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Evidence for a Third, *Ir*-Associated Histocompatibility Region in the *H-2* Complex of the Mouse

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Abstract

Skin grafts transplanted from B10.HTT donors onto (A.TL × B10)F_1 recipients are rapidly rejected despite the fact that the B10.HTT and A.TL strains should be carrying the same *H-2* chromosomes and that both the donor and the recipient contain the B10 genome. The rejection is accompanied by a production of cytotoxic antibodies against antigens controlled by the *Ir* region of the *H-2* complex. These unexpected findings are interpreted as evidence for a third histocompatibility locus in the *H-2* complex, *H-2I*, located in the *Ir* region close to *H-2K*. The B10.HTT and A.TL strains are postulated to differ at this hypothetical locus, and the difference between the two strains is explained as resulting from a crossing over between the $H\text{-}2^{tl}$ and $H\text{-}2^{s}$ chromosomes in the early history of the B10.HTT strain. The *H-2* genotypes of the B10.HTT and A.TL strains are assumed to be $H\text{-}2K^{s}Ir^{s}/^{k}Ss^{k}H\text{-}2D^{d}$ and $H\text{-}2K^{s}Ir^{k}Ss^{k}H\text{-}2D^{d}$, respectively. Thus, the *H-2* chromosomes of the two strains differ only in a portion of the *Ir* region, including the *H-2I* locus. The B10.HTT($H\text{-}2^{tt}$) and B10.S(7R)($H\text{-}2^{th}$) strains differ in a relatively minor histocompatibility locus, possibly residing in the *Tla* region outside of the *H-2* complex.

Introduction

The *H-2* system occupies a relatively small but extremely complex segment of mouse chromosome 17 (linkage group IX). This system controls antigens on various body tissues detectable by serological methods (Gorer 1938); rejection of neoplastic (Gorer 1938) and normal (Amos et al. 1954) tissue transplants; graft-versus-host reaction (Simonsen and Jensen 1959); mixed lymphocyte reaction (Dutton 1966); production of serum proteins (Shreffler and Owen 1963, Passmore and Shreffler 1970); resistance to oncogenic viruses (Lilly et al. 1964); immune response to a

variety of antigens (McDevitt and Chinitz 1969); cell-mediated lymphocytotoxicity in vitro (Brunner et al. 1970); antigens of restricted tissue distribution (Hauptfeld et al. 1973, David et al. 1973); cooperation between thymus and bone marrow-derived lymphocytes in antibody response (Katz et al. 1973), as well as other less well-defined functions. Genetically, the *H-2* complex consists of a large number of loci that are presently grouped into four regions, *H-2K, Ir, Ss,* and *H-2D*. The role of the individual regions in the different *H-2*-associated functions is currently under investigation in several laboratories. We are engaged in a study of the contribution of the different *H-2* regions to skin graft rejection (Klein and Shreffler 1972, Klein 1972 and this communication). During these studies we were confronted with a paradoxical situation in which mice carrying no known histocompatibility difference rapidly rejected each other's skin grafts. We explain this situation by the occurrence of a previously unsuspected genetic recombinational event within the *H-2* complex and by the existence of a third, previously unidentified histocompatibility (*H*) locus in the *H-2* complex.

Materials and Methods

Mice. The following strains were used: B10.A(*H-2*^{a}), B10.D2(*H-2*^{d}), B10.A(5R) (*H-2*$^{i\text{-}2Sg}$), B10.S(7R)(*H-2*th), B10.HTT(*H-2*^{tt}; see Results and Discussion), A.AL(H-2^{al}), A.TL(H-2^{tl}), and A.TH(H-2th). The latter three strains were kindly provided to us by Dr. D. C. Shreffler, Department of Human Genetics, The University of Michigan Medical School; all other strains were bred in our own colonies at the University of Michigan.

