

# MAPPING OF A SECOND RECOMBINATION HOT SPOT WITHIN THE I-E REGION OF THE MOUSE H-2 GENE COMPLEX

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Recombinant mouse strains with crossovers within the H-2 complex have been extremely important in the development of the current genetic map of this important gene complex. By using recombinant mouse strains, the I region of the H-2 complex was originally divided into five subregions: I-A, I-B, I-J, I-E, and I-C (1). Recent molecular genetic analysis (2, 3) has shown that the I region spans a 230 kb stretch of DNA, consisting of six class II genes:  $A_{\beta 2}$ ,  $A_{\beta}$ ,  $A_{\alpha}$ ,  $E_{\beta}$ ,  $E_{\beta 2}$ , and  $E_{\alpha}$ .  $A_{\beta}$  and  $A_{\alpha}$  genes code the two polypeptide chains that form the I-A molecule, while  $E_{\beta}$  and  $E_{\alpha}$  genes code the two polypeptide chains that form the I-E molecule.  $A_{\beta 2}$  and  $E_{\beta 2}$  genes have been shown to produce mRNA, but it is not known whether this mRNA is translated into functional polypeptides (4-6).

To relate the molecular map with the genetic map, Steinmetz et al. (2) and Kobori et al. (7) analyzed six intra-I region recombinants and showed that, in all cases, the crossover point occurred in a 4 kb DNA segment within the  $E_{\beta}$  gene. These results were surprising in two respects. First, it suggested that the I-B and I-J subregions that were defined genetically according to functional and serological phenotype do not encode classical Ia gene products, and therefore the immunological phenomena previously mapped to these subregions must be accounted for by other means. Second, it suggested that recombination within the MHC may not be random, but is localized to specific sites termed recombinational hot spots.

Over the last few years our laboratory has produced additional recombinant mouse strains with crossovers in the H-2 complex. We have recently begun a major effort to determine the crossover points in these recombinants at the DNA level. In this paper we report on the DNA restriction fragment analysis of nine recombinant mouse strains that, by serological analysis, contain crossover points in the I region. Seven of these recombinant mouse strains have crossover points in the I-E subregion within an ~12-14 kb segment of DNA, which contains the  $E_{\alpha}$  gene. This analysis shows that a second recombinational hot spot exists in the I-E subregion.

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## Materials and Methods

*Mice.* All mice used in this study were produced and maintained in our immunogenetics mouse colony at the Mayo Clinic. The recombinant mouse strains analyzed in this paper were derived at the Mayo Clinic from crosses listed in Table I. Mice were serologically typed for H-2K, H-2D, I-A, and I-E antigens by the microcytotoxic dye exclusion assay using normal spleen cells as previously described (8).

*DNA Probes.* DNA probes to the  $E_{\beta 2}$  and  $E_{\alpha}$  genes were obtained from Dr. L. Hood (California Institute of Technology, Pasadena, CA). The  $E_{\alpha}$  probe is a 3.4 kb Sal I fragment from genomic cosmid clone 32.1 (9). This probe contains the second, third, and fourth exons of the  $E_{\alpha}$  gene. The  $E_{\beta 2}$  probe is a 3.2 Eco RI fragment from cosmid clone 8.4 (1). The DNA probes were radiolabeled with  $^{32}\text{P}$  by nick translation and purified by centrifugation through mini-spin columns obtained from Cooper Biomedical (Malvern, PA).

*Isolation of DNA.* High molecular weight DNA were isolated from spleen cells. Spleen cells from three mice were isolated, washed once in PBS, and resuspended in Tris-EDTA containing protease K and 0.50% SDS. The cell suspension was then incubated overnight at 37°C. The DNA was extracted once with phenol, twice with chloroform/isoamyl alcohol (24:1), ethanol precipitated, and resuspended in distilled water overnight. The DNA was then digested with 50  $\mu\text{g}/\text{ml}$  RNase at 37°C for 1 h. The DNA was again extracted with phenol, chloroform/isoamyl alcohol, ether, and ethanol precipitated. DNA was dissolved in Tris-EDTA and stored at 4°C.

