INCLUSION GROUP SYSTEMS AND *CIS-TRANS* EFFECTS IN RESPONSES CONTROLLED BY THE TWO COMPLEMENTING *Ir-GLΦ* GENES*

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The capacity to form antibodies against thymus-dependent antigens is under histocompatibility (H)-linked immune response *(Ir)* gene control in several mammalian species (1, 2). We have recently demonstrated that in mice two genetically distinct *H-2*-linked *Ir* genes are required for responsiveness to a single antigen, the linear random polypeptide of L-glutamic acid, L-lysine, and L-phenylalanine (GLΦ) (3–6). In this report we describe the genetic requirements for control of immune responsiveness for two closely related synthetic linear terpolymers containing L-glutamic acid, L-lysine, and L-tyrosine (GLT^5 and GLT^{15}). Analysis of the immune responses to these polypeptides permitted comparisons of the genetic controls regulating a series of structurally related antigens.

Materials and Methods

Mice. All animals were between 6 and 26 wk of age at the beginning of immunization. Mice were either purchased from The Jackson Laboratory, Bar Harbor, Maine or were raised in the animal facilities of Harvard Medical School.

Antigens. The random linear terpolymer L-glutamic $acid^{53}$-L-$lysine^{36}$-L-$phenylalanine^{11}$ (GLΦ, Sample No. GF6-23-8) was synthesized in Dr. Elkan Blout's laboratory, Department of Biological Chemistry, Harvard Medical School, Boston, Mass. (3). The L-glutamic $acid^{57}$-L-$lysine^{38}$-L-$tyrosine^5$ (GLT, Lot. No. 72-B) and L-glutamic $acid^{51}$-L-$lysine^{34}$-L-$tyrosine^{15}$ (GLT^{15}, Lot. No. M-74-A) were synthesized by one of us (P.H.M.). The polymers were stored at −20°C in saline containing 1% Na_2CO_3 at a concentration of 10 mg/ml (pH 9.5).

The terpolymers were emulsified in complete Freund's adjuvant containing 0.5 mg/ml *Mycobacterium butyrium* (Difco Laboratories, Detroit, Mich.). Primary and secondary immunizations with 0.2 ml emulsion containing 100 μg of antigen were carried out intraperitoneally on days 0 and 21, respectively. Mice were bled on day 28. The sera were stored at −20°C until tested.

The procedure for the antigen-binding assay has been detailed elsewhere (6, 7). Less than 10% antigen binding is considered not significant, since this was the maximum level of binding demonstrated with some samples of normal mouse serum.

Results

The GLT^5, GLT^{15}, and GLΦ Inclusion Group System. The strain distribution pattern of responsiveness to GLT^5, GLT^{15}, and GLΦ after secondary immu-

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TABLE I
Immune Response Inclusion Group System