Skin Grafting. The skin grafting technique employed has been previously described (Klein and Bailey 1971). In one group, the grafts were taken from the ear, in all other groups we used skin from the tail. The grafts were placed in the dorsolateral region of the recipient's trunk. Bandages were removed between seven and nine days after grafting, and the grafts were inspected daily for the first four weeks and once a week thereafter. The recipient and the donor were always of the same sex, not younger than three months and not older than six months at the time of the grafting.

Cytotoxic Test. The two-stage microcytotoxicity test was carried out as described by Amos and his coworkers (1969) with minor modifications. Briefly, twofold serial dilutions of the antiserum were made in Hanks balanced salt solution (HBSS) containing 0.5% bovine serum albumin (BSA). The same medium was used to prepare the suspensions of lymph node lymphocytes (target cells). For testing, 2 μl. of the antiserum and 2 μl. of the target cells at a concentration of 3×10^6/ml were dispensed into plastic microtitre plates (Falcon Plastics, Los Angeles, Ca.) with a Hamilton syringe (Hamilton Co., Inc., Whittier, Ca.). After mixing, the plates were incubated for 20 min at room temperature. The wells were then filled with HBSS and the plates incubated for another 10 min at room temperature. After flicking off the fluid, 2 μl. of complement were added to each well and the plates were incubated for 30 min at 37°C. The reaction was then arrested by cooling at 5°C for 5 min, and the cells were fixed by addition of 2% formaldehyde in HBSS. Cells were transferred to hemocytometers and counted under a phase contrast microscope. Commercially available normal guinea pig serum diluted 1:8 was used as a source of complement (obtained from Grand Island Biological Co., New York, N.Y.).

Results

Background Information. In previous studies (Dĕmant et al. 1971, Klein and Shreffler 1972) it was found that rejection of skin grafts across *H-2* barriers is primarily the function of the peripheral *H-2K* and *H-2D* regions. A considerable portion of the central *H-2* regions, particularly the *Ss* region and the region between *Ss* and *H-2D*, does not appear to be involved in skin graft rejection at all. However, the strain combinations used in these studies did not allow any firm conclusions to be drawn about the involvement of the *Ir* region in skin graft rejection. For this reason, we began an analysis of *H-2* recombinants differing in this region. Two such recombinants, *H-2*th and *H-2*^{tl}, were produced by Stimpfling (Stimpfling and Reichert 1970) and Shreffler (Shreffler and David 1972), respectively. The postulated genetic origin of these recombinants is shown in Fig. 1. The *H-2*th chromosome was transferred by Stimpfling onto the C57BL/10 (abbreviated B10) strain background (congenic line B10.S(7R), abbreviated 7R), and by Shreffler onto the A strain background (congenic line A.TH). The *H-2*^{tl} chromosome was first maintained in an outbred HTT stock, which was then used by P. Iványi and P. Démant (personal communication) for the development of the B10.HTT congenic line. The original *H-2*^{tl} chromosome was also transferred by Shreffler onto the A strain background (congenic line A.TL). Before the establishment of the HTT stock, the *H-2*^{tl} chromosome was maintained for two generations in a heterozygous state with *H-2*^{s}. The A.TL line was established by backcrossing to A.CA(*H-2*^{f}, cf. Meo et al. 1973b).

