H-2d haplotype using co-capping on the lymphocyte membrane (8) and by immunochemical techniques (9).

Sequential precipitations with antigen from the mutant, BALB/c-H-2db, indicated the presence of only two classes of H-2 molecules, those bearing either the H-2K or H-2D private specificity. The third class of molecules found in BALB/c which did not bear private specificities but did react with the antiserum to public specificities, was undetectable in the mutant. This result indicates that there is an antigen which is expressed in the wild-type strain, BALB/c, but not in the mutant, BALB/c-H-2db, and that this antigen is carried on a molecule distinct from the classical transplantation antigens K and D.

Antibodies specific for the molecule undetectable in the mutant were produced by in vivo absorption of the antiserum to public specificities with mutant cells. By inference, these antibodies are specific for alloantigens on the third class of 45,000 mol wt molecules found in the strain of origin but not in the mutant. Using the antiserum absorbed in the mutant and intra-H-2-recombinant strains, the antigenic loss in the mutant was genetically mapped in or to the right of the D region. Recently derived B6-recombinant strains have been used to define the Qa-1 (15) and Qa-2 (16) regions to the right of the H-2 complex, between the D and Tla regions. Using the B6-recombinant strains the antigenic loss in the H-2db mutant was mapped to the left of the Tla and Qa-2 regions. Therefore, the antigen detected in BALB/c but not the mutant is coded for by a gene in the D-Qa-1 interval. Our results confirm and extend the skin graft complementation studies of McKenzie et al. which mapped the mutation of BALB/c-H-2db into or to the right of the D region (11). This agreement in mapping also makes it more likely that the antigenic loss in the mutant detected serologically is on the same molecule as the histocompatibility antigen loss detected by skin grafting.

We tentatively designate the molecule detected in the strain of origin, BALB/c and not the mutant BALB/c-H-2db as D'. This nomenclature is used because no recombinants have been found that separate D' from the D region product carrying the private specificity, and D' is detectable serologically and by immunoprecipitation with conventional anti-H-2 antisera.

There are some data supporting the contention that D' is a histocompatibility antigen similar to the H-2K and H-2D antigens. Like K and D, D' is a molecule of an apparent mol wt of 45,000 daltons. Its affinity for lentil lectin and its detection by incorporation of 3H-leucine indicate that D' is a glycoprotein. Antibodies specific for D' were found to be cytotoxic for greater than 80% of spleen cells, a reaction characteristic of antibodies to K and D and not antibodies to other H-2-associated antigens such as Ia, Ss, G, or TL. It seems likely that the
mutation accounting for the rapid skin graft rejection involves the D' molecule indicating that it is a strong histocompatibility antigen. Of course a second mutation or deletion of multiple genes might also explain the data.

Presumably D' is less polymorphic than the K and D antigens because it was not previously recognized by the strain distribution patterns of H-2 alloantigenic specificities and D' has only been detected because it bears a shared alloantigenic specificity(ies) but lacks a haplotype-specific alloantigen. Since the genes coding for D and D' are tightly linked, antisera produced to detect D region specificities could contain antibodies specific for determinants on D and/or D'. It is therefore unclear which molecule, D or D', or both, bear the public specificities currently assigned to the D region. Further serologic and immunochemical studies utilizing the BALB/c-H-2<sup>th</sup> mutant should help in determining the molecular basis of the D-end specificities. However, recombinants that separate D from D' will be required for accurate assignment of specificities to these molecules. If D' antigens were found to be cross-reactive with either K or D alloantigens, i.e. shared public specificities between D' and K or D, then a structural homology could be implied. Also since considerable chemical homologies have been demonstrated between the partial amino acid sequences of the products of H-2K and H-2D (17-19) it would be informative to sequence D'. Serological and chemical studies are in progress to determine whether D' has structural homologies with D and/or K.

The present data with the BALB/c-H-2<sup>th</sup> mutant suggest that the currently accepted two-gene model for the mouse major histocompatibility complex must be extended at least in certain haplotypes to a "three-gene" model perhaps homologous to the HLA-A, B, and C loci of man (20). Furthermore, the molecular similarities of the products determined by genes mapping between and including H-2 and Tla may indicate multiple duplications of genetic information on this chromosomal segment.

Summary

Sequential precipitations of soluble BALB/c antigen with antisera detecting private and public H-2 specificities indicated three distinct classes of molecules of 45,000 mol wt. However, only two of these classes of molecules were detectable in antigen from the loss mutant, BALB/c-H-2<sup>th</sup>. The class of molecules, detectable in the wild-type strain but missing in the mutant, does not bear private specificities but does react with an antiserum detecting H-2 public specificities. Absorption in mutant mice of the antiserum to public specificities, left antibodies specific for the antigen detectable in BALB/c but not BALB/c-H-2<sup>th</sup>. Genetic mapping studies using this specific antiserum indicated that the antigenic loss of this mutant is in a gene which maps in or close to the H-2D region, separable from the H-2K, S, G, Qu-2, and Tla regions.

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