*Restriction Endonuclease Digestion.* 20  $\mu\text{g}$  of spleen DNA were digested twice with 2 U/ $\mu\text{g}$  of restriction endonucleases for 2 h at 37°C. 3  $\mu\text{g}$  of DNA were removed and electrophoresed in 0.70% agarose gels (15 cm) at 20 V. If digestions were complete, the remaining DNA was electrophoresed in 0.70% agarose gels (25 cm) for 48 h. When the Rsa I restriction endonuclease enzyme was used to digest DNA the fragments were separated on 1.4% agarose gels.

*Southern Blot Hybridization.* DNA were transferred from agarose gels to nitrocellulose filters by blotting. The filters were baked and prehybridized for 2 h at 65°C in 6 $\times$  SSC, 5 $\times$  Denhardt's solution, and 300  $\mu\text{g}$  of salmon sperm DNA denatured by heating at 100°C for 5 min. The filters were hybridized overnight at 65°C in 6 $\times$  SSC, 5 $\times$  Denhardt's solution, 5 mM EDTA, 0.10% sodium pyrophosphate, 300  $\mu\text{g}$  denatured salmon sperm DNA, and 10% dextran sulfate with 3.0–5.0  $\times 10^7$  counts of  $^{32}\text{P}$ -DNA probes. The filters were washed twice for 30 min in 2 $\times$  SSC, 0.10% SDS and two times in 1 $\times$  SSC, 0.10% SDS at 65°C. The filters were then air dried and exposed to x-ray film at –80°C for 3–4 d.

## Results

*Restriction Fragment Analysis of B10.RSB Recombinants.* B10.RSB5, B10.RSB6, B10.RSB8, B10.RSB11, B10.RSB12, B10.RSB14, and B10.RSB15 recombinants were derived in this laboratory from crosses between B10.TL and B10.S(9R) with B10.F(13R) (Table I). Serological typing by microcytotoxicity analysis showed that the K,  $A_{\alpha}$ ,  $A_{\beta}$ , and  $E_{\beta}$  genes were derived from B10.TL ( $K^sA^k$ ) or B10.S(9R) ( $K^sA^s$ ). Typing for the S region–encoded C4 complement components Ss and Slp showed that the S region in these recombinants was derived from the B10.F(13R) parent ( $S^b$ ). Since the D region was also derived from B10.F(13R) ( $D^b$ ), this placed the recombination points between the  $E_{\beta}$  gene and the S region. Further definition of these recombinants by serology could not be done because  $E_{\alpha}$  alleles are mostly nonpolymorphic and serologically identical. To precisely map the recombination points we used restriction fragment polymorphism analysis. DNA from the recombinants were isolated and digested with restriction endonucleases that detect restriction site polymorphism

TABLE I  
*Recombinant Mouse Strains Analyzed*

Recombinants	Parental strains	K	A <sub>β</sub>	A <sub>α</sub>	E <sub>β</sub>	E <sub>α</sub>	S	D
	B10.S(9R)	s	s	s	s/k	k	d	d
	B10.TL	s /	k	k	k	k	d	d
	B10.F(13R)	p	p	p	p	p	b	b
B10.RSB5	B10.S(9R) × B10.F(13R)	s	s	s	s/k	—	b	b
B10.RSB6	B10.S(9R) × B10.F(13R)	s	s	s	s/k	—	b	b
B10.RSB8	B10.S(9R) × B10.F(13R)	s	s	s	s/k	—	b	b
B10.RSB11	B10.TL × B10.F(13R)	s /	k	k	k	—	b	b
B10.RSB12	B10.S(9R) × B10.F(13R)	s	s	s	s/k	—	b	b
B10.RSB14	B10.TL × B10.F(13R)	s /	k	k	k	—	b	b
B10.RSB15	B10.TL × B10.F(13R)	s /	k	k	k	—	b	b
B10.RPD1	B10.F(13R) × B10.S(9R)	p	p	p	p	—	d	d
B10.RPD2	B10.F(13R) × B10.S(9R)	p	p	p	p	—	d	d