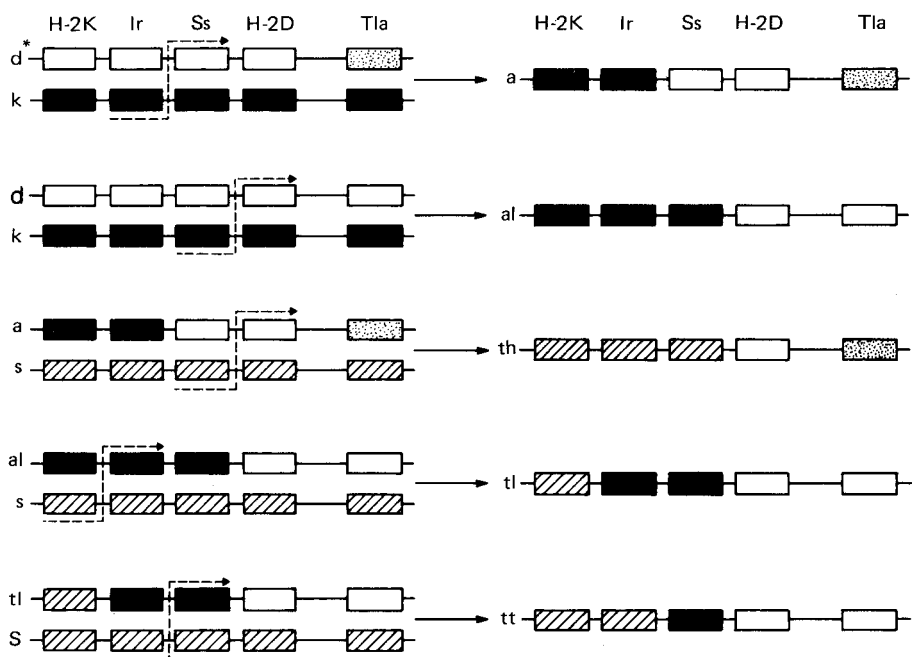

Fig. 1. Postulated Origin of Relevant *H-2* Recombinant Chromosomes.

Skin Grafting Between 7R and B10.HTT. Skin grafts exchanged between the B10.HTT and 7R strains show a peculiar histoincompatibility (Table 1). Some grafts are rejected relatively early after transplantation (between 17 and 30 days); others survive for more than 100 days. The proportion of the early rejected grafts is higher in females than in males and higher in the direction 7R→B10.HTT than in the opposite direction. A similar rejection pattern was described earlier for the histocompatibility locus associated with the *Tla* region (Boyse et al. 1972, Snell et al. 1971) located outside of the *H-2* complex near the *H-2D* end. We wondered, therefore, whether the B10.HTT and 7R strains might differ at this locus. The origin of the B10.HTT and 7R *H-2* chromosomes allows this theoretical possibility (see Fig. 1, and the Discussion). To test this hypothesis, we raised three types of F_1 hybrids–(B10.HTT × 5R)F_1, (7R × B10.D2)F_1, and (HTT × B10.A)F_1–in which the presumed *Tla* difference is "covered" (complemented) by the *H-2* chromosome of the second parent in the F_1 hybrid. The 5R and B10.A strains carry the *Tla* region derived from the $H\text{-}2^a$ chromosome, as does the 7R strain, and both the B10.D2 and the B10.HTT strains carry the *Tla* region of $H\text{-}2^d$. In one combination, (7R→(B10.HTT × 5R)F_1), the grafts were still rejected in the same pattern as in the 7R→B10.HTT combination; in the other two combinations, [B10.HTT→ (7R × B10.D2)F_1 and 7R→(B10.HTT × B10.A)F_1], the early rejection did not occur. Thus, this experiment did not provide *unequivocal* evidence for the involvement of the *Tla* region in the 7R→B10.HTT graft rejection.

For comparison, grafts were exchanged between A.TH and A.TL mice (since we did not have enough of these mice, we used their F_1 hybrids with the B10 strain instead). We expected the same rejection pattern as in the 7R↔B10.HTT combinations since the 7R and A.TH and the B10.HTT and A.TL strains were thought to carry the same *H-2* chromosomes respectively. To our great surprise, the grafts were promptly rejected (Table 1).

F_1 Tests Involving H-2 Chromosomes al, th, tl, tt, and s. To further explore the unexpectedly rapid rejections, we obtained a series of F_1 hybrids and grafted them with 7R, B10.HTT and A.TL skin (Table 2).