Strain	H-2 haplotype	GLT^5	GLT^{15}	GLΦ
A/J	*a*	4.6(5) ± 3.9*	2.3(6) ± 3.7	1.9(11) ± 1.6
C57BL/10J	*b*	−1.2(9) ± 0.2	8.0(5) ± 1.5	0.5(13) ± 2.5
C3H.SW	*b*	−1.0(4) ± 4.4	−3.7(4) ± 2.5	−0.8(3) ± 5.1
BALB/cJ	*d*	NT‡	58.2(4) ± 7.6	59.2(4) ± 2.0
DBA/2J	*d*	79.0(6) ± 4.0	74.2(5) ± 5.2	40.4(4) ± 7.9
B10.M	*f*	1.2(4) ± 2.9	−1.2(4) ± 1.4	11.8(15) ± 3.3
A.CA	*f*	2.3(3) ± 1.5	NT	7.1(7) ± 3.9
HTG	*g*	95.0(5) ± 2.0	69.4(7) ± 7.2	88.5(5) ± 2.6
D2.GD	*g4*	4.0(8) ± 3.0	NT	−3.6(5) ± 5.0
B10.WB	*j*	NT	69.7(5) ± 5.3	79.3(3) ± 7.2
C3H.JK	*j*	91.5(4) ± 6.5	51.2(3) ± 25.2	97.0(4) ± 1.9
CBA/H	*k*	1.6(3) ± 1.6	0.0(3) ± 2.7	1.5(5) ± 3.2
C3H/HeJ	*k*	3.3(4) ± 3.2	6.6(4) ± 3.4	−6.8(5) ± 5.3
B10.AKM	*m*	NT	4.8(4) ± 0.6	4.9(4) ± 6.2
B10.F	*n*	−7.4(5) ± 5.2	4.2(3) ± 1.5	31.7(11) ± 7.8
C3H.OH	*o2*	82.5(2) ± 3.6	77.6(4) ± 3.8	77.1(3) ± 5.7
B10.P	*p*	NT	7.8(3) ± 5.9	79.5(3) ± 1.9
C3H.NB	*p*	5.0(2) ± 3.6	4.3(7) ± 0.8	40.5(4) ± 8.0
BDP/J	*p*	−0.4(7) ± 2.1	−4.4(5) ± 0.8	54.4(5) ± 9.9
P/J	*p*	−4.8(4) ± 2.5	NT	45.4(5) ± 3.3
B10.G	*q*	5.4(5) ± 1.5	32.6(4) ± 8.0	64.3(3) ± 6.3
C3H.Q	*q*	0.5(6) ± 3.2	22.9(3) ± 15.5	85.7(4) ± 8.1
DBA/1J	*q*	3.0(5) ± 3.0	17.0(4) ± 2.7	73.1(5) ± 3.3
T138	*q*	−9.4(4) ± 2.6	38.1(4) ± 7.7	97.1(5) ± 1.2
B10.RIII	*r*	70.6(3) ± 6.6	92.4(4) ± 1.6	68.6(5) ± 4.2
RIII/2J	*r*	93.1(3) ± 4.0	NT	100.0(4) ± 0.6
A.SW	*s*	2.9(4) ± 2.1	−7.3(4) ± 0.9	1.6(5) ± 3.1
SJL/J	*s*	4.4(2) ± 6.4	1.0(5) ± 2.5	−4.3(4) ± 0.9
B10.PL	*u*	57.1(2) ± 0.0	74.1(4) ± 9.2	62.7(3) ± 6.1
PL/J	*u*	NT	69.5(4) ± 5.4	75.7(4) ± 1.0
B10.SM	*v*	3.4(1)	9.3(5) ± 5.7	8.9(3) ± 5.5
SM/J	*v*	5.1(3) ± 5.2	NT	5.1(7) ± 4.5
6R	*y2*	7.1(7) ± 5.2	36.1(5) ± 7.7	69.1(7) ± 9.4

* Mean percentage of radiolabeled GLT^5 ligand bound in Farr assay by a 1:5 dilution of serum ± standard error. Number of mice tested is given in parentheses.
‡ NT, not tested.

nization with 100 μg of terpolymer for 33 inbred and congenic strains carrying 18 different *H-2* haplotypes is given in Table I. There is a striking similarity among the response patterns to these GL-containing terpolymers. Thus, mice carrying the $H\text{-}2^d$, $H\text{-}2^g$, $H\text{-}2^j$, $H\text{-}2^{o2}$, $H\text{-}2^r$, and $H\text{-}2^u$ haplotypes respond to all three terpolymers, while strains bearing the $H\text{-}2^a$, $H\text{-}2^b$, $H\text{-}2^f$, $H\text{-}2^{g4}$, $H\text{-}2^k$, $H\text{-}2^m$, $H\text{-}2^s$, and $H\text{-}2^v$ haplotypes fail to make detectable levels of antibody after immunization with either GLT^5, GLT^{15}, or GLΦ. It is important to note that with these immunization protocols, mice bearing the $H\text{-}2^q$ or $H\text{-}2^{y2}$ haplotypes (the latter is a recombinant *H-2* haplotype carrying an *I*-region allele derived from a $H\text{-}2^q$ parental strain) failed to make detectable levels of antibody after secondary

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immunization with GLT^5, but produced low level antibody responses to the GLT^{15} terpolymer and strong responses after immunization with GLΦ (Table I). In contrast, mice bearing the $H\text{-}2^n$ or $H\text{-}2^p$ haplotypes failed to produce antibody after hyperimmunization with either GLT^5 or GLT^{15}, but made antibody after immunization with GLΦ. In addition, as indicated in Table II, the 9R and B10.HTT recombinant strains responded to GLΦ, but failed to respond to the GLT terpolymers. This hierarchy of immunogenicity for the terpolymers GLT^5, GLT^{15}, and GLΦ can be observed in both the quantitative and qualitative responses of selected inbred strains to these antigens. We shall refer to this type of relationship as an inclusion group system, depicted in Fig. 1.