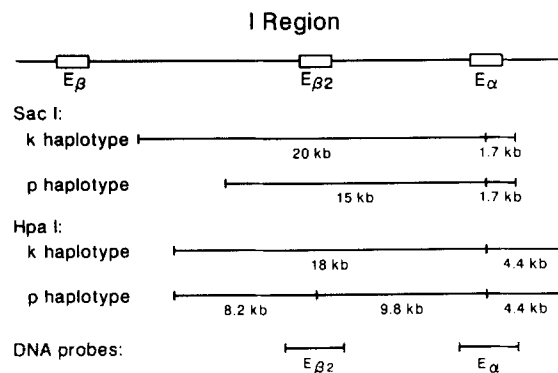


FIGURE 1. Location of polymorphic restriction sites. Sac I endonuclease detects a restriction site polymorphism between the E<sub>β</sub> and E<sub>β2</sub> genes, and Hpa I endonuclease detects a restriction site polymorphism within the E<sub>β2</sub> gene. Also shown are the locations of the E<sub>β2</sub> and E<sub>α</sub> DNA probes used in this study.

between the k and p haplotypes in the DNA segment between the E<sub>β</sub> and E<sub>α</sub> genes (2, 10–11) (Fig. 1).

The first restriction endonuclease enzyme used was Sac I. Sac I has previously been shown (10, 11) to recognize a site in the E<sub>α</sub> gene between the first and second exon. This results in two fragments, a small fragment of ~1.7 kb and a large fragment of ~20 kb in the k haplotype and ~15 kb in the p haplotype. Hybridization with a DNA probe to E<sub>β2</sub> gene showed that both the 15 kb and 20 kb fragments contain the E<sub>β2</sub> gene. This places the site of this restriction site polymorphism between the E<sub>β</sub> and E<sub>β2</sub> genes (Fig. 1). Analysis of the B10.RSB recombinants shows that all have the k haplotype restriction site polymorphism (Fig. 2). This eliminates the E<sub>β</sub> gene, the previously identified hot spot, as the site of crossing over in these recombinants.

The second restriction enzyme used was Hpa I. Hpa I was reported by Steinmetz et al. (2) to also detect a restriction site in the E<sub>α</sub> gene. Hybridization

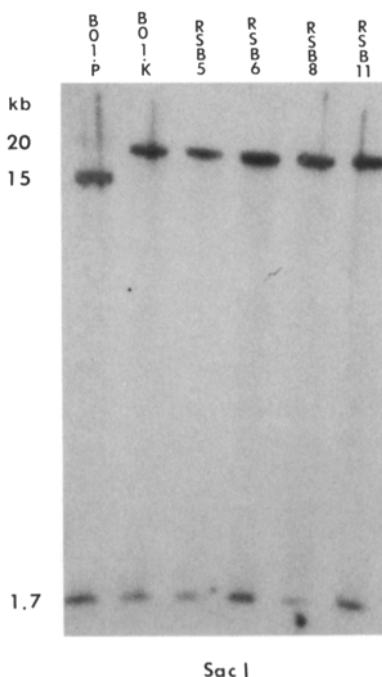


FIGURE 2. Southern blots of DNA digested with *Sac* I and probed with the  $E_{\alpha}$  probe. All B10.RSB recombinants showed the k haplotype restriction fragment pattern. Tested but not shown in this figure are B10.RSB12, B10.RSB14 and B10.RSB15. Also examined were parental strains B10.S(9R) and B10.TL, which gave the k haplotype restriction fragment pattern, and B10.F(13R), which gave the p haplotype restriction fragment pattern.