The (A.AL × B10.S)F_1 hybrids showed no early rejections of either A.TL or B10.HTT grafts, indicating that whatever the difference between the *H-2* chromosomes of the two donor strains, it was not an antigen that these F_1 hybrids lacked. Furthermore, since the F_1 recipients carried the *H-2* chromosomes from which the A.TL and B10.HTT recombinant chromosomes were originally derived, the results also suggested that no other *H-2* chromosome beside *al* or *s* could have contributed the antigen in which the two donor strains differed.

The (A.TH × B10)F_1 hybrids accepted grafts from both B10.HTT and 7R donors (all except one survived for more than 100 days). In sharp contrast, the (A.TL × B10)F_1 hybrids promptly rejected all grafts from these two donor strains. These results clearly indicate that the *H-2* chromosomes of the A.TL and B10.HTT strains, originally believed to be identical, are actually different and that this difference can cause rapid skin graft rejection. Due to the nature of the F_1 test, it is unlikely that the early rejections were due to the difference in the genetic backgrounds of the strains involved. The same difference was apparently also responsible for the rejection of 7R grafts by the (A.TL × B10)F_1 hybrids but was not responsible for the rejection of grafts exchanged between the 7R and B10.HTT strains.

Table 1. Fate of Skin Grafts Transplanted Between Congenic Lines Carrying *H-2*th, *H-2*^{tl}, and *H-2*^{tt} Chromosomes

Donor	Recipient	H-2 Difference	Grafts rejected at day	Grafts healthy at day
HTT	7R ♀	Ss, Tla?	17, 18	100, 100, 60, 60, 40
	♂		55	100, 100, 55
7R	HTT ♀	Ss, Tla?	21, 22, 22, 22, 24, 28	
	♂		18, 20, 35, 50	100, 100, 100, 100, 100
7R	(HTT × 5R)F_1 ♀	Ss	12, 14, 15, 16, 19, 21, 23, 23, 45	60, 90, 90, 90, 90, 90, 90, 100, 100, 100, 100
	♂		11, 19	90, 90, 90, 90, 90, 90, 90, 100, 100, 100, 100,100
HTT	(7R × B10.D2)F_1 ♀	Ss		70
	♂			100, 100, 100
7R	(HTT × B10.A)F_1 ♀	Ss	23	50, 30, 30, 30, 30, 30, 30, 30
	♂		17?	50, 30, 30, 30, 30, 30, 30, 30
(A.TL × B10)F_1	(A.TH × B10)F_1 ♂	Ir, Ss, Tla?	16, 18, 20, 20, 20, 21, 70	80, 80
(A.TH × B10)F_1	(A.TL × B10)F_1 ♂	Ir, Ss, Tla?	12, 13, 16, 16, 18, 18, 21, 50	

Table 2. Fate of Skin Grafts in F_1 Tests Involving H-2 Chromosomes *al, th, tl, tt,* and *s*

Donor	Recipient	H-2 Difference	Grafts rejected at day	Grafts healthy at day
HTT♀ ♂	(A.TH × B10)F_1	Ss, Tla?	12	100, 100, 100 100, 100, 100
7R♀ ♂	(A.TH × B10)F_1	None		100, 100, 100, 100 100, 100, 100, 100
HTT♀ ♂	(A.TL × B10)F_1	Ir?	11, 11, 11, 11, 12, 13, 14, 15, 15 12, 12, 13, 13	
7R♀ ♂	(A.TL × B10)F_1	Ir, Ss, Tla?	12, 13, 14 11, 13, 13, 14, 14	
A.TL♀ ♂	(A.AL × B10.S)F_1	None		50, 50, 50 50, 50, 50, 50, 50
HTT♀ ♂	(A.AL × B10.S)F_1	None		50, 50, 50 50, 50, 50, 50, 50
7R♀ ♂	(A.AL × B10.S)F_1	Tla?	26, 26 10, 10, 20, 24	50