Two H-Linked Ir Genes Control GLT Responsiveness. The patterns of immune responsiveness among the congenic strains listed in Tables I and II indicate that the GLT^5, GLT^{15}, and GLΦ polypeptides are under *H-2*-linked *Ir* gene control.

The B10.A(5R) strain, hereafter termed 5R, has been previously used to demonstrate that the immune response to the GLΦ polymer is controlled by two H-linked *Ir* genes (3–6). The 5R strain carried a recombinant *H-2* haplotype in which crossing over occurred within the *I* region between the GLΦ nonresponder $H\text{-}2^a$ and $H\text{-}2^b$ haplotypes (Table II). 5R mice produced antibody after immunization with GLT^5, GLT^{15}, and GLΦ. Comparisons of the genetic structure of the responder $H\text{-}2^{i5}$ haplotype with the nonresponder $H\text{-}2^a$ and $H\text{-}2^{i18}$ haplotypes localizes one *Ir-GLΦ* or *Ir-GLT* gene, termed α, in the $I\text{-}C^d$ or S^d region and a second *Ir-GLΦ* or *Ir-GLT* gene, termed β, in the K^b, $I\text{-}A^b$, or $I\text{-}B^b$ regions (Table II). The 9R and B10.HTT recombinant lines are additional examples of GLΦ responder strains derived by intra-*I*-region crossover events between two parental nonresponder haplotypes (Table II).

Cis-Trans Effects. To confirm the two gene model, we attempted to complement the $\alpha(+)$ allele of one nonresponder strain with the $\beta(+)$ allele of another strain by the mating of selected nonresponder parental strains. (C57BL/6J × A/J)F_1 and (C3H/HeJ × C57BL/10J)F_1 hybrids immnized with GLΦ produced high levels of anti-GLΦ antibody, yet when immunized with GLT, they failed to make detectble levels of anti-GLT antibodies. An additional significant observation was that F_1 hybrids between nonresponder strains carrying the $H\text{-}2^s$ and $H\text{-}2^a$ or $H\text{-}2^s$ and $H\text{-}2^k$ haplotypes demonstrated quite low level humoral responses to GLΦ, in contrast to the 9R and B10.HTT recombinants which produced substantially higher levels of antibody after immunizaion with GLΦ. It is important to note that the $\alpha(+)$ and $\beta(+)$ alleles which control GLΦ responsiveness are fully dominant, as evidenced by the responses of F_1 hybrids between the B10.HTT responder strain with the $\alpha(-)$ and $\beta(-)$ nonresponder A.CA strain (6) (Table II). Thus, the difference in responsiveness between the above mentioned recombinant mice and the F_1 hybrids must be accounted for by the distinct chromosomal relationships of the relevant genes in the recombinant versus the F_1 hybrid as summarized in Table III and discussed below.

Discussion

Until very recently, it was generally assumed that only one H-linked *Ir* gene was required to control responsiveness to a single antigen. The first clear