with the  $E_{\alpha}$  DNA probe detects 18 and 4.4 kb DNA fragments in the k haplotype and 9.8 and 4.4 kb DNA fragments in the d, b, and s haplotypes. Analysis of DNA from B10.P showed that the p haplotype also has the 9.8 kb fragment with the  $E_{\alpha}$  probe (Fig. 3). The  $E_{\beta 2}$  DNA probe hybridizes with 9.8 and 8.2 kb DNA fragments in the p haplotype. With the k haplotype, the  $E_{\beta 2}$  DNA probe hybridizes only with the 18 kb fragment. This shows that the p haplotype has a *Hpa* I restriction site in the DNA segment covered by the  $E_{\beta 2}$  DNA probe that is absent in the k haplotype (Fig. 1). The B10.RSB recombinants gave the k haplotype restriction pattern (18 kb fragment) with both  $E_{\alpha}$  and  $E_{\beta 2}$  DNA probes (Fig. 3). This shows that the crossover point in these recombinants is located to the right of the *Hpa* I site within the  $E_{\beta 2}$  gene (Fig. 1).

We next searched for restriction endonucleases that might detect polymorphism within the  $E_{\alpha}$  gene. We tried *Rsa* I, *Hae* III, and *Taq* I that recognize four nucleotide sequences and thus cleave the DNA into small fragments. *Rsa* I showed a restriction site polymorphism between the p and k haplotypes (Fig. 4). Hybridization of B10.K DNA with the  $E_{\alpha}$  DNA probe gave three fragments of 2.0, 1.7, and 1.0 kb. Hybridization of B10.P DNA with the  $E_{\alpha}$  probe gave fragments of 2.8, 1.7, and 1.0 kb. The complete DNA sequence for the  $E_{\alpha}$  gene has been reported (12) for the k haplotype. By using this sequence, we were able to map the *Rsa* I restriction sites in the  $E_{\alpha}$  gene (Fig. 5). The 2.0 kb polymorphic fragment contains the first exon of the gene with the polymorphic restriction

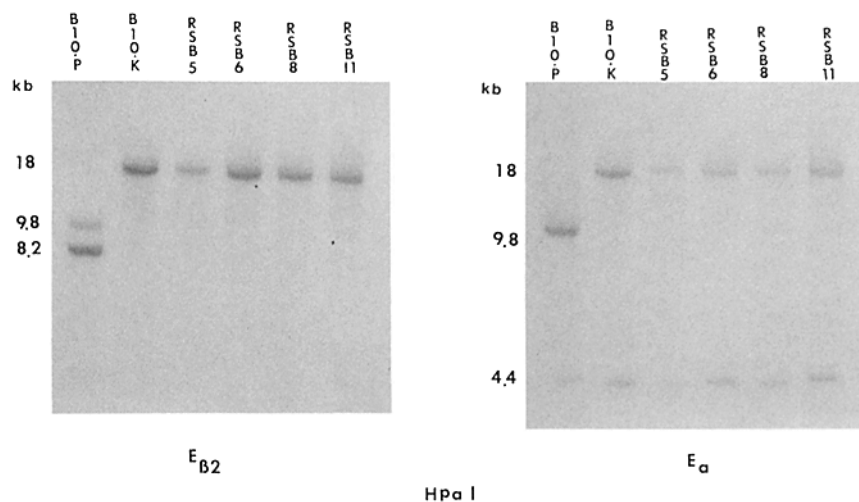


FIGURE 3. Southern blots of DNA digested with Hpa I had probed with  $E_{\beta 2}$  and  $E_{\alpha}$  probes. All B10.RSB recombinants have k haplotype restriction fragment pattern. Tested but not shown in this figure are B10.RSB12, B10.RSB14, and B10.RSB15. Also examined were parental strains B10.S(9R) and B10.TL (k haplotype restriction fragment pattern), and B10.F(13R) (p haplotype restriction fragment pattern).

site located outside the  $E_{\alpha}$  gene on the telomeric side of the gene. Fig. 4 also shows the Rsa I restriction fragment pattern obtained with the B10.RSB recombinants. Two of the recombinants, B10.RSB5 and B10.RSB14 have the k haplotype restriction pattern. This indicates that the  $E_{\alpha}$  gene in these two recombinants is derived from the k haplotype, and places the recombination to the right of the Rsa I site at the 5' end of the  $E_{\alpha}$  gene (Fig. 5). The remaining five recombinants have the p haplotype restriction pattern, placing the recombination point in these recombinants in the I-E region, between the Hpa I and Rsa I polymorphic restriction sites (Figs. 2 and 5). This 12–14 kb DNA segment contains the  $E_{\alpha}$  gene and the intervening sequence between  $E_{\beta 2}$  and  $E_{\alpha}$  genes.