Serological Analysis. Sixteen days after grafting of the (A.TL X B10)F_1 hybrids with B10.HTT skin, the recipients were bled and their sera tested in the microcytotoxic assay against the lymph node cells of the donor. The tests revealed the presence of antibodies reacting with some 45 to 65% of the B10.HTT lymph node cells (Fig. 2). The antisera also reacted with lymphocytes from the spleen but did not react with thymocytes. A positive reaction with 7R, B10.S, and A.TH lymphocytes indicated that the antibodies were directed against antigens shared by the B10.HTT, 7R, A.TH, and B10.S strains but absent in the A.TL strain. Testing of the antiserum against a series of *H-2* recombinants (results to be published elsewhere) suggested that the antigens detected by this antiserum are controlled by the *Ir* region of the *H-2* complex. In all other characteristics, the (A.TL X B10)F_1 anti-B10.HTT serum resembles other sera obtained in our laboratory and several other laboratories by immunization across *Ir* region differences.

Discussion

It appears that there are two types of histocompatibility differences involved in the skin grafting experiments described above. One difference is relatively strong since

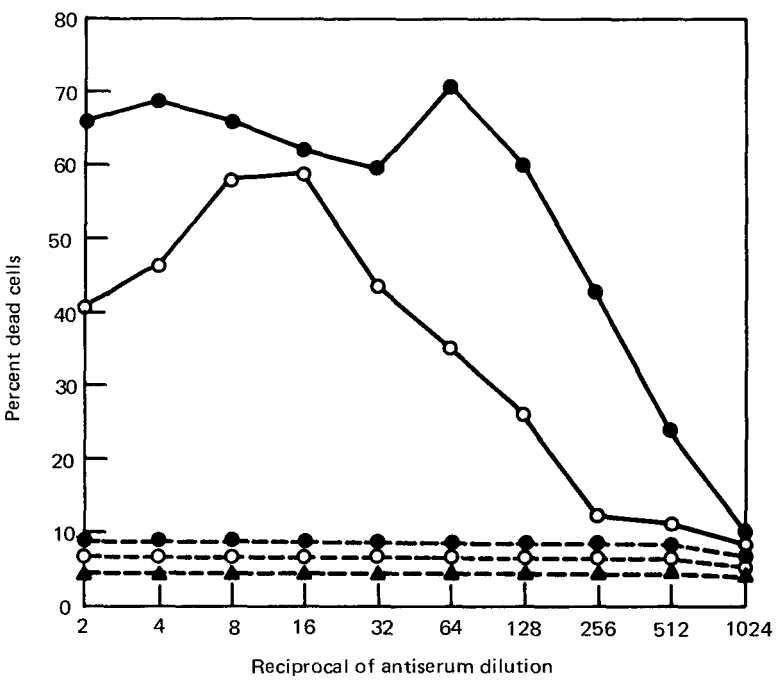

Fig. 2. Reactions in the Microcytotoxicity Test of Antiserum (A.TL X B10)F_1 Anti-B10.HTT with Lymph Node Cells of 7R (●——●), B10.HTT (○——○) (B10 X A.TL)F_1 (▲——▲), and with Thymocytes of 7R (●– – –●) and B10.HTT (○– – –○).

all grafts across this barrier are rejected during the first three weeks after transplantation, by both males and females. The other difference is relatively minor, causing irregular rejections, with some grafts (particularly in females) rejected early, and others (particularly in males) rejected late or not at all.

The strong histoincompatibility is observed when skin grafts are transplanted between the A.TH and A.TL strains, or between the A.TL and B10.HTT strains. The *H* locus responsible for the strong rejection appears to be linked to the *H-2* complex. [The A.TL and A.TH strains have the same genetic background; in the B10.HTT→(A.TL × B10)F_1 combination the background of the donor is contributed by the B10 parent of the F_1 recipient. Thus, the rejections are unlikely to be due to a difference in genes segregating independently of *H-2*.]