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TABLE II

Cis-Trans Effects between Immune Response Genes

<table>
<tr><th rowspan="2">Strain</th><th rowspan="2">H-2 haplotype</th><th colspan="6">H-2 region formulae*</th><th>Ir-GLΦ</th><th colspan="3">Immune response‡</th></tr>
<tr><th>K</th><th>I-A</th><th>I-B</th><th>I-C</th><th>S</th><th>D</th><th>Genotype α,β/α,β</th><th>GLT[5]</th><th>GLT[15]</th><th>GLΦ</th></tr>
<tr><td>C57BL/10J</td><td>b</td><td>b</td><td>b</td><td>b</td><td>b</td><td>b</td><td>b</td><td>−,+/−,+</td><td>−1.2(9) ± 0.2</td><td>8.0(5) ± 1.5</td><td>0.5(13) ± 2.5</td></tr>
<tr><td>B10.BR</td><td>k</td><td>k</td><td>k</td><td>k</td><td>k</td><td>k</td><td>k</td><td>+,−/+,−</td><td>5.3(4) ± 1.2</td><td>NT</td><td>5.1(12) ± 3.0</td></tr>
<tr><td>B10.D2</td><td>d</td><td>d</td><td>d</td><td>d</td><td>d</td><td>d</td><td>d</td><td>+,+/+,+</td><td>74.0(7) ± 4.0</td><td>83.9(5) ± 3.9</td><td>60.9(9) ± 4.7</td></tr>
<tr><td>B10.A</td><td>a</td><td>k</td><td>k</td><td>k |</td><td>d</td><td>d</td><td>d</td><td>+,−/+,−</td><td>3.5(5) ± 3.2</td><td>2.3(6) ± 2.4</td><td>3.9(7) ± 2.1</td></tr>
<tr><td>5R</td><td>i5</td><td>b</td><td>b</td><td>b |</td><td>d</td><td>d</td><td>d</td><td>+,+/+,+</td><td>20.5(5) ± 3.0</td><td>28.6(5) ± 4.4</td><td>73.3(10) ± 5.0</td></tr>
<tr><td>18R</td><td>i18</td><td>b</td><td>b</td><td>b</td><td>b</td><td>b |</td><td>d</td><td>−,+/−,+</td><td>1.4(3) ± 4.0</td><td>6.3(4) ± 3.2</td><td>4.7(5) ± 2.4</td></tr>
<tr><td>7R</td><td>t2</td><td>s</td><td>s</td><td>s</td><td>s</td><td>s |</td><td>d</td><td>−,+/−,+</td><td>NT</td><td>−0.7(3) ± 2.9</td><td>4.4(5) ± 1.8</td></tr>
<tr><td>B10.HTT</td><td>t3</td><td>s</td><td>s</td><td>s |</td><td>k</td><td>k |</td><td>d</td><td>+,+/+,+</td><td>3.4(4) ± 3.9</td><td>8.7(3) ± 3.1</td><td>65.6(6) ± 12.2</td></tr>
<tr><td>9R</td><td>t4</td><td>s</td><td>s</td><td>s |</td><td>d</td><td>d</td><td>d</td><td>+,+/+,+</td><td>NT</td><td>8.5(4) ± 0.7</td><td>70.6(4) ± 9.5</td></tr>
<tr><td>(C57BL/6 × A/J)F1</td><td>b/a</td><td>b/k</td><td>b/k</td><td>b/k</td><td>b/d</td><td>b/d</td><td>b/d</td><td>−,+/+,−</td><td>7.6(4) ± 1.5</td><td>0.8(5) ± 3.1</td><td>85.5(9) ± 3.5</td></tr>
<tr><td>(C3H × C57BL/10)F1</td><td>k/b</td><td>k/b</td><td>k/b</td><td>k/b</td><td>k/b</td><td>k/b</td><td>k/b</td><td>+,−/−,+</td><td>NT</td><td>0.9(5) ± 3.9</td><td>80.0(5) ± 1.7</td></tr>
<tr><td>(B10.HTT × A.CA)F1</td><td>t3/f</td><td>s/f</td><td>s/f</td><td>s/f</td><td>k/f</td><td>k/f</td><td>d/f</td><td>+,+/−,−</td><td>NT</td><td>NT</td><td>57.8(3) ± 8.3</td></tr>
<tr><td>(B10.A × A.SW)F1</td><td>a/s</td><td>k/s</td><td>k/s</td><td>k/s</td><td>d/s</td><td>d/s</td><td>d/s</td><td>+,−/−,+</td><td>NT</td><td>NT</td><td>17.5(6) ± 7.8</td></tr>
<tr><td>(B10.BR × A.SW)F1</td><td>k/s</td><td>k/s</td><td>k/s</td><td>k/s</td><td>k/s</td><td>k/s</td><td>k/s</td><td>+,−/−,+</td><td>NT</td><td>NT</td><td>24.1(8) ± 9.3</td></tr>
<tr><td>(B10.BR × B10.S)F1</td><td>k/s</td><td>k/s</td><td>k/s</td><td>k/s</td><td>k/s</td><td>k/s</td><td>k/s</td><td>+,−/−,+</td><td>NT</td><td>NT</td><td>28.2(5) ± 7.8</td></tr>
</table>

* Vertical bar indicates position of recombination.

‡ NT, not tested.