*Restriction Fragment Analysis of B10.RPD1 and B10.RPD2.* B10.RPD1 and B10.RPD2 recombinants were also derived in this laboratory from crosses between B10.S(9R) and B10.F(13R) (Table I). Serological analysis indicated that the K,  $A_{\beta}$ ,  $A_{\alpha}$ , and  $E_{\beta}$  genes are derived from the B10.F(13R) ( $K^P A^P$ ) parent. Typing for Ss and Slp C4 antigens and H-2D showed the S and D regions to be of d haplotype origin derived from the B10.S(9R) parent. Thus, they are reciprocal to the B10.RSB recombinants with crossover points also between the  $E_{\beta}$  gene and the S region.

B10.RPD1 and B10.RPD2 recombinants were also examined by restriction fragment analysis using the Sac I and Hpa I restriction enzymes (Fig. 6). At both sites, B10.RPD1 and B10.RPD2 show the p haplotype pattern, placing the crossover point to the right of the  $E_{\beta 2}$  gene. These two recombinants were also analyzed using the Rsa I restriction enzyme (Fig. 4). B10.RPD1 and B10.RPD2 have the k haplotype restriction pattern, and thus have crossover points in the I-E region within the same Hpa I–Rsa I DNA fragment as the five B10.RSB

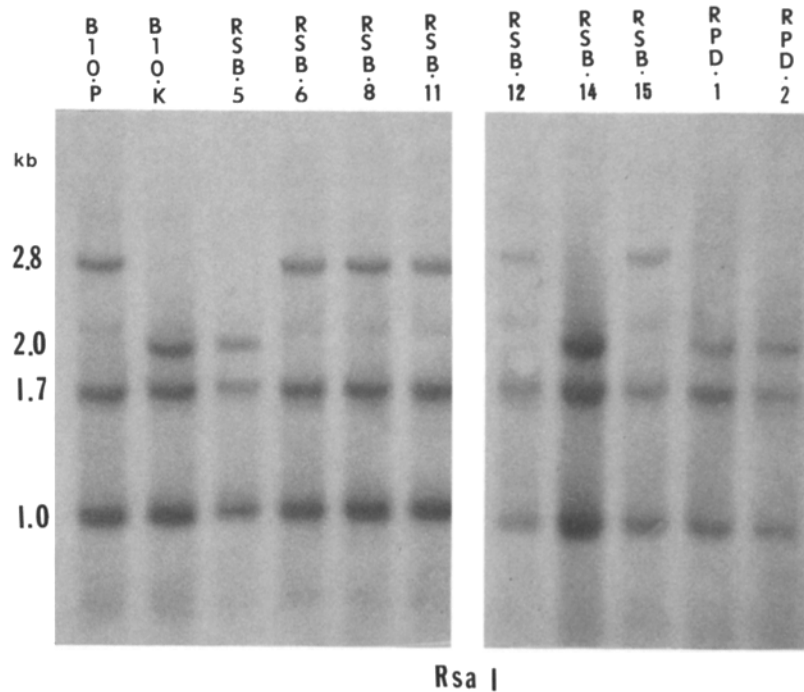


FIGURE 4. Southern blots of DNA digested with *Rsa* I and probed with the  $E_{\alpha}$  probe. Recombinants B10.RSB5, B10.RSB14, B10.RPD1, and B10.RPD2 have the k haplotype pattern. Recombinants B10.RSB6, B10.RSB8, B10.RSB11, B10.RSB12, and B10.RSB15 have the p haplotype pattern. Not shown in this figure are parental strains B10.TL and B10.S(9R), which gave the k haplotype restriction fragment pattern, and B10.F(13R) which gave the p haplotype restriction fragment pattern.