At the outset of the experiments we presumed that the *H-2* chromosomes of the B10.HTT and A.TL strains were the same, since both strains were derived from the same recombinant mouse. However, the fact that B10.HTT and A.TL strains reject each other's skin grafts and that this rejection is not due to background differences indicates that the *H-2* chromosomes *are not the same.* After separation of the strains, a genetic change resulting in a strong *H* locus difference must have occurred in one of them. We suggest that the change represented additional crossing over in the HTT stock, while it was still heterozygous for the $H\text{-}2^{tl}$ and $H\text{-}2^{s}$ chromosomes. We assume that the postulated crossing over between the *tl* and *s* chromosomes occurred in the *Ir* region of the *H-2* complex in such a way that the new recombinant chromosome received the *H-2K* region and part of the *Ir* region from the $H\text{-}2^{s}$ chromosome, and the rest of the *H-2* complex from the $H\text{-}2^{tl}$ chromosome (Fig. 1). The genotype of the new chromosome can, therefore, be written as $H\text{-}2K^{s}Ir^{s/k}Ss^{k}H\text{-}2D^{d}$. It should differ from the genotype of the $H\text{-}2^{tl}$ chromosome ($H\text{-}2K^{s}Ir^{k}Ss^{k}D^{d}$) only in a portion of the *Ir* region. The *tl* and recombinant chromosomes should not differ in the *H-2K, Ss,* and *H-2D* regions. This assumption has been confirmed by testing the B10.HTT and the A.TL mice with monospecific anti-*H-2* sera and with anti-*Ss* and anti-*Slp* sera (J. Klein, unpublished data).

The assumption that B10.HTT and A.TL strains differ in the *Ir* region is supported by the serological analysis of the (A.TL × B10)F_1 anti-B10.HTT serum, which reacts as if directed against antigens controlled by the region between *H-2K* and *Ss* (V. Hauptfeld and J. Klein, in preparation). Strong support for this conclusion comes also from *Ir-1* typing of the B10.HTT strain. According to H. O. McDevitt (personal communication), the B10.HTT mice, like those of the B10.S strain, are low responders to (T,G)-A--L, (H,G)-A--L and (Phe,G)-A--L antigens. The A.TL strain, on the other hand, is a low responder to (T,G)-A--L and a high responder to (H,G)-A--L and (Phe,G)-A--L.

All this confirms the hypothesis that the *H-2* chromosome of the B10.HTT strain arose by crossing over between $H\text{-}2^{tl}$ and $H\text{-}2^{s}$ as shown in Fig. 1. Meo and his coworkers (1973b) recently reached the same conclusion on the basis of their observation that A.TH and B10.HTT stimulate each other strongly in MLR. These authors have assigned the symbol $H\text{-}2^{tt}$ to the new *H-2* chromosome of the B10.HTT strain. We have adopted this symbol.

Alternatively, the $H\text{-}2^{tt}$ chromosome may have originated by a mutation in the *Ir* region. However, this interpretation would require that the mutation occurred, by chance, in the direction $Ir^{k}\rightarrow Ir^{s}$. The probability of this happening is extremely small,

particularly if one assumes that the change involved more than one gene.

No matter what the origin of the *H-2*^{tt} chromosome, it is clear that its difference from the *H-2*^{tl} chromosome exerts a relatively strong effect on skin graft rejection and that this difference is located in the *Ir* region. The postulated *H* gene(s), which we designate *H-2I*, is probably located in close proximity to the *H-2K* region within the *H-2* complex.

The contention that the *H-2I* gene is located close to the *H-2K* region is supported by the finding that B10.A(2R) and B10.A(4R) strains, which differ only in the portion of the *Ir* region proximal to *Ss* (Lieberman et al. 1972), are histocompatible (Stimpfling and Reichert 1970, Livnat et al. 1973). In another pair of *Ir* congenic lines, B10.T(6R) and B10.AQR, rapid rejection of skin grafts is known to occur in at least one direction [B10.T(6R)→B10.AQR (cf. Livnat et al. 1973]. This rejection can also be attributed to the *H* locus in the vicinity of *Ir-1*.