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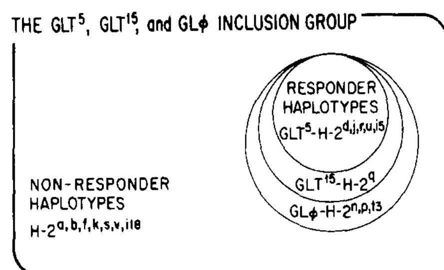

FIG. 1. The GLT^5, GLT^{15}, and GLΦ inclusion group. All *H-2* haplotypes associated with responsiveness to GLT^5 are included among all the haplotypes that respond to GLT^{15}, which in turn are included among all the *H-2* haplotypes associated with responsiveness to GLΦ. The haplotypes that fail to respond are indicated in the area outside the circles.

TABLE III
Cis-Trans Effects for GLΦ and GLT Responses

H-2 haplotype	Genotype $\alpha,\beta/\alpha,\beta$	Antibody response*		Chromosomal relationship‡
		GLΦ	GLT	
a or *k*	+,−/+,−	−	−	
b or *s*	−,+/−,+	−	−	
f	−,−/−,−	−	−	
i5	+,+/+,+	+++	+	*Cis* (2×)
b × *a*	−,+/+,−	+++	−	*Trans* 1×)
b × *k*	−,+/+,−	+++	−	*Trans* (1×)
t3 (s/k)	+,+/+,+	+++	−	*Cis* (2×)
t4 (s/a)	+,+/+,+	+++	−	*Cis* (2×)
t3 × *f*	+,+/−,−	+++		*Cis* (1×)
s × *a*	−,+/+,−	+		*Trans* (1×)
s × *k*	−,+/+,−	+		*Trans* (1×)

* Summary of data presented in Table II.

‡ Relationship of $\alpha(+)$ and $\beta(+)$ alleles, gene dose indicated in parentheses.

example of dual H-linked *Ir* gene control of immune responsiveness was demonstrated with the GLΦ terpolymer (3–6). In the present study the response of the 5R strain to the GLT^5 and GLT^{15} terpolymers also illustrates the requirement of two *H-2*-linked *Ir* genes for the response to these antigens. Thus, as shown in Table II, strains carrying either the $H\text{-}2^a$ or $H\text{-}2^b$ haplotypes are phenotypic nonresponders to the GLT^5, GLT^{15}, or GLΦ polymers. However, the 5R strain whose *H-2* haplotype was derived by recombination of the $H\text{-}2^a$ and $H\text{-}2^b$ types respond to all three polymers. The (C57BL/6J × A/J)F_1 hybrids ($H\text{-}2^a/H\text{-}2^b$) responded to GLΦ, but failed to make detectable levels of antibody after immunization with GLT. Thus, for both GLT^5 or GLT^{15}, the α and β *Ir* genes interact successfully in the *cis* position (i.e. on the same chromosome), but not in the *trans* configuration of the F_1 hybrid (i.e. each gene on separate chromosomes). In addition, a quantitative *cis* effect was observed when comparing the responses of the 9R and B10.HTT recombinant strains with the responses of the (B10.A ×

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A.SW)F_1, (B10.BR × A.SW)F_1, or (B10.BR × B10.S)F_1 hybrids (Table II). The infrequent ability to observe complementation for other antigens under *H-2*-linked *Ir* gene control in F_1 hybrids between parental nonresponders may in part reflect strong *cis-trans* effects. These *cis* effects have been summarized in Table III. These *cis* phenomena are not related to a gene dose effect since the $\alpha(+)$ and $\beta(+)$ alleles for GLΦ are fully dominant when tested in a (B10.HTT × A.CA)F_1 hybrid between a responder $\alpha(+)$ $\beta(+)$ and an $\alpha(-)$ $\beta(-)$ nonresponder strain (see Tables II and III) (6). In addition, the *cis* effects appear to be more apparent for particular combinations of haplotypes inasmuch as the $\alpha(+)$ and $\beta(+)$ alleles are completely dominant in the *trans* configuration in selected F_1 combinations, such as the (C57BL/6 × A/J)F_1 response to GLΦ. However, the *cis* effect can also be demonstrated in the anti-GLΦ response of cells from (C57BL/6 × A/J)F_1 hybrids in transfer experiments (8).