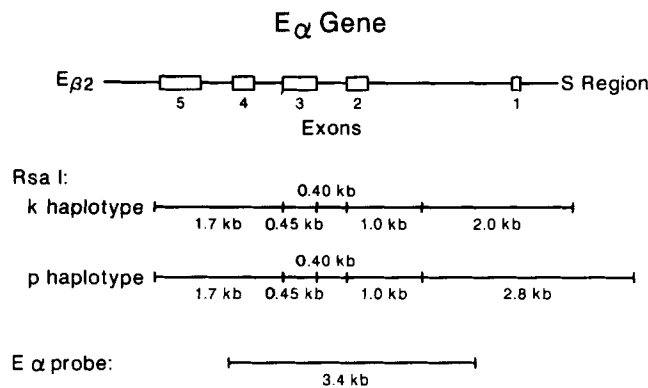


FIGURE 5. Location of *Rsa* I restriction sites within the  $E_{\alpha}$  gene. Southern blot analysis detects DNA fragments of 2.0, 1.7, and 1.0 kb in the k haplotype, and 2.8, 1.7, and 1.0 kb in the p haplotype. DNA fragments of 0.4 and 0.45 kb predicted by the DNA sequence were not observed in Southern blots. The polymorphic restriction site maps to the right of the  $E_{\alpha}$  gene. Also shown in this figure is the  $E_{\alpha}$  DNA probe used to detect these DNA fragments.

recombinants described above. Thus, seven of the nine recombinant mouse strains have crossover points within the 12–14 kb segment defined by RFLP analysis.