The relationship of the *H-2I* locus to the *Ir-1* gene and to the gene(s) responsible for *Ir* region-associated MLR (Bach et al. 1972, Meo et al. 1973a) and GVHR (Klein and Park 1973) is not clear. However, since there is enough room in the *Ir* region for some 500 genes, mapping of two genes in this same chromosomal segment by no means indicates identity of the genes.

On the other hand, the possibility that the *H-2I* locus is identical with the recently discovered *IrR-1* (Hauptfeld et al. 1973) or *Lna* (David et al. 1973) loci must be seriously considered. The antibodies produced by immunization of the (A.TL × B10)F_1 recipients with the B10.HTT tissue seem to be directed against the *IrR-1* (*Lna*) antigens. The fact that such antibodies accompany skin graft rejection suggest that the *IrR-1* (*Lna*) antigens are also present on epithelial cells. However, it is also possible that the antibodies are produced against passenger lymphocytes of the skin graft (Steinmuller and Hart 1971) rather than against the skin itself. Experiments are in progress to distinguish between these two possibilities.

The existence of the *H-2I* gene could explain why skin grafts exchanged between congenic lines differing in the *H-2K* subdivision (*H-2K+Ir*) are rejected more rapidly than those transplanted across the *H-2D* subdivision (Klein 1972, McKenzie and Snell 1973): The prompt rejection could be due to a cumulative effect of *H-2K* and *H-2I* in the *H-2K* subdivision.

The existence of a third histocompatibility region in the *H-2* complex could also explain the asymmetry in the formation of hemizygous variants from *H-2* heterozygous tumors (for discussion see Klein 1972).

The minor histoincompatibility observed when grafts are exchanged between the B10.HTT and 7R strains could be due to either a residual background difference between these two strains (i.e., to a minor *H* gene unlinked to *H-2*) or to an *H* gene in the *H-2* chromosome. In the latter case, the *H* gene could be located either in the *Ss* region or anywhere to the right of *Ss* (between *Ss* and *H-2D*, in *H-2D*, or outside of *H-2D*). If *H-2*-associated, the minor barrier could result from the difference in origin of the *D* subdivisions in the *th* and *tt* chromosomes. The *th* chromosome is derived from an *a/s* heterozygote, and the *a* chromosome, in turn, is believed to have originated from the *d* and *k* chromosomes. However, since it is not known when the *a* recombination took place and which strains it involved, it is conceivable that the *d* chromosome that gave rise to *a* was not identical with the currently known *d*. (This is indicated by the *d** symbol in Fig. 1). Since the *D* subdivision of the *tt* chromosome is ultimately derived

(through *tl* and *al*) from the *d* (and not from the hypothetical *d**) chromosome, one may speculate that the different origin of the *d* and *d** chromosomes is responsible for the observed histoincompatibility. The two chromosomes may differ, for example, in the *Tla* region, as we have indicated. Unfortunately, in an attempt to prove this, the results were inconclusive. In one strain combination, complementation with *Tla* had no effect on the rejection, whereas in the other two combinations it seemed to have eliminated the incompatibility. Perhaps in the former case the rejections were due to residual background differences.

Finally, it is interesting to note that if the *H-2*^{tt} chromosome indeed arose by crossing over, it represents another case in which this relatively rare event (the overall frequency of intra-*H-2* crossing over is 0.3%) occurred twice in rapid succession; other instances are described (Shreffler and David 1972). It appears that occurrence of one crossing over increases the chances of additional crossing over in the same chromosomal region. Such chromosomal instability could be due to the presence of a high number of duplicated genes in the *H-2* complex.

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