The *cis-trans* effects described in this report are similar in many respects to the gene interactions noted between the C_H and V_H immunoglobulin genes. Thus, in F_1 hybrid mice heterozygous for the heavy chain allotypes, the V_H allotypic and idiotypic markers are generally associated with the allotypic markers of the C_H alleles found in the *cis* configuration (9, 10). It is tempting to extend the analogies of the *cis-trans* effects found in the H-linked *Ir* genes and the immunoglobulin genes.

The *cis* effects may also explain the selective forces which maintained the close linkage among individual *Ir* genes during mammalian evolution. Since the most efficient mechanism for *Ir*-gene interaction appears to require that complementary genes reside on the same haplotype, strong selective pressures would favor the maintenance of a cluster of *Ir* genes within the genome.

The *H-2*-controlled immune response patterns to the three structurally related synthetic linear random terpolymers, GLT^5, GLT^{15}, and GLΦ is best described as an inclusion group relationship. Fig. 1 depicts this inclusion group relationship. All the *H-2* types associated with responsiveness to GLT^5 are included among all the haplotypes that respond to GLT^{15}, which in turn are included among all the *H-2* types associated with responsiveness to GLΦ (i.e. $GLT^5 \supset GLT^{15} \supset GL\Phi$). This inclusion group relationship suggests that at least some common *Ir* gene products may be involved with responsiveness to the three related polypeptides.

It has been difficult to determine whether different responder *H-2* haplotypes carry identical *Ir* alleles for each antigen or if the *Ir* allele for a specific antigen carried by one *H-2* haplotype differs from the *Ir* allele coding for responsiveness to the same antigen in another haplotype. The quantitative differences in the GLT^5 and GLT^{15} responses of B10.D2 and 5R mice (which share identical $\alpha(+)$ alleles, derived from the $I\text{-}C^d\text{–}S^d$ regions) suggest that these strains carry different $\beta(+)$ alleles (Table II).

A characteristic feature of H-linked immune response genes is their exquisite degree of antigen specificity. Genetically, this can be attributable to: *(a)* the existence of a large number of specific *Ir* loci each controlling responsiveness to a very limited set of antigens; *(b)* the possibility that each *Ir* allele controls responsiveness to a variety of different antigens (such alleles could be highly polymorphic, each haplotype carrying a different set of *Ir* alleles coding for

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responses to a diverse set of antigens); *(c)* the hypothesis that a large number of interacting *Ir* genes are required in the genetic control of responsiveness to each antigen; or *(d)* some combination of the above. The available data suggest the latter possibility, i.e., a limited number of *Ir* loci with polymorphic alleles and a requirement for at least bigenic control for responsiveness to certain antigens.

Using the branched synthetic polypeptide (T,G)-A- -L, Munro and Taussig (11) have also obtained evidence for the dual gene control of immune responsiveness. These authors have suggested that one gene codes for the ability to produce an antigen-specific T-cell factor, while the second gene acts at the level of the B cell, presumably by coding for a B-cell acceptor molecule. However, experiments presented in one of the accompanying manuscripts (8), which were designed to determine in which cell types the GLΦ $\alpha(+)$ and $\beta(+)$ alleles are expressed, indicate that both the $\alpha(+)$ and $\beta(+)$ alleles are required in the B-cell population for cooperative immune responses to DNP-GLΦ. In addition, data are presented in the following manuscripts which indicate that both the $\alpha(+)$ and $\beta(+)$ alleles are also required for T-cell function (8, 12). Thus, the two *Ir-GLΦ* genes behave differently by functional analysis than the manner interpreted by Munro and Taussig for the two genes concerned with responses to (T,G)-A- -L (11).

Summary

The immune responses to the random linear terpolymers of L-amino acids, poly-(glu^{57}, lys^{38}, tyr^{5}), poly-(glu^{51}, lys^{34}, tyr^{15}), and poly-(glu^{53}, lys^{36}, phe^{11}) are each controlled by two dominant H-linked *Ir* genes. The immune responses to these three related terpolymers demonstrate different *H-2* distributions, however, the *H-2* patterns are part of a single inclusion group system. The α- and β-genes are dominant; however, most effective gene interactions occur when the two genes are in the *cis* configuration. The potential significance of this *cis-trans* effect is discussed.

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