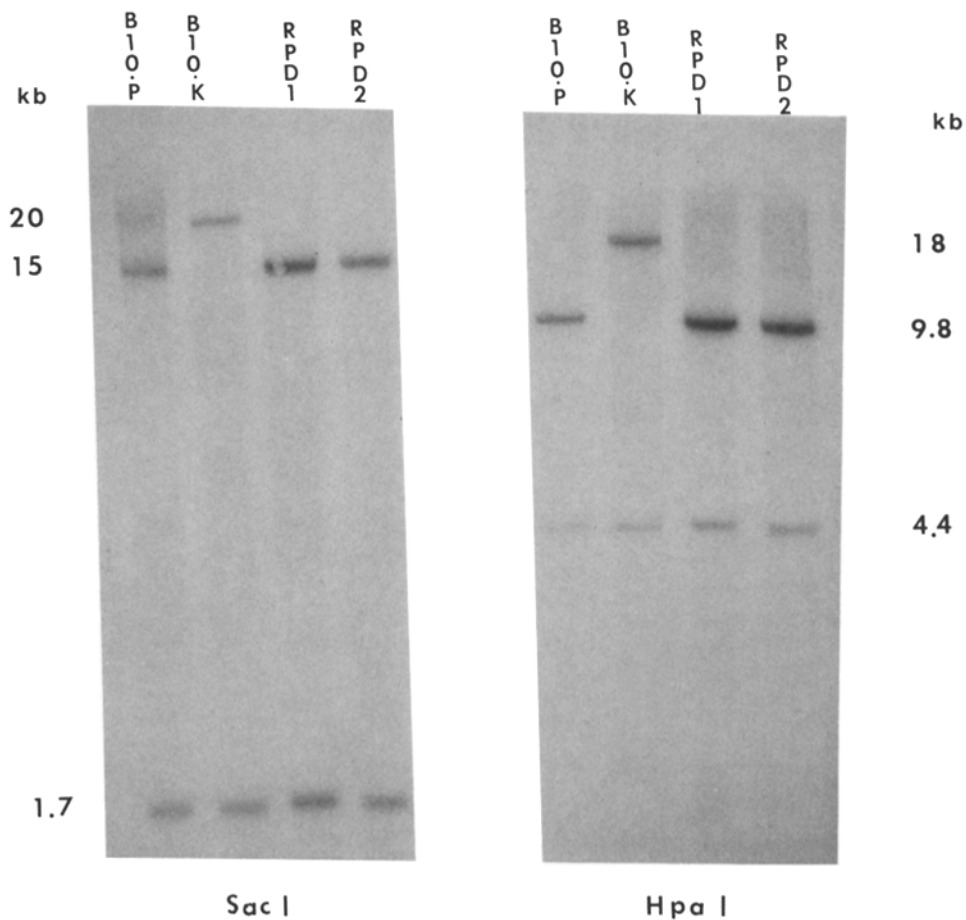


FIGURE 6. Southern blots of B10.RPD1 and B10.RPD2 DNA digested with *Sac* I and *Hpa* I and probed with the  $E_{\alpha}$  probe. B10.RPD1 and B10.RPD2 have the p haplotype restriction pattern with both *Hpa* I and *Sac* I endonucleases.

### Discussion

Previous studies by Steinmetz et al. (2) and Kobori et al. (7) mapped the crossover point of six mouse I-region recombinants to a 4.0 kb region within the  $E_{\beta}$  gene. This 4.0 kb region encompasses part of the intron between the first- (B1) and second (B2) domain exons, and the second-domain exon (B2) of the  $E_{\beta}$  gene. One conclusion of these studies is that recombination in the mouse H-2 complex is not random, but rather, occurs in specific hot spots. We were interested to know whether there are additional recombination hot spots. In this paper we analyzed nine additional intra-I region recombinant mouse strains. These recombinants were examined by restriction fragment analysis using three different restriction endonucleases that direct restriction site polymorphism in the I region. The recombination sites of these mouse strains are shown in Table II. Seven of these recombinant mouse strains have crossovers in the I-E region within the same DNA segment. This DNA segment comprises ~12–14 kb, and contains the  $E_{\alpha}$  gene and the intervening sequence between the  $E_{\beta 2}$  and  $E_{\alpha}$  genes.

TABLE II  
Location of Recombination Sites

Strain	K	A <sub>β</sub>	A <sub>α</sub>	E <sub>β</sub> Sac I	E <sub>β2</sub> Hpa I	E <sub>α</sub> Rsa I	S	D
B10.RSB6	s	s	s	s/k	k——p		b	b
B10.RSB8	s	s	s	s/k	k——p		b	b
B10.RSB11	s	/	k	k	k——p		b	b
B10.RSB12	s	s	s	s/k	k——p		b	b
B10.RSB15	s	/	k	k	k——p		b	b
B10.RPD1	p	p	p	p	p——k		d	d
B10.RPD2	p	p	p	p	p——k		d	d
B10.RSB5	s	s	s	s/k	k	k——b		b
B10.RSB14	s	s	s	s/k	k	k——b		b

Solid lines indicate DNA segments that contain crossover points.

At present we do not know whether this hot spot is within the E<sub>α</sub> gene or in the intervening sequence. We also do not know whether all seven of these strains have exactly the same recombination site. To answer these questions, we are currently producing genomic phage libraries from these recombinants as the first step in cloning these DNA segments. Two other recombinant mouse strains were found in this paper to have crossovers between the E<sub>α</sub> gene and the S region. We plan to do additional restriction fragment analysis of these recombinants using DNA probes mapping between the E<sub>α</sub> gene and the S region.

It is surprising that none of the recombinants derived from the parental strain combinations listed in Table I map in the previously described (2, 7) E<sub>β</sub> recombination hot spot. The recombinants analyzed in this paper involve B10.F(13R) as one of the parental stains. It is possible that, in this strain, the recombination site in the E region is more fragile than in the E<sub>β</sub> recombination site. It is also possible that the recombination hot spot we have found in this paper is unique to recombinants involving E<sub>α</sub> genes from the p and k haplotypes.

An important question is why recombination in the MHC occurs in these specific sites. Steinmetz et al. (13) have reported that the I region consists of tracts of high and low sequence variability with the region of A<sub>β</sub>, A<sub>α</sub> and E<sub>β</sub> being highly polymorphic, the region of E<sub>β2</sub>-E<sub>α</sub> being highly conserved. The E<sub>β</sub> recombination site occurs at the boundary between high and low polymorphism. Whether this contributes to the recombination hot spot is debatable. The studies presented in this paper indicate a recombination hot spot in the more highly conserved region.

Recombination hot spots are not unique to the mouse H-2 complex. Similar sites have been found in the human β globin gene cluster (14, 15) and the human insulin loci (16). Chakravarti et al. (15) found 75% of the recombinations in the 63 kb β gene cluster to occur within a 9.1 kb region. They estimate that the recombination rate in this segment is ~3-30 times greater than the expected rate. Restriction fragment analysis of the human insulin gene (16) has shown significant polymorphism in restriction fragment lengths in both the 3' and 5' regions of the insulin gene, indicating that recombination occurred 33 times more frequently than expected to generate this polymorphism.



Within both of these recombination hot spots, there are repeated sequences that vary in the number of copies present. In the insulin gene, this sequence consists of 14–16 bp tandem repeats located 5' to the insulin gene (17–19). Lebo et al. (16) have shown that these repeat sequences are palindromic in a single strand, and they propose that this characteristic is likely to promote unequal recombination resulting in new numbers of this repeat sequence in each daughter chromosome. In the  $\beta$  globin gene, Spritz (20) has identified an array of tandemly repeated sequences of ATTTT located 1.5 kb 5' to the  $\beta$  globin gene. This sequence is usually present in five copies, but chromosomes containing of four and six copies have also been observed and are thought to have occurred by unequal crossing over. Computer search of the  $\beta$  globin gene sequences has also revealed the presence of a chi sequence (5'-GCTGGTGG-3') within the  $\beta$  globin gene (15). This sequence is a general promoter of recombination in  $\lambda$  phage (21), increasing recombination leftward by 10–20-fold (22). This sequence exists in the mouse genome at the rate of once every 17 kb, and has been found in Ig genes (23).

Evidence suggests that haplotype specific DNA sequences are involved in H-2 recombination hot spots. All of the recombinants analyzed in this paper involved  $E_\alpha$  genes from the p and k haplotypes. Also, the large majority of strains with crossovers in the  $E_\beta$  hot spot involve the k and b haplotypes. Sequence analysis of the  $E_\beta$  gene has been published (24, 25) for the b and d haplotypes. A tandemly repeated sequence (CAGG) is also present in the  $E_\beta$  recombination hot spot. This sequence, located in the large intron between the exons encoding the first and second external domains of  $E_\beta$  polypeptide chain, is present in the d haplotype as 18 tandemly repeated copies with some minor variations. The b haplotype has only seven copies of this repeat sequence, as a result of a 36 bp deletion. One possible explanation for this deletion is unequal crossing over. Thus, a possible hypothesis for the haplotype specificity of the  $E_\beta$  recombination hot spot is unequal recombination between haplotypes with variable numbers of this repeat sequence. It has also been observed by Shiroishi et al. (26) that crosses between B10.MOL-SGR (H-2<sup>wm7</sup>), a strain derived from Japanese wild mice *Mus musculus molossinas* and inbred B10 congenic strains result in high incidence of recombination between K and I-A regions. This suggests the presence of a DNA sequence in *Mus musculus molossinus* that is absent in the B10 congenic strains derived from *Mus musculus domesticus* and which promotes recombination between the K and the I-A region. Sequence analysis of these recombination hot spots may identify the DNA sequences involved in these site specific recombinational events.

### Summary

The crossover points of nine intra-I region recombinant mouse strains were determined by restriction fragment analysis. The recombinants were examined for the presence of k and p haplotype specific DNA restriction endonuclease sites. These restriction sites were a Sac I site between the  $E_\beta$  and  $E_{\beta 2}$  genes, a Hpa I site within the  $E_{\beta 2}$  gene, and a Rsa I site ~1 kb to the right of the  $E_\alpha$  gene. Seven of the recombinants were found to have crossovers between the Hpa I and the Rsa I site. This analysis suggests that a recombination hot spot exists

within this segment. This segment is ~12–14 kb, and contains the  $E_\alpha$  gene and the intervening sequence between the  $E_{\beta 2}$  and  $E_\alpha$  genes.

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